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Aspects of bleeding complications and hemostasis at central line insertion and mild induced hypothermia

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Aspects of bleeding complications and hemostasis at central line insertion and mild induced hypothermia

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av

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Abstract

Bleeding complications range from 0.5 to 1.6% in connection with central venous catheter insertions but are more frequent in patients with bone marrow failure and severe thrombocytopenia. Although supportive evidence is scarce, prophylactic platelet transfusion is sometimes performed in these patients before catheter insertion. Furthermore, the ideal threshold platelet count and timing of transfusion remain controversial among clinical studies.

Hypothermia is generally considered to reduce coagulation and platelet function. However, studies performed in animals, healthy volunteers, and patients have shown conflicting results.

Paper I, a retrospective study in non-intensive care unit patients, showed that serious bleeding complications in association with central line insertions were uncommon and that insertion of a large bore catheter may be an independent risk factor for mild bleeding complications in this population.

Paper II, a prospective observational study, evaluated the efficacy of prophylactic platelet transfusions in thrombocytopenic patients with bone marrow failure. Transfusion improved hemostatic parameters in ROTEM and Multiplate tests by increasing the number of platelets and not by enhancing platelet function. Improved clotting parameters persisted between 1 and 4 hours after transfusion.

Paper III demonstrated increased platelet aggregation and strengthened clot formation over time in out-of-hospital cardiac arrest patients treated with hypothermia. In patients with dual platelet inhibition, including ticagrelor and aspirin, this effect was offset by powerful $P2Y_{12}$ blockade, confirmed by analysis of vasodilator-stimulated phosphoprotein. The effect of ticagrelor was delayed in survivors of cardiac arrest, probably due to slow gastric emptying.

Paper IV demonstrated prolonged clot initiation and decreased clot propagation in ROTEM testing (EXTEM, FIBTEM, and APTEM assays) in whole blood from out-of-hospital cardiac arrest patients treated with mild induced hypothermia.

Paper V investigated whole blood from acute coronary syndrome patients treated with ticagrelor and aspirin. *In vitro* applied hypothermia to 33°C markedly increased platelet activity measured by flow cytometry, whereas a viscoelastic coagulation test (Sonoclot) revealed a hypocoagulative response.

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Aspects of bleeding complications and hemostasis at central line insertion and mild induced hypothermia

Doctoral dissertation

by

Thomas Kander
MD

LUND UNIVERSITY
Faculty of Medicine

2014

To my family

Table of contents

Original studies

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals.

I	Kander T, Frigyesi A, Kjeldsen–Kragh J, Karlsson H, Rolander F, Schött U. Bleeding complications after central line insertions: Relevance of pre–procedure coagulation tests and institutional transfusion policy. Acta Anaesthesiol Scand 2013;57:573–79.

II	Kander T, Tanaka K, Nordström E, Persson J, Schött U. The effect and duration of prophylactic platelet transfusions before insertion of a central venous catheter in patients with bone marrow failure evaluated with point of care methods and flow cytometry. Anest Analg 2014;119:882–90.

III	Kander T, Dankiewicz J, Friberg H, Schött U. Platelet aggregation and clot formation in comatose survivors of cardiac arrest treated with induced hypothermia and dual platelet inhibition with aspirin and ticagrelor; a prospective observational study. (Epub ahead of print). Critical Care 2014;18:495.

IV	Kander T, Brokopp J, Friberg H, Schött U. Wide temperature range testing with ROTEM coagulation analyses. Ther Hypothermia Temp Manag 2014;4:125-30.

V	Kander T, Brokopp J, Erlinge D, Lood C, Schött U. Temperature effects on hemostasis in whole blood from ticagrelor and aspirin treated patients with acute coronary syndrome. Accepted for publication in Scand J Clin Lab Invest September 11th, 2014.

Abbreviations

ACT   Activated clotting time (Sonoclot parameter)
ADP   Adenosine diphosphate
ADPtest   Platelet aggregation in response to ADP, 6.5 µM
APC   Activated protein C
aPTT   Activated partial thromboplastin time
ASPItest Platelet aggregation in response to arachidonic
          acid, 0.5 mM
AUC   Area under the curve
CDC   Central dialysis catheter
CFT   Clot formation time (ROTEM parameter)
COLtest   Platelet aggregation in response to collagen, 3.2
          µg/ml
CR   Clot rate (Sonoclot parameter)
CT   Clotting time (ROTEM parameter)
CVC   Central venous catheter
F   Coagulation factor
GP   Glycoprotein
MCF   Maximum clot firmness (ROTEM parameter)
MIH   Mild induced hypothermia
OHCA   Out-of-hospital cardiac arrest
PAC-1   Procaspase Activating Compound 1. Antibody for
          flow cytometry analyses
PF   Platelet function (Sonoclot parameter)
PRI   Platelet reactivity index
PT/INR   Prothrombin time/international normalized ratio
TF   Tissue factor
TRAPtest Platelet aggregation in response to thrombin
          receptor agonist peptide, 32 µM
VASP   Vasodilator-stimulated phosphoprotein

Background

History of hemostasis

The study of blood coagulation can be traced back to approximately 400BC, when the father of medicine, Hippocrates, observed that the blood of a wounded soldier congealed as it cooled; he also found that bleeding from a small wound stopped when it was covered with skin but started again when the skin was removed. Later, Aristotle noted that blood cooled when removed from the body, initiating decay that resulted in congealing, but if fibers were removed there was no clotting. It was not until 1627, however, that Mercurialis observed clots in veins at body temperature.

Platelets

The description of particles in the blood that are smaller than leukocytes and erythrocytes, today called platelets or thrombocytes, dates back to the end of the 18th century. Between 1865 and 1877, these corpuscles were clearly described, although without an understanding of their origin, significance, or function. There is general agreement that Giulio Bizzozzero was the first person to establish the central role of platelets, not only in physiological hemostasis but also in thrombosis. In 1883, Bizzozzero used a microscope to perform *in vivo* investigations of the outcomes of penetrating vessel walls with a needle. He observed that platelets adhered to the vessel wall, changed their shape by emitting protrusions of various lengths, and then induced the aggregation of other elements (including red and white cells). This process continued until the formation of a network of fibrin fibrils, after which platelets lost the appearance of distinct cellular elements. This was the first clear demonstration in the history of medicine of the physiological role of platelets in hemostasis[1, 2].

Platelet transfusion

In 1910, a case study by Duke was among the earliest evidence that platelets are crucial for human hemostasis and that platelet transfusions can restore hemostatic competence in individuals with low platelet counts [2]. A 20-year-old man was

admitted with a platelet count of 6×10^9/L and profound mucocutaneous bleeding. He developed uncontrollable epistaxis that left him nearly moribund. In desperation, a donor was selected from among his young friends, and a direct blood transfusion was arranged. A "large" amount of blood was transfused, as judged by an increase in the pulse of the donor and a rise in the platelet count in the recipient to 123×10^9/L. All overt signs of bleeding disappeared immediately after the transfusion. To monitor the patient's response to transfusion, Duke used a bleeding time assay that he had developed, which included analyzing the time for bleeding to stop from a standardized wound in the earlobe.

Despite this remarkable success, many obstacles to routine platelet transfusion remained. It was mainly radiation-induced thrombocytopenia resulting from the Hiroshima and Nagasaki atomic bombings and secondary thrombocytopenia following chemotherapy that drove the development of safe platelet transfusions to our current standard.

Coagulation

In 1905, Paul Morawitz's theory of coagulation was that in the presence of calcium and thromboplastin, prothrombin was converted to thrombin, which in turn converted fibrinogen into a fibrin clot. This idea persisted for 40 years until Paul Owren, in 1944, found that a four-factor concept of clotting did not apply to a bleeding patient, thus leading to the discovery of coagulation factor V (FV) [3].

In the 1960s, two groups proposed a waterfall-cascade model of coagulation involving intrinsic and extrinsic pathways that lead to a burst of thrombin generation [4, 5]. Although this model explains the results of laboratory coagulation tests such as activated partial thromboplastin time (aPTT) and prothrombin time/international normalized ratio (PT/INR), it is not sufficient to explain the pathways leading to hemostasis *in vivo*. For this reason, in the last decade, Hoffman and Monroe proposed a new model of hemostasis [6] in which coagulation is regulated by cell surface properties and specific receptors for coagulation factors. This cell-based model of coagulation provides a description of coagulation that more likely reflects hemostatic processes as they occur *in vivo*.

Thus, hemostasis research has flourished the last decades and led to today's view which will be presented briefly, without too many details, below.

Normal hemostasis

The theory described in this section of the thesis is described in textbooks and compendiums [7, 8] as well as in journal articles [6, 9].

Hemostasis is a process that causes bleeding to stop to retain blood within a damaged blood vessel, whereas hemorrhage is the opposite of hemostasis. Hemostasis is the first stage of wound healing. It involves a balance of coagulation and natural inhibitors of platelets, which protects the vasculature from uncontrolled bleeding and excessive clotting.

Hemostasis occurs when blood is present outside of the body or blood vessels; it is an innate response of the body that stops the loss of blood. During hemostasis, three steps occur in a rapid sequence. First, vascular spasm occurs as blood vessels constrict to slow blood loss. Second, during platelet plug formation, platelets stick together to form a temporary seal that covers the break in the vessel wall. Third, coagulation, or blood clotting, reinforces the platelet plug with fibrin threads that act as "molecular glue".

The two first steps - vascular spasm and platelet plug formation - are sometimes referred to as primary hemostasis.

Within seconds of blood vessel epithelial wall disruption, platelets begin to adhere to the subendothelium surface (i.e., platelet plug formation). It takes approximately 60 seconds until the first fibrin strands begin to form within the wound (i.e., coagulation). After several minutes, the platelet plug is completely interspersed by fibrin (Figure 1).

Rheology also influences hemostasis. Under conditions of normal concentration of hemoglobin (Hb), flow is maximal at the center of the vessel, and platelets are marginalized toward the periphery close to the site of the injury, thus promoting platelet-endothelial interaction [10]. In arterioles this rheological effect of Hb can increase platelet concentration near the injured vessel wall by as much as seven times normal, and can therefore enhance thrombus formation.
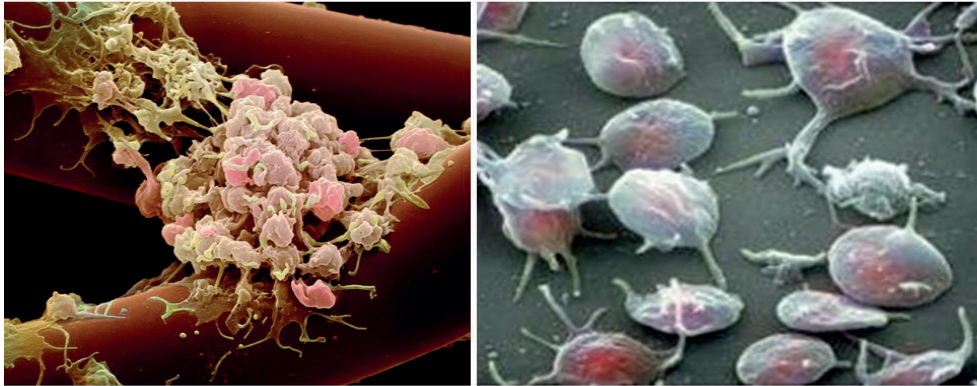
Figure 1. Left: Colored scanning electron micrograph (2000× magnification) of activated platelets (pink/grey) on a cellular filter (brown). Mediators from activated platelets convert soluble fibrinogen to the insoluble protein fibrin. The resulting fibrin mesh traps platelets and red and white blood cells, forming a plug that seals the damaged vessel. Right: Activated platelets become "sticky" and form pseudopodia

Vasospasm.

Damaged blood vessels constrict. This vascular spasm is the blood vessel's first response to injury, which reduces the amount of blood flow through the area and limits the amount of blood loss. This response is triggered by direct injury to vascular smooth muscle and vasoconstrictors released from activated platelets that are adherent to the subendothelial structures. The spasm response becomes more effective as the amount of damage increases.

Platelet plug formation

When the vessel is damaged, collagen from the subendothelial tissue is exposed to blood. This activates platelets that bind to the exposed collagen both directly and indirectly via von Willebrand factor (vWF), which itself has collagen binding ability. The platelets roll onto the damaged endothelium and bind to vWF and collagen via specific platelet receptors, ultimately covering the exposed subendothelial tissue—a phenomenon called platelet adhesion (Figures 1 and 2).

As platelets adhere to the collagen fibers, they become activated, turn "sticky", and form pseudopodia (Figures 1 and 2).

Activated platelets express glycoprotein receptors on their surface as well as emptying alpha-granulae and dense bodies. Alpha granulae release vWF, FV, FXIII, and fibrinogen, and dense bodies release tromboxan A2, serotonin, and adenosine diphosphate (ADP). The release of these chemical messengers causes more platelets to stick to the area and release their contents and enhances vascular spasm. As more chemicals are released, more platelets stick and release their chemicals, which creates a platelet plug and continues the process via a positive feedback loop, called platelet aggregation. Platelets then bind together via glycoprotein (GP) IIb/IIIa and fibrinogen (Figures 1 and 2), and the platelet membrane is inverted to create a negatively charged phospholipid surface on which coagulation continues to progress. Leukocytes also interact with platelet adhesion and aggregation [11].
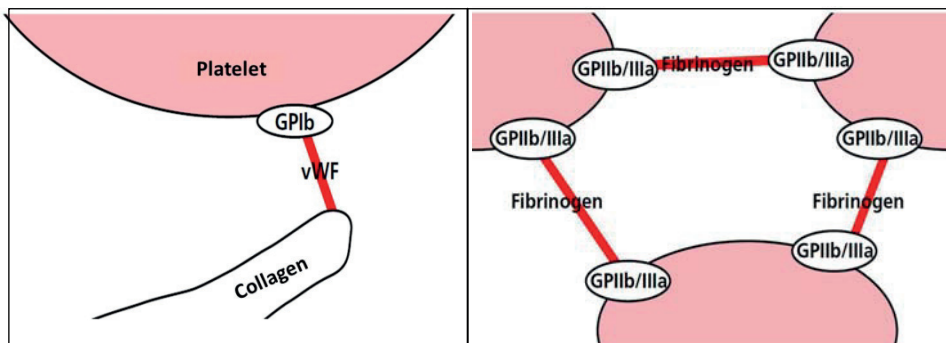
Figure 2. Left: Platelets adhere to collagen in the vessel wall. vWF = von Willenbrand factor. Right: Platelets form a plug by binding together with fibrinogen. GP = glycoprotein. (Figure reproduced with permission from authors of "Hemostas vid allvarlig blödning" [7])

Coagulation

Coagulation, the final step in this hemostatic rapid response system, reinforces the platelet plug with stabile fibrin strands. This is achieved through activation of coagulation factors, which are pro-enzymes produced in the liver. The activation of coagulation factors occurs in a specific order close to the damaged endothelium in an *initiation phase* and continues on the negatively charged surface of the activated platelets in an *amplification and propagation phase*. This coagulation process is offset by natural anticoagulation processes.

Initiation phase
Tissue factor (TF), also known as FIII, is a cell-bound transmembrane glycoprotein that is constitutively expressed by fibroblasts and smooth muscle

cells of the vessel wall. When the vessel wall is injured, the expression of TF induces the activation of FVII to FVIIa. The coagulation factor complex FVIIa/TF then activates other coagulation factors and finally leads to the activation of a small amount of thrombin (Figure 3).
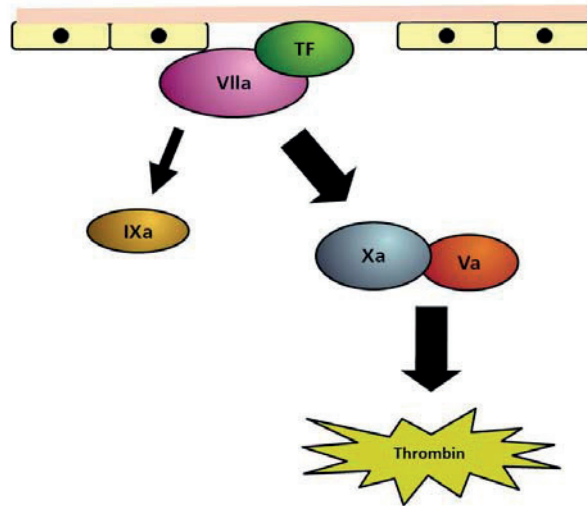
Figure 3. Initiation phase. Activation of coagulation factors by injured endothelium leading to the activation of a small amount of thrombin. TF = tissue factor. (Figure reproduced with permission from authors of "Hemostas vid allvarlig blödning" [7]).

Amplification and propagation phase

The small amount of thrombin generated during the initiation phase activates several coagulation factors, which in the presence of calcium ions cause a massive activation of prothrombin to potent thrombin (i.e., thrombin burst) via a positive feedback loop (Figure 4). Thrombin converts fibrinogen to soluble fibrin that stabilizes the platelet plug. Soluble fibrin has no ability to form the network that is essential for a stable clot. Through the actions of FXIII, soluble fibrin strands bind together by covalent bonds to form a stable network.

The amplification phase occurs mainly on activated platelets, although it may also occur on activated endothelium and other cells.
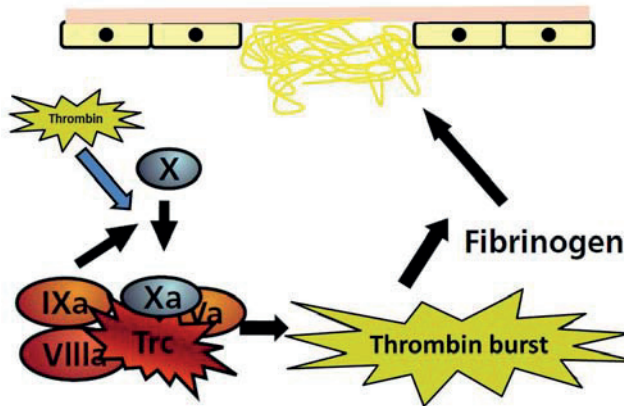
Figure 4. Amplification and propagation phase. The small amount of thrombin generated during the initiation phase activates (blue arrow) several coagulation factors and causes a thrombin burst. (Figure reproduced with permission from authors of "Hemostas vid allvarlig blödning" [7]).

Natural inhibitors of hemostasis

The pro-hemostatic processes described above are offset by natural inhibitors of hemostasis that can be divided into inhibitors of platelet activation and inhibitors of coagulation.

Inhibitors of platelet activation

Platelet plug formation stops when it reaches the intact endothelium due to the presence of prostacyclin, heparan sulfate, and nitrogen monoxide, which prevent activation of platelets (Figure 5).
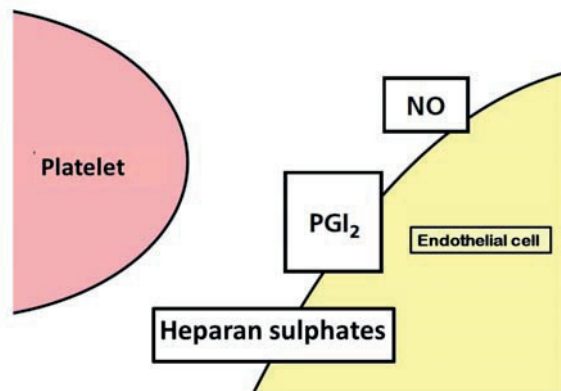
Figure 5. Platelet aggregation is stopped when platelets come into contact with intact endothelium expressing nitrogen monoxide (NO), prostacyclin (PGI2), and negatively charged heparan sulphates. (Figure reproduced with permission from authors of "Hemostas vid allvarlig blödning"[7]).

Inhibitors of coagulation

Natural inhibitors of coagulation limit the coagulation cascade to the injured vessel wall and the activated platelets in the wound. The most important inhibitors are antithrombin, protein C, and protein S (Figure 6).

Antithrombin inactivates thrombin, FXa, and other clotting enzymes in a reaction that is accelerated by heparin. *In vivo*, vessel wall heparan sulfate may substitute for medicinal heparin as an antithrombin activator.

Thrombomodulin, expressed on the surface of endothelial cells, serves as a receptor for thrombin. The thrombomodulin-thrombin complex activates endothelial-bound protein C in the presence of protein S as a cofactor. The activated protein C, and its cofactor protein S, inactivates FVa and FVIIIa, blocking amplification of the coagulation system and thereby limiting further thrombin formation.
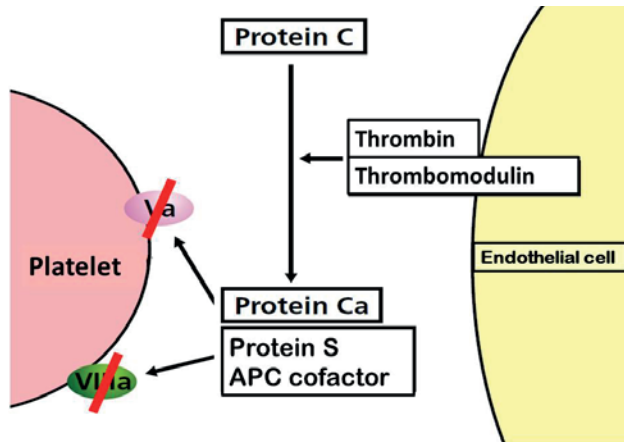
Figure 6. Inactivation of coagulation factors by protein C. APC = activated protein C. (Figure reproduced with permission from authors of "Hemostas vid allvarlig blödning" [7]).

Fibrinolysis

Fibrinolysis is an enzymatic dissolution of blood clots. When there is a balance between fibrin formation and fibrinolysis, the vasculature is protected from blood loss at the injured area, and blood flow through the vessels is preserved. The most important factor of the fibrinolytic system is plasminogen. Plasminogen is produced in the liver and binds to the surface of clots, where it is activated to plasmin by plasminogen activator from the endothelium or urinokinas-type plasminogen activator. Plasmin cuts the fibrin mesh at various places, leading to the production of fibrin degradation products that are cleared by other proteases or by the kidneys and the liver.

Fibrinolysis is offset by anti-fibrinolytic processes involving plasminogen activator inhibitor 1, which inactivates plasminogen activator.

Medications that convert plasminogen to plasmin are used to treat acute, life-threatening thrombotic disorders such as myocardial infarction and ischemic stroke.

Central venous catheters

Central venous catheters (CVCs) are mainly used for reliable infusion of fluids and potentially irritant drugs for assessment of central venous pressure and for hemodialysis. Bleeding complications are reported to range from 0.5 to 1.6% in connection with CVC insertion, but these are rarely fatal [12]. Fatal bleeding complications include hemothorax, artery dissection and airway compression [13].

Bleeding complications

Bleeding complications can be graded according to the Common Terminology Criteria for Adverse Events (Version 4.0, National Cancer Institute, USA) [14] (Table 1).

Table 1. Grading of bleeding complications.

Grade 0	No bleeding
Grade 1	Slight oozing from the insertion site not requiring any intervention
Grade 2	Mild symptoms (e.g., hematoma) at the insertion site requiring external compression
Grade 3	Severe symptoms requiring blood transfusion
Grade 4	Life-threatening bleeding

Platelet transfusion

Patients with bone marrow failure and severe thrombocytopenia are frequently given prophylactic platelet transfusion before invasive interventions. The threshold platelet count and the timing of transfusion remain controversial among clinical studies [15].

Platelets for transfusion can either be prepared from buffy coats from several donors (generally four) or by the apheresis technique from a single donor.

In contrast to other blood components, platelets have a limited shelf-life (5-7 days) for transfusion purposes because they must be stored at room temperature and are thus prone to microbial contamination. This method of storage is necessary because refrigeration of platelets, even for short periods, leads to their rapid clearance from the circulation upon transfusion [16]. For proper oxygenation and

carbon dioxide escape through the storage bag a gentle agitation of the bag is necessary [17].

Mild induced hypothermia

Mild induced hypothermia (MIH) is indicated for comatose survivors of out-of-hospital cardiac arrest (OHCA) to improve neurological outcomes [18-20]. However, in a recent multicenter study of OHCA patients (the Target Temperature Management trial) [21], a targeted temperature of 33°C did not confer a benefit compared with a targeted temperature of 36°C, a practice that challenges current guidelines.

Hemostasis in mild induced hypothermia

Conventional wisdom holds that hypothermia reduces coagulation and platelet function and impairs primary and secondary hemostasis. Whether this is also true during MIH is still debated [22]. A few animal studies support weakened markers of hemostasis during hypothermia [23-27], whereas others do not [28-30]. Several studies of blood from healthy volunteers incubated at different temperatures provide contradictory findings, with some showing that hypothermia decreases hemostasis [31-37], and others showing the opposite [38-43]. Studies including patients treated with MIH after OHCA are more infrequent. In two such studies [44, 45], thromboelastography analyses showed evidence of decreased coagulation with prolonged clot initiation during hypothermia.

These discrepant results may be explained by differences in methods used to study various aspects of hemostasis. Furthermore, it is necessary to separate effects on platelet function from effects on coagulation. Studies that focus on coagulation generally show weakened coagulation ability in mild hypothermia, whereas studies evaluating platelet function are more likely to show enhanced coagulation ability [23-46].

Platelet inhibitory drugs

Cardiac arrest patients often undergo emergency coronary interventions with a metal stent in one or several coronary arteries. This stent may stimulate platelet aggregation and patients must receive antiplatelet therapy during and after this procedure to avoid stent thrombosis. Current guidelines recommend dual antiplatelet therapy with aspirin and $P2Y_{12}$ inhibitors for 1- 6 months after stent

implantation [47]. See table 2 for pharmacokinetic specifications and principle mechanism of action.

Pharmacodynamics

Aspirin permanently inhibits cyclooxygenase and thereby blocks formation of prothrombotic thromboxane A2.

When the $P2Y_{12}$ receptor is stimulated by ADP, intracellular messenger systems in the platelet, including vasodilator-stimulated phosphoprotein (VASP) and cAMP, stimulate the expression of activated glycoprotein GPIIa/IIIb on the platelet surface. Thus, when the $P2Y_{12}$ receptor is blocked, the expression of GPIIa/IIIb decreases, and the activation by ADP is effectively blocked (Table 2).

		Table 2. *Properties of platelet inhibitors*			
Drug	**Mechanism of action**	**Administration**	**Active metabolites**	**Elimination**	**Platelet recovery time**
Aspirin	Irreversible cyclo-oxygenase enzyme inhibition	Oral	No	Liver, by deacetylation to salicylic acid	30% at 48 hours
Clopidogrel	Irreversible $P2Y_{12}$ receptor blockade by metabolite	Oral	Yes	Liver, involving CYP3A5/2CD19 to active metabolite	40% at 3 days
Prasugrel	Irreversible $P2Y_{12}$ receptor blockade by metabolite	Oral	Yes	Liver, by CYP3A4 to active metabolite	2-3 days
Ticagrelor	Reversible $P2Y_{12}$ receptor blockade	Oral	No	Liver	57% at 24 hours

It is still unclear how MIH influences the effect of platelet inhibitory drugs but several clinical studies have demonstrated decreased clopidogrel generated platelet inhibition during mild hypothermia [43, 48-50]. Two investigations in patients treated with clopidogrel and aspirin during MIH report a high incidence of stent thrombosis after percutaneous coronary intervention [50, 51]. However, at many

hospitals, clopidogrel has been replaced by ticagrelor because it is associated with better survival rates [52], probably due to its stronger and more predictable platelet inhibiting effect [53].

Regarding ticagrelor and hypothermia, a recent study [54] demonstrated a high rate of ticagrelor non-responders among patients treated with MIH. However, this effect may be related to insufficient intestinal absorption of the orally administered drug in comatose survivors and not an effect of hypothermia [55, 56].

Aims and methods

Paper	Aim	Methods	n
	Table 3. *Aims and methods*		
I	To map pre-procedural coagulation status, use of prophylactic blood components, technical problems encountered during central venous catheter insertion, and bleeding complications, and to identify independent risk factors for bleeding complications associated with central venous catheter insertion.	Retrospective register study	1,789 patients receiving central venous catheter
II	To measure coagulation enhancement in thrombocytopenic patients at 1 and 4 hours after platelet transfusion in connection with insertion of central venous catheter.	Conventional platelet and coagulation analyses ROTEM MultiPlate Flow cytometry	39 patients with bone marrow failure and platelet count $< 50 \times 10^9$
III	To assess hemostasis over time in comatose out-of-hospital cardiac arrest survivors and investigate the relationship between gastric emptying and the efficacy of oral platelet inhibiton.	Conventional platelet and coagulation analyses MultiPlate Sonoclot Vasodilator-stimulated phosphoprotein	23 patients treated with mild induced hypothermia at 33 C for 24 hours.
IV	To investigate the temperature-dependent effects on ROTEM (EXTEM, FIBTEM, and APTEM assays) using whole blood isolated from out-of-hospital cardiac arrest survivors.	ROTEM	10 patients treated with mild induced hypothermia at 33°C for 24 hours
V	To investigate the effect of *in vitro* applied hypo- and hyperthermia on ticagrelor- and aspirin-mediated platelet inhibition.	Conventional platelet and coagulation analyses MultiPlate Sonoclot Flow cytometry	15 patients with acute coronary syndrome and 8 healthy volunteers

Materials and methods

All studies were approved by the Regional Ethical Review Board, Lund, Sweden. Signed informed consent was received from all patients and/or their closest next-of-kin.

Measuring hemostasis

Conventional platelet and coagulation analyses

Platelet count

In general a low platelet count is associated with an increased risk of bleeding. It is a purely quantitative measure and cannot detect platelet dysfunction [57].

Prothrombin, (PT/INR)

PT/INR analysis, which has been in use in Sweden since 1999, is performed with instruments and reagents calibrated against international standards. INR (Owren) measures the sum of the vitamin K-dependent coagulation factors prothrombin (FII), FVII, and FX, but not FIX. INR is most sensitive to reductions in FVII, which has the shortest half-life (7 hours) of the measured factors.

Activated partial thromboplastin time (aPTT)

aPTT measures the sum of all coagulation factors except FVII and FXIII. The reference value is reagent-dependent and varies slightly for different laboratories.

Fibrinogen

Fibrinogen is an acute phase reactant that naturally increases after major surgery or trauma. Decreased values can be seen in conditions such as liver failure, ablatio placentae, trauma, and sepsis, along with consumption and increased fibrinolysis. Colloids, which are plasma volume expanders, may give falsely elevated values when analyzed by the commonly used optical Clauss method.

Viscoelastic tests

Viscoelastic tests asses the formation of blood clots and clot strength by measuring fibrin polymerization and interaction among platelets, fibrin, and fibrinolysis. Viscoelastic tests have been shown to reduce transfusions compared with conventional platelet and coagulation tests [58]. Although results can be obtained within 30 minutes, they are insensitive to warfarin and platelet inhibitory drugs

Thromboelastometry with ROTEM

The ROTEM® Delta (trademark of TEM International GmbH; www.rotem.de) [59] is a point-of-care analyzer that uses thromboelastometry, a viscoelastic method, to test hemostasis in whole blood. It is performed near the patient during surgery or when the patient is admitted following trauma. It is used to assist with the diagnosis, management and monitoring of hemostasis disorders during and after surgery associated with high blood loss during and after surgery. It is an integrated all-in-one system that analyzes the coagulation status of a blood sample (Figure 7) to differentiate between surgical bleeding and a hemostasis disorder. It uses a combination of five assays to characterize coagulation profile (Table 4), and the results are presented as a graph with defined parameters (Figures 8 and 9). The typical test temperature is 37°C, but different temperatures can be selected.
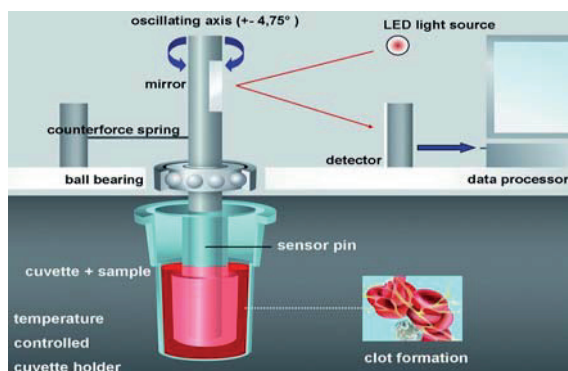
Figure 7. The ROTEM system. Citrated whole blood is placed into the disposable cuvette using an electronic pipette. The degree of restriction from the oscillating pin is transmitted via an optical detector system. The test is started by adding the reagents described above. While the blood remains liquid, the movement is unrestricted; as the blood starts clotting, the clot restricts the rotation of the pin with increasing resistance as the firmness of the clot increases. This is measured by the ROTEM system and translated to the output, which consist of graphical displays and numerical parameters.

Assay	Activator	Additives	Information
EXTEM	Tissue factor		Global test. Plasmatic coagulation factors, fibrin polymerization, platelet function and count.
FIBTEM	Tissue factor	Platelet inhibition, by cytochalacin D	Fibrin status. Identification of polymerization disorders or deficiency.
APTEM	Tissue factor	Fibrinolysis inhibition by aprotinin	Detection or exclusion of hyperfibrinolysis.
INTEM	Contact activator		Global test. Plasmatic coagulation factors, fibrin polymerization, platelet function and count.
HEPTEM	Contact activator	Heparin inactivation by heparinase	Screening test in the presence of heparinase.
NATEM			Global test without activator. High sensitivity. Time consuming

Table 4 Overview of ROTEM assays
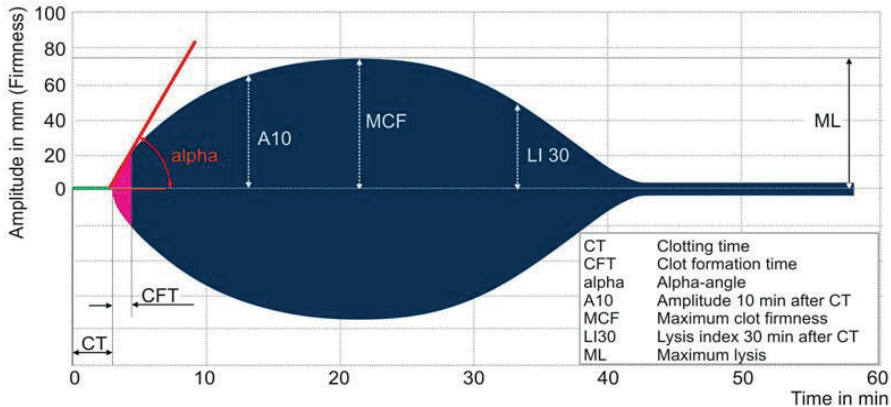
Figure 8. ROTEM parameters.

CT = Clotting time. The time from adding the start reagent until the blood starts to clot. Reflects initiation of clotting with thrombin and fibrin formation and the start of clot polymerization.

CFT = Clot formation time. The time after CT until a clot firmness of 20 mm is detected on the thromboelastogram. Reflects clot formation dynamics (clot propagation) and depends on fibrin polymerization, stabilization of the clot with platelets, and FXIII.

Alpha angle = The angle of the tangent of the curve. Alpha angle and CFT indicate the speed at which the clot is forming and are influenced by platelet function, fibrinogen and coagulation factors.

A10 = Amplitude 10 minutes after the start of clot formation. Used to predict MCF at an earlier stage.

MCF = Maximum clot firmness. The greatest vertical amplitude of the trace. A low MCF value suggests decreased platelet numbers/function or decreased fibrinogen levels/function.
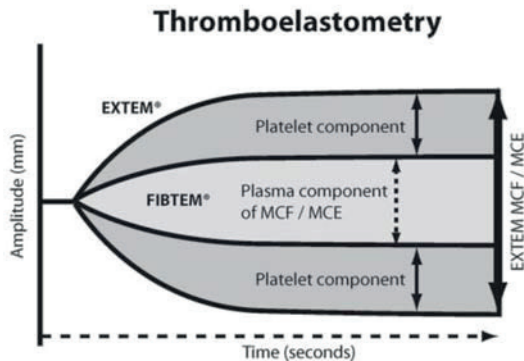
Figure 9. Diagram illustrating thromboelastometry with ROTEM. FIBTEM inhibits platelets and only measures the plasma component of clotformation whereas EXTEM provides a global measurment of coagulation ability.Figure from Lang et al. [60]; reused with permission from Lippincott Williams & Wilkins.

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Sonoclot Coagulation Analyzer

Another method that uses viscoelastometry to measure coagulation is the Sonoclot coagulation analyzer (Sienco Inc., Arvada, CO, USA) [61] which uses a temperature-regulated heating or cooling plate. It provides information on the hemostasis process including coagulation, fibrin gel formation and fibrinolysis. According to recently published guidelines the Sonoclot system is only recommended for use in research [58].

Whole blood is added to a cuvette containing the reagents. As with ROTEM, a probe moves within the sample; however, rather than rotating, the probe moves up and down along the vertical axis (Figure 10). As the sample starts to clot, changes in impedance to movement are measured. The time-based graph (Sonoclot signature) that is generated reflects different steps in the clotting of the whole blood sample with defined parameters (Figure 11).
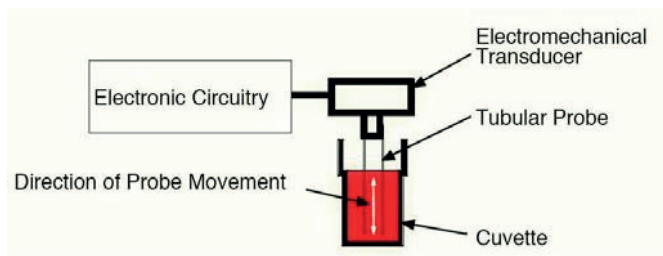
Figure 10. The Sonoclot Coagulation Analyzer.
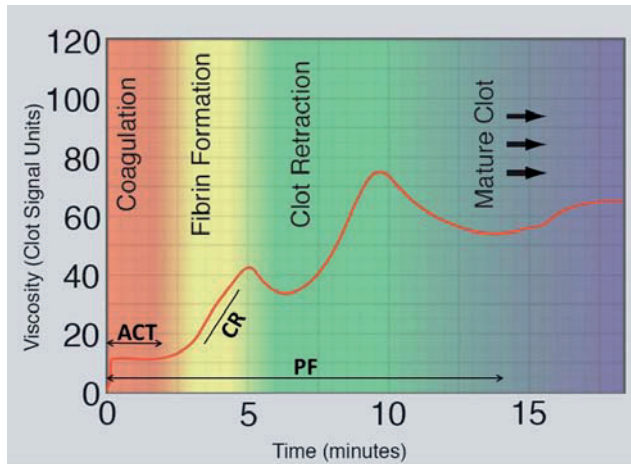
Figure 11. The Sonoclot signature.

ACT = Activated clotting time. The time required for the first fibrin to form. ACT corresponds to aPTT and traditional ACT tests.

CR = Clot rate. The rate of increase in the clot impedance due to fibrin formation and polymerization. The CR is the maximum slope of the Sonoclot signature during initial gel formation.

PF = Platelet function. A calculated parameter including, among others, the point at which the squeezing out of trapped serum in the contracting clot-clot retraction (sign of functioning platelets) exceeds the accumulation of clot bulk on the probe.

Multiple electrode aggregometry

Aggregometry measures the degree of platelet aggregation after adding platelet activating agonists to whole blood or platelet-rich plasma. The original method is Born light transmission aggregometry which is based on the principle that aggregated platelets in platelet-rich plasma, absorb less light than non-aggregated platelets. In the multiple electrode impedance aggregometry analyzer (Multiplate; Roche Diagnostics, Basel, Switzerland) [62, 63], analysis of whole blood takes place in a single-use test cell, which incorporates two dual electrodes and a coated stirring magnet, (Figure 12). When platelets are activated by various platelet agonists (see below) they stick to the electrodes and enhance the electrical impedance between the electrodes. The impedance is plotted as a function of time. The area under the curve is proportional to the degree of platelet aggregation. The analysis temperature can be changed.

Examples of platelet agonist for the Multiplate instrument are:

- ADPtest (platelet aggregation in response to ADP, 6.5 µM).

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- COLtest (platelet aggregation in response to collagen, 3.2 µg/ml).

- TRAPtest (platelet aggregation in response to thrombin receptor agonist peptide, 32 µM).

- ASPItest (platelet aggregation in response to arachidonic acid, 0.5 mM).
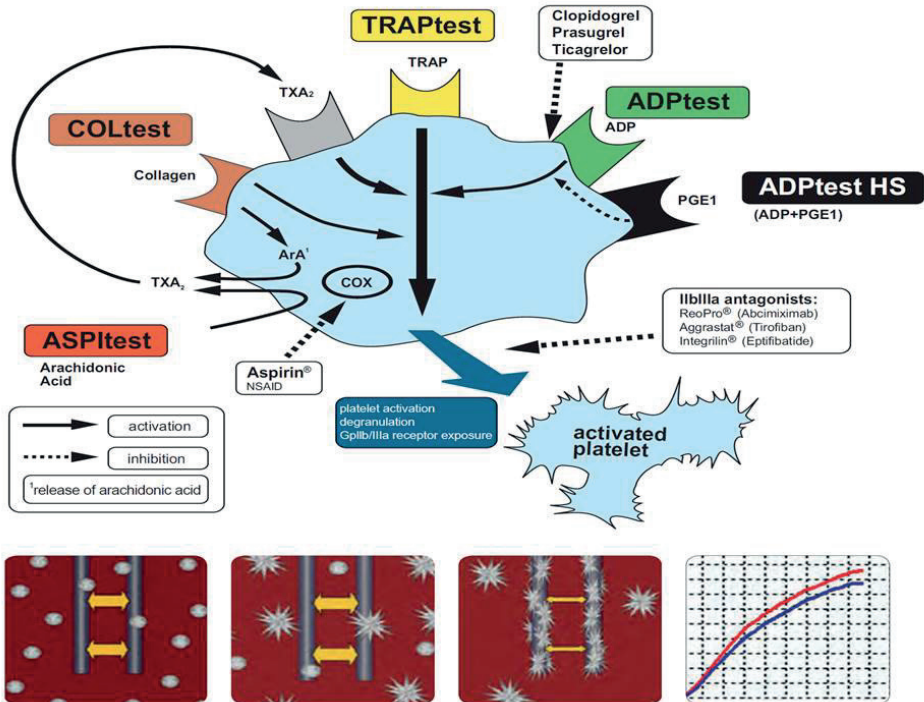
Figure 12. Schematic illustration of how different platelet agonists activate platelets and stick to the electrodes in the test cell of the Multiplate instrument and give rise to increased impedance, which is plotted over time in a graph.

Flow cytometry

Flow cytometry is a technique for examining microscopic particles, such as platelets, through their suspension in a stream of fluid passed by an electronic detection instrument. When analyzing platelets, the sample is usually spiked with a platelet agonist and then analyzed in the flow cytometer after adding fluorescent antibodies specific for proteins on the surface of the activated platelets. A laser beam of a single wavelength is directed through the stream of fluid. Several

detectors are aimed at the point where the stream passes through the sample and register the absorbed light, which is proportional to the number of positive cells (i.e., activated platelets).

Agonists similar to those used in the Multiplate test can be used to activate platelets before flow cytometry analysis.

The procaspase activating compound 1 antibody (PAC-1) is used for measuring the activation of membrane bound GPIIb/IIIa [64].

Following platelet activation, α-granules exocytose and release the unique P-selectin protein CD62P, which becomes expressed on the platelet surface and can be detected with fluorescent antibodies in the flow cytometry instrument [65].

Vasodilator stimulated phosphoprotein (VASP)

VASP is an intra-cellular protein that exists both in a phosphorylated (VASP-P) and a dephosphorylated (VASP) state in platelets. When ADP activates the $P2Y_{12}$ receptor, VASP-P is dephosphorylated to VASP. If the $P2Y_{12}$ receptor is blocked the dephosphorylation process is inhibited thus increasing VASP-P concentration and decreasing VASP concentration.

VASP and VASP-P can be measured by flow cytometry, and the ratio between VASP and VASP-P is used to calculate the platelet reactivity index (PRI), which reflects the effect of $P2Y_{12}$ inhibitory drugs. A low value represents robust inhibition of $P2Y_{12}$ receptors. According to previous studies, a PRI <50% is regarded as a satisfactory effect of $P2Y_{12}$ inhibitors, a PRI ≥50% is regarded as a unsatisfactory effect of $P2Y_{12}$ inhibitors [66], and a PRI ≥70% is considered a normal value for untreated patients.

Paper I

In this retrospective study, 1737 consecutive insertions of CVCs in 1444 patients were investigated. Pre-procedural coagulation status, blood component use, type of catheter, insertion site and complications during insertion were recorded and compared with bleeding complications documented on electronic charts.

Paper II

Thirty nine adult patients with bone marrow failure and platelet count below 50 x 10^9/L were consecutively enrolled before prophylactic platelet transfusion for

subclavian central venous catheter insertion. Blood samples were drawn from the patients before platelet transfusion, 1 hour, and 4 hours after completion of the transfusion. The coagulation profile was assessed according to table 3. Bleeding complications were classified with a 4-grade scale, according to the Common Terminology Criteria for Adverse Events (Table 1).

Paper III

Twenty-three comatose survivors of OHCA were divided in two groups depending on whether or not dual platelet inhibition with peroral ticagrelor and aspirin was administered. The first blood samples (T1) were collected 12-24 hours after reaching target temperature (33°C) and were compared to blood samples collected 12-28 hours after reaching normothermia (37°C) (T2) within each group (Figure 13). All samples were analyzed with the instruments set to *in vivo* temperature. Gastric secretion from the nasogastric tube was measured to assess absorption of orally administered antiplatelet drugs. Differences between T1 and T2 within each group as well as the relationship between gastric secretion and VASP were calculated.
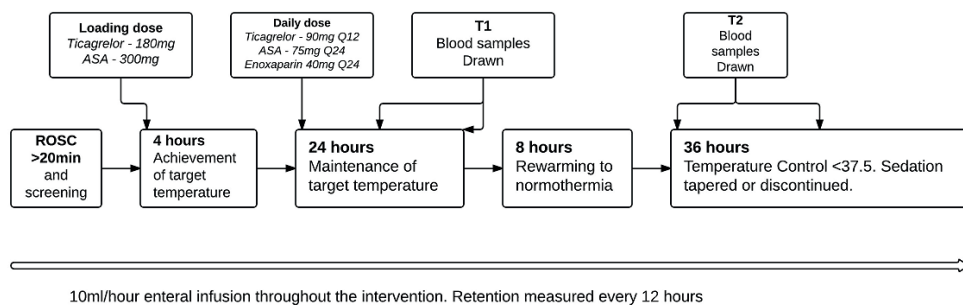
Figure 13. Flowchart. OHCA = Out-of-hospital cardiac arrest. MIH = Mild induced hypothermia. Loading and daily doses of ticacrelor and aspirin at the clinicians' discretion (n=14). Nine patients did not receive ticagrelor and aspirin. All patients received enoxaparin. T1 blood samples were taken 12-24 hours after reaching hypothermia. T2 blood samples were taken 16-28 hours after reaching normothermia.

Paper IV

Ten patients with OHCA who underwent induced hypothermia were studied during stable hypothermia at 33°C. ROTEM temperature effects with EXTEM, FIBTEM and APTEM assays were studied at temperatures between 30 and 42°C.

Paper V

Whole blood samples from 15 patients with acute coronary syndrome who were treated with ticagrelor and aspirin and eight healthy volunteers were incubated for 1 hour at 28, 33, 37, and 39°C and analyzed for platelet activation and clotting ability using the tests specified in table 3.

Statistics

Paper I

To identify risk factors for bleeding complications, Fisher's exact tests were used to analyze single independent variables for their association with bleeding complications. Due to the complex interdependence of the independent variables, a multivariate logistic regression was performed. The data in Paper I were analyzed using R Core Team (2012; R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/).

Paper II-V

Distribution measures

Results from conventional platelet and coagulation tests (platelet count, PT/INR, aPTT, and fibrinogen) and VASP were considered parametric (Gaussian distribution assumed) and were summarized using the mean and standard deviation as distribution measures.

Results from viscoelastic testing (ROTEM and Sonoclot), Multiplate testing, and flow cytometry were considered non-parametric (Gaussian distribution not

assumed) and were summarized using the median and range (min-max or 25-75th percentiles) as distribution measures.

Hypothesis testing

Differences between groups were calculated using two-tailed paired t-tests for means, two-tailed Wilcoxon matched-pairs signed-ranks tests for medians, and Fischer's exact tests for categorical variables.

To reduce the risk of type I error due to multiple comparisons, p-values were adjusted using Bonferroni corrections in Paper III and V.

In Paper II and III, correlation coefficients were calculated using Spearman's rank correlation coefficients.

Software

All statistical analyses were performed using GraphPad Prism (version 6.02 for Windows, GraphPad Software, La Jolla, CA, USA).

Databases containing data from all papers were created in Microsoft Excel (2010, version 14.0) or Microsoft Access (2010, version 14.07; Microsoft Corp., Redmond, WA, USA).

Results and comments

Paper I

No serious bleeding complications were associated with the insertion of CVCs. Sixteen of 1,769 (0.9%) insertions caused grade 2 bleeding, defined as bleeding requiring prolonged compression at the insertion site. Insertion of a large bore central dialysis catheter was found to be an independent risk factor for bleeding complications. Neither conventional coagulation tests nor accidental arterial puncture or the number of needle passes predicted bleeding complications (Table 4).

This retrospective study of non-intensive care unit patients showed that serious bleeding complications in association with central line insertions are uncommon and suggests that insertion of a large bore catheter is an independent risk factor for mild bleeding complications in this population.

Table 4. Risk factors for grade 2 bleeding complications.			
CATEGORY	**Total, n**	**Grade 2 bleeding complication, n (%)**	**p-value**
Coagulopathic patients*	204	2 (0.9%)	p = 0.47
Non-coagulopathic patients	634	9 (1.4%)	
CDC	88	7 (7.9%)	p < 0.0001
Non-CDC	1571	12 (0.8%)	
Arterial puncture	46	2 (4.3%)	p = 0.77
Non-arterial puncture	1691	15 (0.9%)	
Ultrasound guidance	859	11 (1.3%)	p = 0.14
No ultrasound guidance	878	5 (0.5%)	
Needle passes ≤ 2	973	9 (0.9%)	p = 0.094
Needle passes ≥ 3	49	2 (4.1%)	

Table 4. Risk factors for grade 2 bleeding complications according to univariate analysis (Fisher's exact tests). *Coagulaopathic patients were defined as those having a platelet count $< 50 \times 10^9$/L, PTT > 45 s, or PT > 1.8. The study included only cases for which the results of complete routine coagulation tests were available before insertion. CDC = central dialysis catheter.

Paper II

Seventeen women and 22 men were included in the study. The main results are presented in Figure 14. Platelet count increased by 74% 1 hour post-transfusion (p < 0.001) and remained elevated 4 hours post-transfusion. Maximal clot firmness EXTEM (MCF_{EXTEM}) increased by 21% 1 hour post-transfusion (p < 0.001) and remained elevated 4 hours post-transfusion. Clotting time EXTEM (CT_{EXTEM}) decreased by 10% 1 hour post-transfusion (p < 0.001) and remained low 4 hours post-transfusion. FIBTEM values were unchanged after transfusion. All Multiplate measures were significantly increased 1 and 4 hours post-transfusion. Four grade 1 bleeding episodes but no grade 2-5 bleeding episodes occurred. Flow cytometry analyses showed mixed results with no overall trends.

These results indicate that prophylactic platelet transfusions in thrombocytopenic patients with bone marrow failure improved hemostatic ROTEM and Multiplate parameters by increasing the number of platelets but not by enhancing platelet function. Improved ROTEM clotting parameters and Multiplate platelet aggregation appeared to persist between 1 and 4 hours after transfusion.
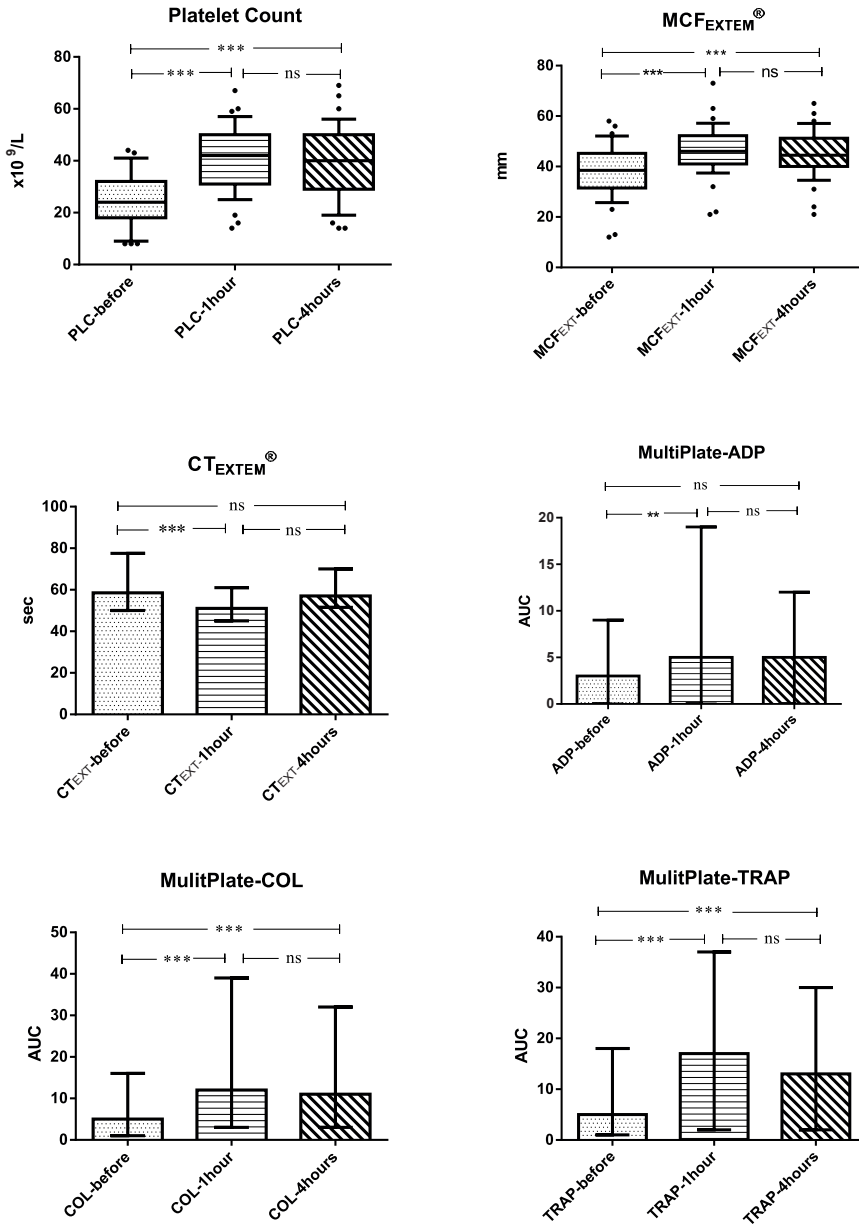
Figure 14. Platelet count, ROTEM and Multiplate test results. MCF = maximal clot firmness. CT = clotting time. ADP = adenosine diphosphate agonist. COL = collagen agonist. TRAP = thrombin agonist. AUC = area under the curve. Before, 1 hour, and 4 hours after completion of platelet transfusion. Bar graphs show median and interquartile range. **p < 0.01, ***p < 0.001.

Paper III

Patients with dual platelet inhibition (n = 14): Multiplate tests showed no changes in ADP-stimulated platelets. COL, TRAP, and ASPI aggregations were higher at T2 than at T1. Sonoclot tests showed that ACT was unchanged, but both clot rate and platelet function were higher at T2 than at T1 (Figure 15). Fifty percent of patients on oral ticagrelor did not reach the target VASP PRI of < 50% at the first sampling 12-24 hours after reaching hypothermia. VASP decreased from 53 ± 28 at T1 to 24 ± 22 at T2 ($p < 0.001$). The average volume of gastric secretion aspirated before T1 was highly correlated with VASP at T1 ($r = 0.81$, $p < 0.001$) (Figure 16).

Patients with no platelet inhibition (n = 9): Similar changes between T1 and T2 were seen in patients with no dual platelet inhibition, although VASP did not change between T1 and T2.

This paper demonstrates increased platelet aggregation and strengthened clot formation over time in OHCA patients treated with hypothermia. In patients with dual platelet inhibition, including those treated with ticagrelor, this effect was offset by powerful $P2Y_{12}$ blockade as confirmed by VASP analysis. The effect of ticagrelor was delayed in survivors of cardiac arrest, probably due to slow gastric emptying.
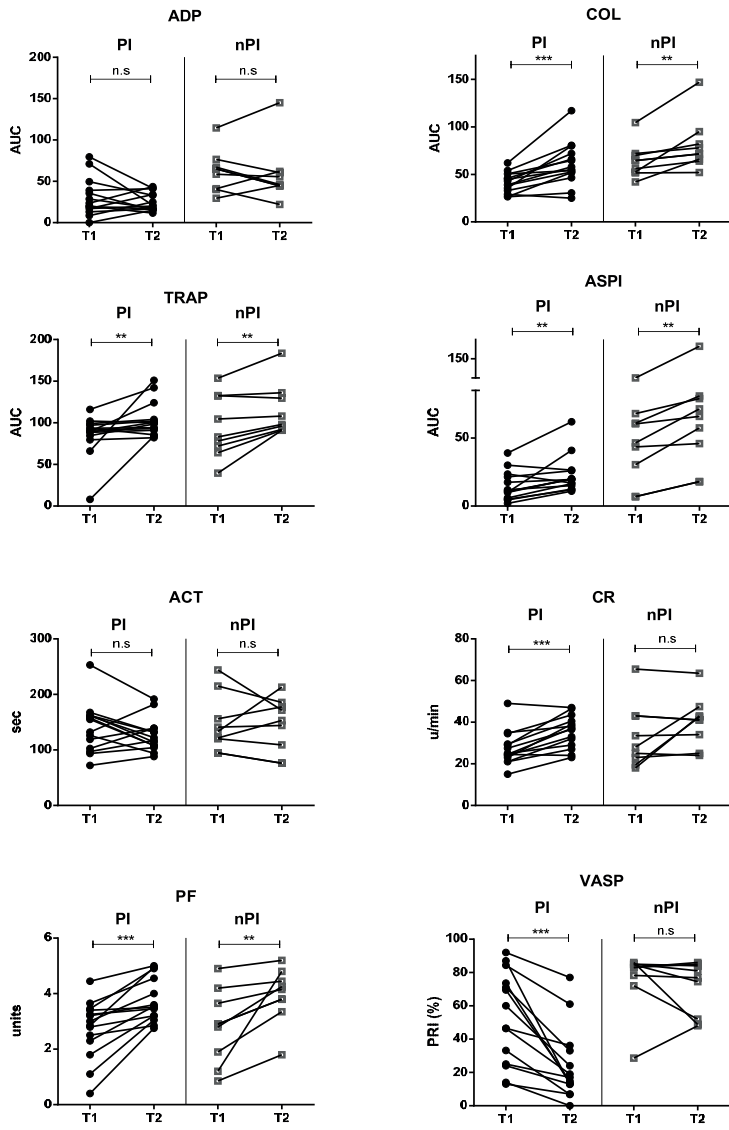
Figure 15. Results of blood analysis for individual patients. PI = Patients with dual platelet inhibition, n=14. nPI = Patients with no platelet inhibition, n=9. T1 = Blood sampling 12-24 hours after reaching 33°C body temperature. T2 = Blood sampling 16-28 hours after reaching normothermia. Multiple electrode aggregometry and Multiplate tests: ADP = adenosine diphosphate agonist, COL = collagen agonist, TRAP = thrombin agonist, ASPI = arachidonic acid agonist. Sonoclot tests, ACT = activated clotting time, CR = clot rate, PF = platelet function. AUC = area under the curve. Multiplate and Sonoclot instruments were set to *in vivo* temperature. **p < 0.01, ***p < 0.001.
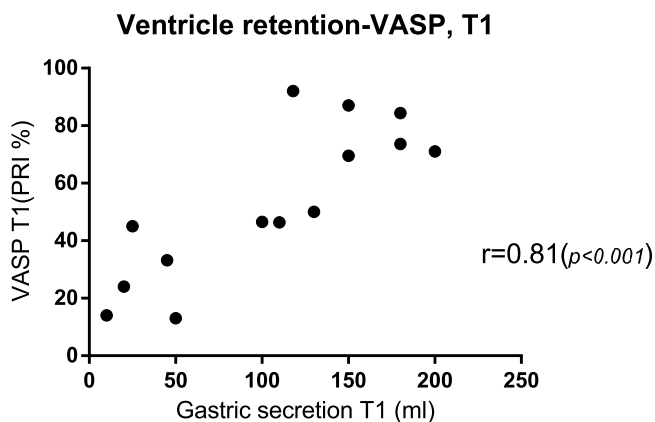
Figure 16. Correlation between aspirated gastric secretion and VASP (vasodilator-stimulated phosphorylated phosphoprotein). Patients with dual platelet inhibition, n = 14. Gastric secretion T1 = the median volume of gastric secretion aspirated from the nasogastric tube 0-24 hours after reaching 33°C body temperature. VASP T1 = VASP 12-24 hours after reaching 33°C body temperature. PRI = platelet reactivity index.

Paper IV

Results are presented in Figure 17. CT_{EXTEM} and CT_{APTEM} were prolonged by *in vitro* applied hypothermia at 30 and 33°C, whereas hyperthermia at 42°C shortened both CT_{EXTEM} and CT_{APTEM}.

Clot formation time $(CFT)_{EXTEM}$ and CFT_{APTEM} were prolonged by hypothermia at 30, 33, and 35°C and shortened by hyperthermia at 40 and 42°C.

α-angle$_{EXTEM}$ and α-angle$_{APTEM}$ was decreased at 30, 33, and 35°C but increased at 40 and 42°C.

MCF did not change depending on the temperature for EXTEM, APTEM and FIBTEM.

This paper demonstrates that ROTEM (EXTEM and APTEM assays) reveal a hypocoagulative response to *in vitro* applied hypothermia in the blood of cardiac arrest patients, reflected by prolonged clot initiation and decreased clot propagation. Hyperthermia had the opposite effects. Clot firmness was not affected by temperature.
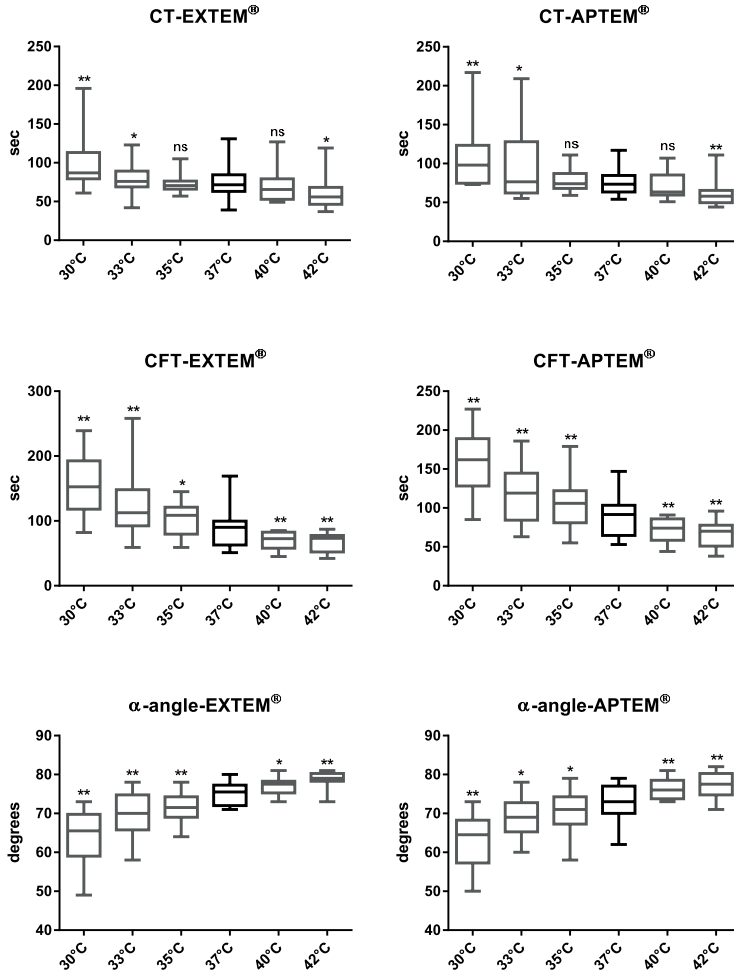
Figure 17. ROTEM (EXTEM and APTEM assays) on whole blood from cardiac arrest patients under stable hypothermia. Blood was incubated at 30, 33, 35, 37, 40, or 42°C prior to analysis. Analyses were performed with the ROTEM instrument set to the incubation temperature. CT = clotting time. CFT = clotting formation time. Boxplots show the median and interquartile range with min-max whiskers. $*p < 0.05$, $**p < 0.001$.

Paper V

The most important results are presented in Figures 18 and 19. In blood from patients with acute coronary syndrome, Sonoclot ACT was prolonged in mild

hypothermic (33°C) compared with normothermic (37°C) samples. Sonoclot clot rate and platelet function were decreased in hypothermic compared with normothermic samples. Platelet-induced activation and aggregation (Multiplate) were similar between mild hypothermic and normothermic samples. By contrast, mild hypothermia increased platelet activation as measured by flow cytometry, with up-regulation of PAC-1 and P-selectin on the platelet surface.

In acute coronary syndrome patients treated with ticagrelor and aspirin, *in vitro* hypothermia to 33°C markedly increased platelet activity as measured by flow cytometry, whereas Sonoclot tests revealed a hypocoagulative response to hypothermia. These results are in agreement with previous investigations reporting weakened coagulation ability and strengthened platelet function in response to mild hypothermia [23-46].
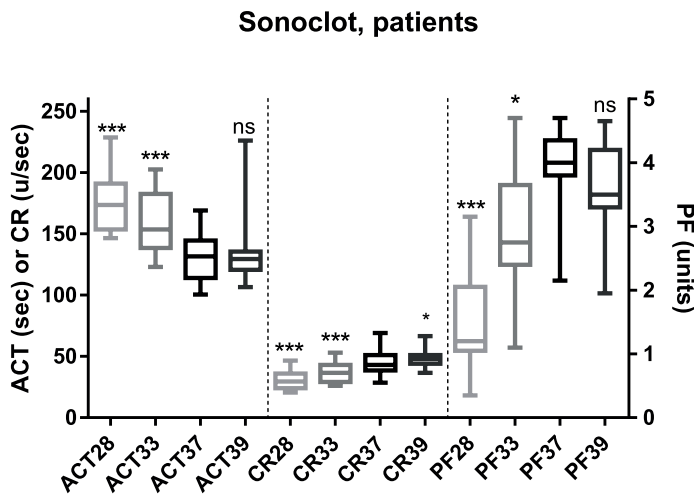
Figure 18. Sonoclot analysis of blood samples from patients with acute coronary syndrome. Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C and analyzed with the instrument set to the corresponding temperature as indicated by the numbers on the x-axis. ACT = activated clotting time. CR = clotting rate. PF = platelet function. The 28, 33, and 39°C samples were compared with the normothermic blood sample (37°C) using two-tailed Wilcoxon matched-pairs signed-ranks tests. Boxplots show median and interquartile range with min-max whiskers. ns = not significant, *p < 0.017, ***p < 0.001.
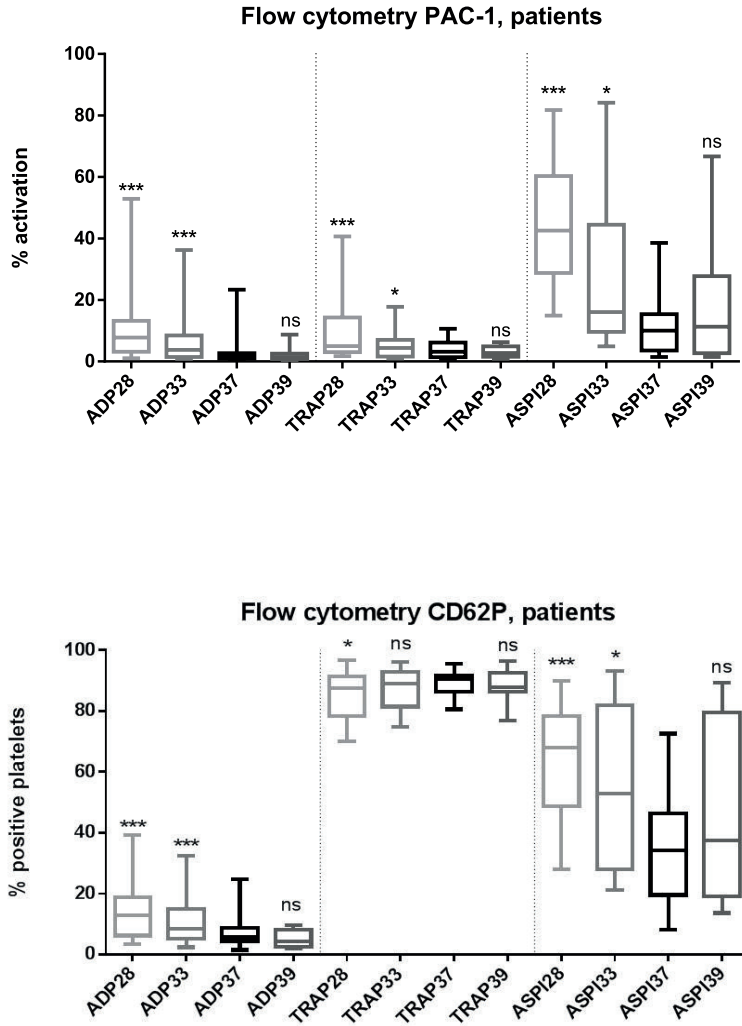
Figure 19. Results from flow cytometry analysis with PAC-1 (indicates activation of GPIIb-IIIa) or CD62P (indicates activation of P-selectin) in blood samples from patients with acute coronary syndrome. Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C prior to analysis as indicated by the numbers on the x-axis. ADP = adenosine diphosphate agonist. TRAP = thrombin agonist. ASPI = arachidonic acid agonist. The 28, 33, and 39°C samples were compared to the normothermic blood sample (37°C) using two-tailed Wilcoxon matched-pairs signed-ranks tests. Boxplots show median and interquartile range with min-max whiskers. ns = non-significant, *p < 0.017, ***p < 0.001.

Discussion

Central venous catheters and bleeding complications

Paper I shows that serious bleeding complications in association with central line insertions are uncommon and that insertion of a large bore catheter may be an independent risk factor for mild bleeding complications.

Although bleeding complications during insertions of CVCs are uncommon (0.5-1.6%) [12], they may be dangerous or even fatal. Paper I presents a safe concept for CVC insertion, including a trigger point of 50×10^9 platelets/L for platelet transfusions. Nevertheless, some patients are probably still given unnecessary plasma or platelet transfusions with the risk, albeit small, of infections, transfusion-related immunomodulatory effects (TRIM), allergic reactions, transfusion-related acute lung injury (TRALI), and transfusion associated circulatory overload (TACO).

The optimal threshold for platelet transfusion before insertion of CVCs is still debated and it is unclear whether prophylactic platelet transfusion before an intervention is superior to a strategy of therapeutic transfusion only [15, 67-71]. Although there is some evidence for the effectiveness of prophylactic platelet transfusion in terms of threshold for bleeding and dose for hemostasis [72-75] these studies did not evaluate platelet transfusions before intervention but rather prophylactic platelet transfusions in leukemia patients when platelet count was < 10×10^9/L.

To avoid uncommon but sometimes dangerous bleeding complications when inserting CVCs in patients with hemostatic disturbances several factors should be considered. The site of insertion is of major importance. If the bleeding site is compressible (e.g., the internal or external jugular vein), the demand on normal hemostasis is reduced compared with non-compressible bleeding sites (e.g., the sublcavian vein). Other factors of proven importance are the caliber of the inserted catheter, the experience of the operator, and the use of real-time ultrasound guidance [13, 76]. Taken together, prospective studies are needed to define optimal threshold values to trigger hemostatic improvement measures before insertion of a CVC in coagulopathic patients.

Efficacy of platelet transfusions

Paper II describes the efficacy of platelet transfusion in thrombocytopenic patients with bone marrow failure and comprehensively evaluates the time-dependence of platelet function and count after transfusion.

It seems obvious that platelet transfusion enhances both primary and secondary hemostasis by raising the number of platelets. However, some studies indicate that platelet function is diminished in patients with hematological diseases, [77-79] and that the enhanced coagulation observed after platelet transfusions can only partly be explained by an increase in platelet count [80].

In Paper II, the efficacy of platelet transfusion was measured before as well as 1 and 4 hours after transfusion, using platelet count, thromboelastometry (ROTEM; EXTEM and FIBTEM), multiple electrode aggregometry (Multiplate; ADP, COL and TRAP), and flow cytometry. Thromboelastometry was chosen as a reference because this method was previously used, although not over time, in a similar study [81]. Multiplate testing was chosen because this method measures platelet function. However, both thromboelastometry and Multiplate tests are dependent on platelet count [82-85]. Therefore, increased clot stability measured with thromboelastometry and increased platelet aggregation measured with Multiplate in blood from thrombocytopenic patients could be due to either improved platelet function, increased platelet count, or both. To address this, we also performed flow cytometry which assessed the functional status of individual platelets independent of platelet count.

The results of Paper II demonstrate that an increase in hemostasis after transfusion is not explained by better functioning platelets but rather by an increase in platelet count.

When evaluating coagulation using thromboelastometry (ROTEM), we found that MCF_{EXTEM} increased 1 hour after transfusion and persisted for 4 hours. Platelet transfusion also accelerated clot formation measured by CFT_{EXTEM}. This finding is in agreement with Larsen et al. [83], who demonstrated that the maximum rate of clot formation is dependent on platelet count in thromboelastometry. Unexpectedly, the onset of tissue factor-induced coagulation (CT_{EXTEM}) also was shortened after platelet transfusion. CT_{EXTEM} is a sensitive parameter that detects the initiation of the coagulation cascade. One therapeutic unit of platelet transfusion contains approximately 100 ml plasma, which despite the low volume may partially explain the transient decrease in CT_{EXTEM} observed 1 hour after transfusion. Platelet aggregation in response to three different agonists in Multiplate testing also demonstrated a steady increase in aggregation throughout the entire time of observation. These results indicate that hemostatic effects of

platelet transfusion last at least 4 hours, thus suggesting that 4 hours may be an optimal window for performing an invasive procedure in patients with bone marrow failure.

The finding of no major bleeding complications suggests that the carefully designed clinical practice at our institution is safe. However, the study was not sufficiently powered to make a conclusive statement on this clinical end point. It would be of great importance to conduct a clinical randomized trial for thrombocytopenic patients with bleeding complications as an end point and two groups that either do or do not receive pre-procedural platelet transfusions.

Effects of mild hypothermia on hemostasis

As previously described in the Background section (p. 15) earlier studies on the effect of hypothermia on hemostasis are contradictory [23-46]. These varying results may be explained by differences in methods of studying platelet function and coagulation. Furthermore, many studies only investigated mild hypothermia, whereas others tested a broader spectrum of temperature ranges, including deep hypothermia.

However, many surgeons can testify that patients have a greater tendency to bleed during hypothermia than after rewarming, and the results of some studies also support that hypothermia increases perioperative blood loss. In a study by Schmied et al. [86], patients were randomly assigned to normothermia or mild hypothermia during elective primary hip arthroplasty. A reduction of just 1.6°C in core temperature increased blood loss by 500 ml (30%) and significantly increased the need for allogeneic transfusion. By contrast, another study of blood loss during hip arthroplasty failed to identify a temperature-dependence of blood loss [87]. The reason for these differing results between similar and apparently well conducted studies remains unclear, but it should be noticed that the temperature differences between the groups in the latter study was smaller than in the study by Schmied et al. Winkler et al. [88] confirmed in a randomized controlled trial that a decrease in core temperature of only 0.5°C increased blood loss by 200-300 ml in patients undergoing hip arthroplasty under spinal anesthesia. Furthermore, in 2008, Rajagopalan et al. [89] conducted a meta-analysis of 14 randomized controlled trials that compared normothermic patients with those who experienced mild hypothermia (34-36°C), which indicated that mild hypothermia increases blood loss by approximately 16%.

In a recent large randomized clinical trial, the Target Temperature Management trial (TTM) [21] 950 comatose survivors of OHCA were randomized to either 33

or 36°C body temperature conditions for 24 hours. This study, which was powered to detect differences in mortality and neurological outcome, concluded that 33°C did not confer a benefit compared with 36°C. However, the frequency of bleeding complications was also recorded for both groups, and interestingly, no differences in serious bleeding complications between groups were detected. This can be taken as evidence that MIH is safe with regard to bleeding complications. However, when aiming to detect hemostatic impairments during hypothermia, bleeding volume during surgery is probably more sensitive than the frequency of bleeding complications during MIH. Furthermore, this study was not powered to detect differences in bleeding complications, and there was a tendency toward significantly more bleeding complications from insertion sites in the 33°C group.

If hypothermia impairs hemostasis, several mechanisms are possible, such as impaired platelet function, reduced coagulation ability, and increased fibrinolysis. Each of these possible mechanisms will be discussed separately.

Impaired platelet function

Paper V showed markedly increased platelet activity measured by flow cytometry in response to *in vitro* applied hypothermia to 33°C on whole blood from acute coronary syndrome patients treated with ticagrelor and aspirin. This finding is in agreement with several other studies, in which *in vitro* incubation at mild hypothermia of whole blood from healthy volunteers resulted in increased platelet reactivity. Scharbert et al [40, 41] used Multiplate to exhibit increased platelet aggregability in response to *in vitro* applied mild hypothermia (33°C). These findings, however, were not confirmed by the Multiplate-results of Paper V. Högberg et al [38] found an increase in ADP-stimulated platelet aggregation, after temporary clopidogrel treatment, in hypothermic (33°C) compared with normothermic blood. Xavier et al [42] showed that cooling blood to 28°C increasesed platelet aggregation in whole blood, independent of the anticoagulant used. Ferreiro et al [43] used Multiplate testing to investigate the effect of *in vitro* applied hypothermia on blood from clopidogrel-treated patients; they concluded that mild therapeutic hypothermia is associated with impaired response to clopidogrel therapy.

Reduced coagulation ability

Conventional coagulation tests (i.e. PT/INR, aPTT, platelet count) seem to be unaffected by mild hypothermia when analyzed at normothermia. Rohrer et al. [90] analyzed PT/INR and aPTT at 37- 28°C temperatures and showed a progressively hypocoagulative response. Wohlberg et al. [36] performed similar

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experiments and only demonstrated a hypothermic effect at temperatures below 33°C. Using the Sonoclot instrument, Shimokawa et al. [26] demonstrated a significant hypocoagulative response when analyzed at the subject's own hypothermic body temperature.

Paper IV demonstrated delayed clot initiation and propagation at *in vitro*-applied MIH to 33°C as measured by thromboelastometry (ROTEM). This finding is in agreement with the results of previous studies [23, 24, 26, 33, 37, 44, 45] using other reagents.

Paper V investigated the effects of hypothermia using the Sonoclot viscoelastic test. The results, which agree with previous studies (see Paper IV), show that *in vitro* applied hypothermia increases time to clot initiation and impairs clot propagation.

Increased fibrinolysis

Preliminary data suggest that fibrinolysis remains normal during mild hypothermia, but is significantly increased during hyperthermia, suggesting that hypothermia-induced coagulopathy does not result from excessive clot lysis [91], which is in agreement with results from paper V.

Conclusion

Impaired platelet function does not seem to be the cause of the tendency for increased bleeding under mild hypothermia; instead, there is evidence to the contrary. Mild hypothermia increases time to clot initiation and impairs clot propagation in viscohemostatic tests. Also, increased fibrinolysis does not seem to be responsible for the tendency for increased bleeding observed under mild hypothermia.

Hemostasis in OHCA patients

In Paper III, platelet function and coagulation over time were measured in OHCA patients treated with MIH. This study did not include a normothermic control group, thus it cannot be evaluated whether the increased platelet aggregability and viscoelastic clot formation depends on hypothermia. Nevertheless, this study is important because it demonstrates that OHCA patients treated with MIH exhibit increased platelet aggregation and strengthened clot formation over time,

independently of the cause. Additionally, the effect of ticagrelor was delayed in survivors of cardiac arrest, probably due to slow gastric emptying, and 50% of patients on oral ticagrelor did not reach the target for platelet inhibition at the first sampling occasion 12-24 hours after reaching hypothermia, thus placing them at risk for thromboembolic events.

Measuring hemostasis

Measuring hemostasis is very complex, and different *in vitro* tests only reflect very limited aspects of how platelets adhere to damaged endothelium and how coagulation proceeds and ultimately leads to stable clot formation *in vivo*. Most *in vitro* tests are performed under static conditions without natural venous or arterial flow, and therefore complex interactions among erythrocytes, platelets, white cells, and coagulation factors under various shear rates cannot be evaluated. Recently, Ogawa et al [92, 93] reported the effects of flow conditions on hemodilution-induced changes in coagulation. The authors used a new commercialized automated microchip flow chamber technique called the Total Thrombus-formation Analysis System (T-TAS), which assesses coagulation processes in recalcified/corn trypsin-inhibited whole blood inside collagen/tissue factor-coated capillaries under arterial and venous flow conditions. This is an interesting technology that might approach *in vivo* conditions, at least regarding blood flow.

Also, the activated endothelium plays a vital role in *in vivo* hemostasis and is difficult to simulate in test systems outside the body.

Thus, neither our commonly used conventional coagulation tests nor point-of-care coagulation tests reflect the complexity of hemostasis *in vivo*. This does not mean that the tests are useless. Rather, we need to understand what they can and cannot tell us. Any laboratory test requires skilled interpretation and clinical correlations for evaluation of its results.

Key messages

- Serious bleeding complications in association with central line insertions are uncommon. *Paper I.*

- Insertion of a large bore central catheter may be an independent risk factor for mild bleeding complications. *Paper I.*

- Prophylactic platelet transfusions in thrombocytopenic patients with bone marrow failure improve hemostatic parameters in ROTEM and Multiplate tests by increasing the number of platelets and not by enhancing platelet function. *Paper II.*

- Platelet count and improved clotting parameters in ROTEM tests and platelet aggregation in Multiplate tests appear to persist between 1 and 4 hours after platelet transfusion. *Paper II.*

- Platelet aggregation and clot formation demonstrated by Multiplate and Sonoclot tests are strengthened over time in OHCA patients treated with hypothermia. *Paper III.*

- Fifty percent of patients on oral ticagrelor do not reach the target VASP PRI of < 50% at the first sampling occasion 12-24 hours after reaching hypothermia, thus placing them at risk for thromboembolic events. *Paper III.*

- The effect of ticagrelor is delayed in survivors of cardiac arrest, probably due to slow gastric emptying. *Paper III.*

- ROTEM testing shows a hypocoagulative response to *in vitro* applied hypothermia in the blood of cardiac arrest patients, reflected by prolonged clot initiation and decreased clot propagation. Hyperthermia has the opposite effects. Clot firmness is not affected by temperature. *Paper IV.*

- In acute coronary syndrome patients treated with ticagrelor and aspirin, *in vitro* hypothermia to 33°C markedly increases platelet activity measured by flow cytometry, whereas viscoelastic coagulation testing (Sonoclot) shows a hypocoagulative response. *Paper V.*

Sammanfattning på svenska/Summary in Swedish

Blodlevring

Blodlevring är en samlingsterm för en serie reaktioner som medför att blod omvandlas från vätska till en geléliknande massa. I koagulationsprocessen samverkar de minsta cellerna i blodet, trombocyterna (blodplättarna), med ett stort antal blodproteiner. Koagulationens viktigaste uppgift är att stoppa blödningar som uppkommer vid skador på blodkärlen.

Central venkateter

Central venkateter (CVK) behövs ibland för att ge kärlretande läkemedel såsom cellgifter direkt i blodbanan. CVK kan läggas i flera olika vener varav de vanligaste går på halsen och under nyckelbenen. Vid inläggning av CVK förekommer komplikationer, i sällsynta fall livshotande blödningar som t.ex. kan komprimera luftstrupen eller ge upphov till blödningschock om blodet får rinna utan motstånd t.ex. ut i ena lungsäcken, Kärlen på halsen har fördelen att de, vid händelse av blödning kan komprimeras genom tryck på huden, vilket kärlen under nyckelbenen inte kan.

I *studie I* undersöktes 2009-2010 års CVK-inläggningar på patienter utanför intensivvårdsavdelningen på kliniken för intensiv- och perioperativ vård, Skånes Universitetssjukhus, Lund med avseende på blödningskomplikationer. Resultaten innefattar analys av 1 789 CVK-inläggningar och visar att gällande rutiner vid kliniken är säkra samt att det inte förekom någon allvarlig blödningskomplikation. Sexton inläggningar ledde emellertid till lindrig blödningskomplikation av typen lokal blodutgjutning vid insticksstället. Vidare identifierades inläggning av grov dialyskateter som den enda oberoende riskfaktorn för lindrig blödningskomplikation vid CVK-inläggning.

I *studie II* undersöktes blod från 39 cellgiftsbehandlade patienter med lågt antal trombocyter som planerades för CVK inläggning. Blodet analyserades dels innan och dels vid olika tidpunkter efter transfusion av trombocyter. Syftet var att

undersöka vilka mätbara effekter trombocyttransfusion har och hur länge de effekterna varar. Resultaten visar att den starkare blodlevringsförmåga som transfusion av trombocyter ger hos dessa patienter, beror på att antalet trombocyter blir fler och inte på bättre fungerande trombocyter, samt att denna effekt varar i minst 4 timmar efter avslutad transfusion.

Mild kylbehandling

Personer som drabbas av hjärtstopp riskerar grava hjärnskador på grund av reducerat blodflöde till hjärnan. För att minska skadorna i de områden av hjärnan som är lindrigt skadade hos patienter som är medvetslösa efter hjärtstopp, inleds ofta aktiv kylbehandling till 33°C i 24 timmar. Denna behandling sker under djup sömn på intensivvårdsavdelning. Eftersom det varit känt sedan länge att patienter som är kalla blöder ymnigare under operationer än patienter med normal kroppstemperatur, har det ibland ifrågasatts om denna kylbehandling är säker ur ett blödningsperspektiv. Forskare har länge försökt beskriva varför kalla patienter uppvisar blödningsbenägenhet, men resultaten är motsägelsefulla. *Studie III-V* har adresserat denna fråga och beskriver bland annat hur kyla påverkar resultaten av flera olika analyser.

I *studie III* har blod från 23 hjärtstoppspatienter som behandlades med aktiv kylbehandling till 33°C i 24 timmar undersökts. Resultaten visar stärkt blodlevringsförmåga när blodprov 2 togs vid normal kroppstemperatur, ca 2 dygn efter hjärtstoppet jämfört med blodprov 1 som togs vid kylbehandling ca 1 dygn efter hjärtstoppet. Denna effekt kan bero på att kylbehandlingen gett sänkta basvärden vid 33°C (blodprov 1). Effekten kan emellertid också förklaras av naturligt stärkt blodlevringsförmåga i samband med den systemiska inflammation som naturligt utvecklas efter hjärtstopp och sammanfaller med tidpunkten ca 2-3 dygn efter hjärtstoppet då blodprov 2 togs.

Många hjärtstoppspatienter undersöks avseende kranskärlsförträngningar i hjärtat och erhåller vid behov en sträckmetallcylinder, s.k. stent, i förträngt kärlområde. Denna armerar kranskärlet inifrån och möjliggör blodpassage. Stenten kan aktivera trombocyter och ge upphov till blodproppar. Alla patienter som erhåller stent måste därför behandlas med läkemedel som hämmar trombocyterna och därmed förebygger att blodproppar bildas i stenten. Man har i tidigare studier visat att hjärtstoppspatienter har dålig effekt av dessa trombocythämmande läkemedel och har spekulerat i möjliga förklaringar till detta. Trombocythämmande läkemedel finns för närvarande bara tillgängliga som tabletter, vilket gör att patienten är beroende av fungerande mag-tarmkanal för upptag av läkemedlen. Detta är inte alltid fallet i en intensivvårdssituation. I *studie III* visades för första

gången ett samband mellan dåligt fungerande mag-tarmkanal och dålig effekt av trombocythämmande läkemedel.

I *studie IV* undersöktes blod från 10 hjärtstoppspatienter som behandlades med aktiv kylbehandling till 33°C i 24 timmar. Blodprover som togs under kylbehandlingen sattes i värmeblock, inställda till olika temperaturer, under 30 – 60 minuter innan analys vid samma temperatur. Resultaten beskriver hur tiden till start av blodlevring fördröjs och att hastigheten med vilken blodet levras sjunker av kyla.

I *studie V* undersöktes blod från 15 patienter med akut kranskärlssjukdom som behandlades med läkemedel som hämmar trombocyternas funktion och från 8 friska frivilliga. Blodproverna hölls vid 28, 33, 37 och 39°C under 1 timma innan de analyserades. Även analyserna utfördes vid dessa temperaturer. Resultaten visar något oväntat att kyla ökade trombocyternas aktivitet men att tiden till blodlevringsstart var fördröjd.

Studie *III-V* visar att det inte verkar vara dålig trombocytaktivitet som förklarar varför kyla ökar blödningsbenägenheten. Tvärtom ökar trombocytaktiviteten av mild kyla. Däremot beskrivs hur tiden till start av blodlevring fördröjs och att hastigheten med vilken blodet levras sjunker av kyla. Möjligen kan detta fenomen vara en delförklaring till varför kyla försämrar blodlevringsförmågan men fler studier behövs för att bekräfta detta.

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Appendix

I   Kander T, Frigyesi A, Kjeldsen–Kragh J, Karlsson H, Rolander F, Schott U. Bleeding complications after central line insertions: Relevance of pre–procedure coagulation tests and institutional transfusion policy. Acta Anaesthesiol Scand 2013;57:573–79.

II Kander T, Tanaka K, Nordström E, Persson J, Schött U. The effect and duration of prophylactic platelet transfusions before insertion of a central venous catheter in patients with bone marrow failure evaluated with point of care methods and flow cytometry. Anest Analg 2014;119:882–90.

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Paper I

Bleeding complications after central line insertions: relevance of pre-procedure coagulation tests and institutional transfusion policy

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Background: The aim of this study was to map pre-procedural variables for insertion of a central venous catheter, prophylactic blood component use and to investigate whether any independent variable could be identified as an independent risk factor for associated bleeding complications in patients outside the intensive care unit.

Methods: In this retrospective study, we investigated 1737 consecutive insertions of central venous catheters in 1444 patients in a large university hospital during 2009–2010. Pre-procedural coagulation status, blood component use, type of catheter, insertion site and complications during insertion were recorded and compared with bleeding complications documented on electronic charts.

Results: No serious bleeding complications were recorded in connection with the insertion of central venous catheters. Sixteen of 1769 (0.9%) insertions caused grade 2 bleeding,

defined as bleeding requiring prolonged compression at the insertion site. Insertion of a large bore central dialysis catheter was found to be an independent risk factor for bleeding complications. Neither conventional coagulation tests nor accidental arterial puncture or the number of needle passes could predict bleeding complications in this study.

Conclusion: This retrospective study, in non-ICU patients, shows that serious bleeding complications in association with central line insertions are uncommon and that insertion of a large bore catheter is likely to be an independent risk factor for mild-bleeding complications in this population.

BLEEDING complications are reported to range from 0.5% to 1.6% in connection with central venous catheter (CVC) insertions, but they are rarely fatal.[1] Blood components are administered before CVC procedures to correct coagulation defects or low platelet counts. Cut-off levels for transfusing plasma and/or platelet concentrates vary between institutions, and the scientific evidence is low for these prophylactic transfusion procedures prior to inserting CVCs.[2–6]

To our knowledge, there is no report on bleeding complications after CVC insertions after correction of severe coagulopathy measured with routine coagulation tests including activated partial thromboplastin time (APTT), prothrombin time (PT) and platelet count (PLC).

The aim of this retrospective, single-centre study was to map the pre-procedural coagulation status, use of prophylactic blood components, technical problems encountered during CVC insertion and bleeding complications, and to investigate whether any variable could be identified as an independent risk factor for bleeding complications associated with CVC insertion.

Methods

This study was approved by the Regional Ethical Review Board in Lund (Dnr 2011/626) and included 1737 consecutive CVC insertions in 1444 adult patients at the Department for Intensive and Perioperative Care, Skåne University Hospital, Lund, Sweden between 2009 and 2010. This is a retrospective evaluation, but data were prospectively and very carefully recorded according to clinical practice in this institution.

The anaesthesiologist documented the cannulation procedure with conventional coagulation laboratory results (APTT, PT and PLC), insertion site, type of catheter, number of attempts, accidental arterial puncture, and whether or not ultrasound guidance was used. All bleeding complications in connection with CVC insertion were either recorded on the CVC chart by the anaesthesiologist or documented in a standardised way on the patient's electronic chart by the nurse or doctor on the regular ward. The bleeding complications recorded on the electronic chart were identified using database queries up to 5 days past the insertion date for all 1737 insertions. The pre-procedural transfusion of plasma or platelet concentrates was checked by examining the Regional Blood Centre transfusion register.

CVC placements
Records from patients receiving CVC or central dialysis catheters (CDC; 11.5–13 French) at a centralised CVC clinic and in the operating theatre were included. Indications for CVC insertion at the CVC clinic involved nutrition, administration of drugs such as chemotherapy and blood sampling. Indications for CVC insertion in the operating theatre involved measurement of central venous pressure, administration of vasoactive drugs and anticipated need for long-term postoperative nutrition.

The preferred site of insertion for CVCs was the internal jugular vein. However, if the CVC was expected to be in situ for a longer period and/or the patient was considered to be immune-incompetent, the subclavian vein was preferred. CVCs were inserted by anaesthesiologists with varying degrees of experience (residents and specialists). Residents were supervised or assisted by a specialist during the CVC or CDC cannulation. All operators had access to ultrasound equipment for vascular access if so desired. Ultrasound may have been used prior to the procedure or during cannulation.

Bleeding complications
The frequencies of local haematoma and haemothorax after CVC insertion were retrospectively identified through database searches of the hospital's electronic charts. Local haematomas were evaluated daily by ward and post-operative care unit nurses, and documented on the electronic charts under set headings. Bleeding was classified according to the Common Terminology Criteria for Adverse Events (Version 4.0).[7] Grade 1 bleeding was characterised by mild symptoms, such as slight oozing from the insertion site, not requiring any intervention. Such bleedings are considered of little clinical significance and were not always documented in a standardised manner, and are therefore not included in the present report. Grade 2 bleeding has mild symptoms that require invasive interventions. In the present, study grade 2 bleeding also included bleeding requiring prolonged compression without invasive intervention as in proper grade 2 bleeding. In grade 3 bleeding, transfusion, radiologic, endoscopic or elective operative interventions are indicated, and in grade 4 bleeding, life-threatening consequences call for prompt and urgent intervention.

Coagulopathy and laboratory analysis
APTT, PT and PLC were measured prior to CVC insertion. Patients with PLC $< 50 \times 10^9$/L routinely received 1 unit of platelets (approximately 240×10^9 platelets) before CVC insertion. No new PLC was measured after transfusion prior to or after cannulation. Patients were considered coagulopathic if PT > 1.8, APTT > 45 s or PLC $< 50 \times 10^9$/L according to international guidelines.[6] For perspicuity reasons we also present the results from the conventional coagulation tests with these boundaries.

APTT was assayed using the hospital's routine PTT-Automate method (Stago, Asnière sur Seine, France). The locally established reference range is 28–45 s. PT was performed using a combined thromboplastin reagent (prothrombin complex assay, SPA+, Stago, Asnières, France). The Owren PT assay was calibrated using international normalised ratio (INR) calibrators certified by the Swedish external quality assessment organisation (Equalis, Uppsala, Sweden). The reference range for PT (INR) is 0.9–1.2. PLC was measured using a Sysmex XE 5000 cell counter (Sysmex Corp., Kobe, Japan). The locally determined reference range for platelets is 165–387×10^9/L for adult women and 145–348×10^9/L for adult men.

Statistics
Fisher's exact test was used to analyse single independent variables for association with bleeding complications. Because of the complex interdependence of the independent variables, we also performed a multivariate logistic regression using the generalised linear model routine of R (http://www.r-project.org). The type of catheter (groups 1, 2, 3, 4 or CDC), side of puncture (left or right), site of puncture (with a group 'not available'), number of needle passes, use of ultrasound, arterial puncture and coagulation parameters (PLC, APTT and PT)

Table 1

Types of catheter and insertion sites.

Site of insertion	Catheter type					
	Single lumen	Double lumen	Multi-lumen	CDC	NA	Total (%)
IJV	764	166	131	77	43	1181 (68.0%)
EJV	101	17	19	0	7	144 (8.3%)
Subclavian	198	38	25	0	6	267 (15.4%)
Femoral	3	1	2	5	1	12 (0.7%)
Cubital veins (PICCLINE)	39	0	0	0	0	39 (2.2%)
NA	44	16	7	6	21	94 (5.4%)
Total	1149 (66.1%)	238 (13.7%)	184 (10.6%)	88 (5.1%)	78 (4.5%)	1737 (100.0%)

CDC, central dialysis catheter; IJV, internal jugular vein; EJV, external jugular vein; NA, not available.

were used as independent variables. The side of puncture, site of puncture, use of ultrasound and arterial puncture were treated as categorical variables (as factor in R, see previous discussion). All other independent variables were treated as continuous independent variables. A drawback of this method is that it requires a value for every independent variable for every observation, that is, if one PT value is missing, all other variables related to this procedure will be excluded from the model. This problem was alleviated by performing several different analyses using different selections of independent variables, thus reducing the number of missing observations.

Results

The type of catheters and insertion sites are shown in Table 1. The overall rate of grade 2 bleeding complications was 0.9% (16/1737). There were no grade 3 or 4 bleeds. Three CVC insertions led to pneumothorax, and one of these required chest tube drainage.

Fourteen percent of the CVC insertions were performed on patients considered to be coagulopathic ($PLC < 50 \times 10^9/L$, $APTT > 45\,s$ or $PT > 1.8$). Combined defects in APTT, PT and PLC are illustrated in Figs 1 and 2. Eighty-nine percent of referrals with $PLC < 50 \times 10^9$ were transfused with one or more platelet units within 1 h prior to cannulation. Eight percent with prolonged APTT/PT received plasma transfusion with one or more units of fresh frozen plasma (FFP) or wet stored plasma (stored at 4°C for ≤ 2 weeks) prior to insertion. Two of 36 patients with $PT > 1.8$ received prothrombin complex concentrate (Fig. 2).

Fisher's exact test (Table 2) revealed that the only risk factor for bleeding complication was the insertion of an 11.5–13 F CDC (p < 0.001). This was also confirmed by the multivariate logistic regression

using all independent variables (odds ratio 3.2, $P < 0.0009$). In this analysis, however, 1282 observations were excluded because of missing independent variables.

PLC was almost significant ($P = 0.057$), but subsequent analyses using only coagulation status as the independent variable did not confirm this finding. Exclusion of the number of needle passes (accounting for the majority of the missing values) gave the same result; only the type of catheter was an independent risk factor. Not using the number of needle passes and coagulation parameter as independent variables excluded 364 observations and again confirmed the result (the type of catheter as an independent risk factor). Coagulation independent variables alone (896 missing observations) yielded no significant differences (Fisher's exact test $P = 0.47$).

Inadvertent arterial puncture in this material was not an independent risk factor for bleeding complications ($P = 0.77$ from Fisher's exact test and $P = 0.45$ from multivariate logistic regression).

Abnormal coagulation analyses did not affect the propensity to use ultrasound-guided insertion. An ultrasound device was used in 49% of all insertions and did not significantly reduce the risk of bleeding complications ($P = 0.14$ from Fisher's exact test and $P = 0.56$ from multivariate logistic regression).

Discussion

This retrospective single-centre study evaluated bleeding complications after CVC insertion in 1737 CVC placements in non-ICU patients in daily practice, indicating that severe bleeding is a rare event irrespective of clinical and laboratory bleeding risk factors.

The only independent risk factor for grade 2 bleeding complications in this series was the 11.5–13
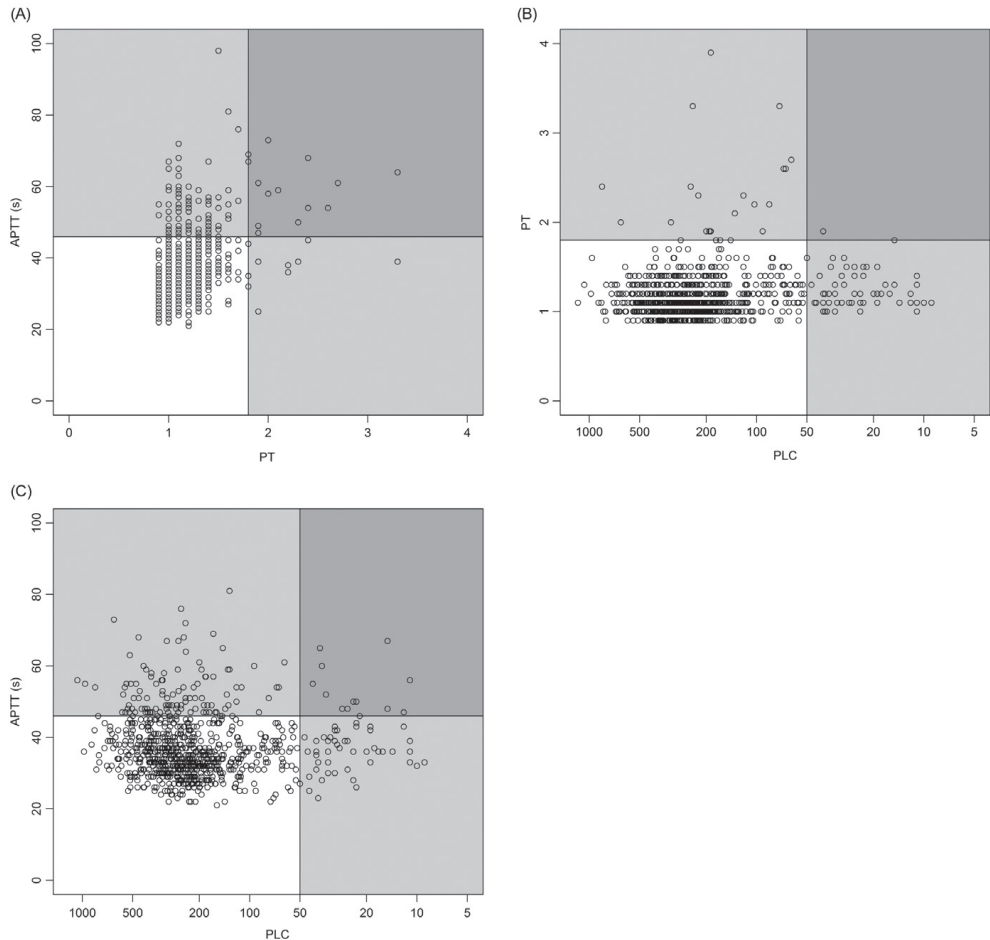
Fig. 1. Pre-procedural laboratory results. Combined coagulation defects. The lines in the scatter plots mark the borders for the chosen limits for coagulopathy. PLC < 50 × 10⁹/L, APTT > 45 s or PT >1.8. (A). APPT and PT. Lines at APTT 46 s and PT 1,8. (B). Platelet count ×10⁹/L (PLC) and PT. Note the use of logarithmic scales for PLC. (C). Platelet count ×10⁹/L (PLC) and APTT.

F CDC insertion. This is an anticipated result. A bigger venous tube requires a bigger wound that is likely to cause bleeding more often. In agreement with our findings, Haas et al. found no serious bleeding complications in connection with large bore (8–14.5 F) CVC insertions in patients with a PLC between 25 and 50×10^9/L.[8] Mild bleeding complications such as grade 2 bleeds were excluded in their study.

Our study was warranted because contradictory results appear from previous studies.[3,9] In a retrospective study, Zeidler et al.[10] showed that a PLC as low as 20×10^9/L did not increase bleeding complications during CVC insertions in patients with acute leukaemia. However, two independent studies, one prospective audit[11] and one retrospective analysis,[12] indicated that a PLC < 50×10^9/L increases the risk for minor haemorrhagic complications.

In this study, severe coagulopathy was corrected, before insertion in accordance with existing routines and at the discretion of the practitioner. Patients could receive platelet transfusion, plasma transfusion or PCC (Fig. 2). We present a safe concept for CVC insertion including trigger points for transfusions that provide safe insertion conditions. Nevertheless, this concept also means that some patients are probably given unnecessary plasma or platelet transfusions with the risk, albeit small, of infections, negative immunomodulatory effects, allergic reactions, and transfusion-related acute lung injury.[6,13] The optimal pre-procedural plasma and platelet transfusion thresholds are still under debate and remain to be determined.[2–6,14] We do not know what

Fig. 2. Combined coagulation defects, Venn diagram. Number of cases receiving any pro-coagulative treatment before insertion within brackets. () = Cases receiving pre-procedural platelet transfusion with approximately 240×10^9 platelets (n = 70). ()* Cases receiving pre-procedural plasma transfusion with one or more unit of plasma (n = 14). Two received fresh frozen plasma (FFP), and 12 received wet stored plasma. ()** Cases receiving pre-procedural prothrombin complex concentrate.

would have occurred if no compensation had been done in our study. Foster et al.[15] did not use any correction in patients with prolonged preprocedural APTT/PT and low PLC. Many of the patients in the study by Foster et al. had liver failure and may actually be at an increased risk for thrombosis even with prolonged PT-INRs because of defects in glycocalyx and decreased plasma levels of protein C and antithrombin.[16]

The present study includes CVC insertions both at a centralised CVC clinic and in the operating theatre. Patients being directed to the CVC clinic are likely to have a pre-procedural conventional coagulation test result before insertion. Unfortunately, this does not hold true for CVC insertions in the operating theatre where the majority of missing values for coagulation tests arise. This is a weakness with this study. However, CVC insertions in the operating theatre are performed on patients prior to large elective surgery, who have undergone large preoperative investigation and without a bleeding history or anticoagulant medication; hence, they are unlikely to be coagulopathic and lack a clinical indication for conventional coagulation tests.

Patients with a PLC below 50×10^9/L are candidates for platelet transfusions before central line insertion at our centre. Eighty-nine percent of these cases received pre-procedural platelet transfusion. Transfusion guidelines recommend checking the transfusion response after platelet concentrate transfusion with a new PLC within an hour after the end of transfusion.[6] For logistic reasons, it is difficult to wait for a new PLC before the insertion of a CVC/CDC, and we do not usually adopt this recommendation. This routine, in a previous publication from our centre, has shown to increase PLCs from median 32 (range 20–44) to 44 (range

Table 2

Risk factors for grade 2 bleeding complications according to univariate analysis (Fisher's exact test).

Category	Total	Grade 2 bleeding complication	P value
Coagulopathic patients*	204	2 (0.9%)	P = 0.47
Non-coagulopathic patients*	634	9 (1.4%)	
CDC	88	7 (7.9%)	P < 0.0001
Non-CDC	1571	12 (0.8%)	
Arterial puncture	46	2 (4.3%)	P = 0.77
Non-arterial puncture	1691	15 (0.9%)	
Ultrasound guidance	859	11 (1.3%)	P = 0.14
No ultrasound guidance	878	5 (0.5%)	
Needle passes ≤ 2	973	9 (0.9%)	P = 0.094
Needle passes ≥ 3	49	2 (4.1%)	

*Coagulopathic patient defined as platelet count < 50×10^9/L, APTT > 45 s or PT > 1.8. Includes only insertions where results from complete routine coagulation tests were available before insertion. CDC = central dialysis catheter.

$38–71) \times 10^9/L$ after platelet transfusion and also improved clot propagation and elastic modulus (47% improvement) in rotational thrombelastometry with no change in APTT/PT.[17]

APTT still remains a routine test in our institution but is of questionable value in patients without positive bleeding history or risks of disseminated intravascular coagulation, acute coagulopathy of trauma or circulating anticoagulants. An APTT at least up to 1.3 times the upper reference level, without suspicion of defective haemostasis, does not increase the risk of bleeding, and it should not be the cause of specific corrective treatments.[3,12] In the present study, six patients with isolated increased APTT (range 52–156 s) received plasma.

The number of inadvertent arterial punctures in patients with coagulation defects in our study was 3% for internal jugular vein insertion and 2% for the subclavian approach, and no serious complications were recorded. This is comparable with Ruesch et al.,[18] who reported 3% and 0.5%, respectively. We did not detect an increased risk for bleeding complications associated with either coagulopathy or accidental arterial puncture or the number of needle passes.

Previous studies have convincingly shown that real-time ultrasound guidance significantly reduces complications when cannulating the internal jugular vein.[19] Ultrasound guidance in this study was used in 61% of the internal jugular vein insertions and in 15% of the subclavian insertions. We found no evidence that the use of ultrasound guidance reduced the risk of bleeding complications. Contrary, ultrasound-guided insertions resulted in twice as many grade 2 bleedings as non-ultrasound guided insertions. This may be due to the fact that ultrasound guidance was performed at the discretion of the practitioner and was not always used in real time as it was in the studies mentioned earlier.

Even though documentation of bleeding complications is standardised in this hospital, being a retrospective investigation, there is a risk for under-reporting of bleeding complications in this study. There is also a risk for under powering of this investigation in the sense that if more patients were investigated, maybe more of the independent variables studied would be identified as risk factors for bleeding complications.

Conclusion

This retrospective study, in non-ICU patients, shows that serious bleeding complications in association with central line insertions are uncommon and that insertion of a large bore catheter is likely to be an independent risk factor for mild bleeding complications in this population.

Acknowledgement

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Paper II

The Effect and Duration of Prophylactic Platelet Transfusions Before Insertion of a Central Venous Catheter in Patients with Bone Marrow Failure Evaluated with Point-of-Care Methods and Flow Cytometry

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Johan Persson, MD, PhD,* and Ulf Schött, MD, PhD*

BACKGROUND: Patients with bone marrow failure and severe thrombocytopenia are frequently given prophylactic platelet transfusion before interventions. The clinical effects of such transfusions, however, are poorly defined. We performed a prospective observational study on patients with bone marrow failure scheduled for prophylactic platelet transfusion before the insertion of a central venous catheter. The objectives were to evaluate the effect and duration of prophylactic platelet transfusions on central venous catheter insertion in thrombocytopenic patients with bone marrow failure.

METHODS: Thirty-nine adult patients with bone marrow failure and platelet counts below 50×10^9/L were consecutively enrolled before prophylactic platelet transfusion for subclavian central venous catheter insertion. Blood samples were drawn from the patients before platelet transfusion, 1 hour, and 4 hours after completion of the transfusion. The coagulation profile was assessed by conventional hematological tests, thromboelastometry (ROTEM®) assays (EXTEM® and FIBTEM®), multiple electrode aggregometry (Multiplate®) assays including adenosine diphosphate, collagen, and thrombin receptor agonist peptide, and by flow cytometry for the platelet expression of P-selectin (CD62P) and activated glycoprotein IIb–IIIa (PAC-1). Bleeding complications were classified with a 5-grade scale, according to the Common Terminology Criteria for Adverse Events.

RESULTS: Seventeen women and 22 men were included in the study. Platelet count was increased from 24×10^9/L (18–32) before to 42×10^9/L (31–50) 1 hour after transfusion ($P < 0.0001$) and was not significantly different 4 hours after transfusion (40×10^9/L (29–50), $P = 0.047$). Maximal clot firmness EXTEM was increased from 38 mm (32–45) before to 46 mm (41–52) 1 hour after transfusion ($P < 0.0001$) and did not change 4 hours after transfusion. Clotting time EXTEM was decreased from 58.5 seconds (50–78) beforehand to 53 seconds (45–61) 1 hour after transfusion ($P = 0.0006$) and was not significantly different 4 hours after transfusion (57 seconds (52–70, $P = 0.025$). FIBTEM results were all unchanged after transfusion. All Multiplate analyses were significantly increased after 1 hour and were not diminished 4 hours after transfusion. Four grade 1 bleeding episodes occurred, but no grade 2 to 5 bleeding could be detected. Flow cytometry analyses showed mixed results with no overall trend.

CONCLUSIONS: Prophylactic platelet transfusions in thrombocytopenic patients with bone marrow failure improve hemostatic parameters on ROTEM and Multiplate by increasing the number of platelets, and not through enhancement of platelet function. Improved clotting parameters on ROTEM and platelet aggregation on Multiplate appear to persist between 1 and 4 hours after transfusion. (Anesth Analg 2014;119:882–90)

The risk of bleeding complications is high in patients with bone marrow failure and severe thrombocytopenia. Although supportive evidence is scarce,[1] prophylactic platelet transfusion is routinely performed in these patients before vascular and surgical interventions.[2–5]

The threshold platelet count and the timing of transfusion remain controversial among clinical studies.[6–8] Moreover, many patients suffer from bleeding despite prophylactic platelet transfusion. Therefore, platelet function testing could be important in assessing the therapeutic responses to platelet transfusions. A few studies have addressed this issue,[9–12] but to our knowledge, no study has comprehensively evaluated the time-dependence of platelet function after transfusion.

It seems obvious that platelet transfusions enhance both primary and secondary hemostasis by increasing the

number of platelets. However, some studies indicate that platelet function is diminished in patients with hematological diseases,[13–15] and that enhanced coagulation observed after platelet transfusion are only partly explained by increases in platelet count.[9] To further clarify the impact of platelet transfusion, we performed a prospective observational study on patients with bone marrow failure who were scheduled for prophylactic platelet transfusion before the insertion of a central venous catheter (CVC). The aim was to measure the coagulation enhancement at 1 and 4 hours after transfusion using thromboelastometry (ROTEM®; EXTEM® and FIBTEM®), multiple electrode aggregometry (MEA) (Multiplate®; adenosine diphosphate [ADP], collagen [COL] and thrombin receptor agonist peptide [TRAP], and flow cytometry.)

METHODS

This study was approved by the Regional Ethical Review Board in Lund (registration number 2011/626) and included 39 consecutive adult patients at the Department for Intensive and Perioperative Care, Skåne University Hospital, Lund, Sweden from September 2011 to March 2013. Written informed consent was obtained from all patients before inclusion.

The inclusion criteria were thrombocytopenia $<50 \times 10^9$ /L due to bone marrow failure (chemotherapy, malignancy, or both) and scheduled platelet transfusion for planned CVC insertion. Exclusion criteria included patients with hepatic or renal failure, heparin-induced thrombocytopenia, idiopathic thrombocytopenic purpura, and those receiving mechanical ventilation or anticoagulant therapy (except for thromboprophylactic low molecular weight and unfractionated heparin [subcutaneous]).

The indication for CVC insertion was chemotherapy in all patients. The CVC was placed either in the subclavian or internal jugular vein at the discretion of the performing physician. All patients received 1 therapeutic unit of platelets, containing approximately 260 to 270×10^9/L platelets. One therapeutic unit of platelets could be either whole blood pooled buffy coat platelets from 4 donors or apheresis platelets from 1 donor (Table 1). One therapeutic unit contained approximately 300 mL of which 75 mL was plasma in the blood pooled buffy-coat platelets, and 100

mL was plasma in the apheresis platelets. Samples for bacterial culture were taken from each therapeutic unit on the first day after collection and were proven negative before the unit was transfused in a patient. All transfused platelets were ≤7 days old and leukocyte reduced to 0.001×10^9 leukocytes per unit.

Blood sampling was performed using a vacutainer system (BD, Plymouth, UK) before the platelet transfusion, 1 hour, and 4 hours after the completion of transfusion. The 1-hour sampling point was chosen according to the recommended Hemovigilance protocol,[16] and the sampling at 4 hours after transfusion was the extended time limit to perform analyses during office hours. Pretransfusion blood samples were collected from a peripheral vein within 4 hours before the platelet transfusion. Posttransfusion blood samples were collected from the CVC after the pertinent catheter was flushed with normal saline, and after 10 mL blood was discarded. No heparin or fibrinolytic drug was administered through CVCs.

Blood samples were analyzed at each time point by conventional coagulation tests; prothrombin time (PT)/ international normalized ratio (INR), activated partial thromboplastin time (aPTT), platelet count, and fibrinogen along with ROTEM and Multiplate. The flow cytometry analysis was performed in the last 17 of 39 patients. Blood samples for ROTEM and flow cytometry analysis were collected in a 4.5-mL tube containing 0.109 M citrate (BD, Plymouth, UK). Blood for Multiplate analysis was collected in a 3.0-mL tube containing recombinant hirudin (Dynabyte GmbH, Munich, Germany).

Conventional Hematological Tests

PT/INR, aPTT, fibrinogen and platelet count analyses were performed at the accredited hospital laboratory. PT/INR was performed using a combined thromboplastin reagent (Stago prothrombin complex assay, SPA+, Stago). The Owren PT assay was calibrated using INR calibrators certified by the Swedish external quality assessment organization (Equalis, Uppsala, Sweden). The reference range for PT/INR is 0.9 to 1.2.

APTT was analyzed with an aPTT reagent from Actin FSL (Siemens Healthcare Diagnostics) Plasma fibrinogen concentration was measured using the Dade Thrombin reagent (Siemens Healthcare Diagnostics. CS-5100). The reference range for aPTT has been established locally to 26 to 33 seconds and for fibrinogen to 2-4 g/L.

Platelet counts were measured using the Sysmex XE 5000 cell counter (Sysmex Corp., Kobe, Japan). The locally determined reference range for platelets is 165 to 387×10^9/L for adult women and 145 to 348×10^9/L for adult men.

Rotational Thromboelastometry

(ROTEM; Pentapharm, Munich, Germany) was used for viscoelastic evaluation of clot formation in the recalcified whole blood at 37°C. The test was performed within 2 hours from blood sampling. Tissue factor was used to trigger coagulation for EXTEM and FIBTEM. Thrombin-mediated platelet activation and fibrin polymerization are reflected on EXTEM, while fibrin polymerization is selectively shown on FIBTEM by inhibiting platelet–fibrin interactions using cytochalasin D.[17] The latter correlates with plasma fibrinogen levels.[18] The

Table 1. Baseline Characteristics	
Age	57(36–66)
Male	17
Female	22
Body mass index	25 (23–28)
Body temperature, °C	37.2 (36.7–37.6)
Diagnosis	
Acute myeloid leukemia	13
Acute lymphatic leukemia	8
Chronic lymphoid leukemia	2
Chronic myeloid leukemia	2
Myelodysplastic syndrome	6
Acute onset leukemia	8
Platelet transfusions	
Apheresis/ buffy-coat platelets	2/37
ABO-match/non-ABO-match	8/31
Age of transfused platelets (days)	6 (4–7)

Medians with interquartile range (Q1–Q3).

limited number of glycoprotein IIb/IIIa receptors due to thrombocytopenia results in decreased platelet–fibrin interactions, and reduced clot formation time (CFT, seconds), α angle (°), maximum clot firmness (MCF, mm).[19,20]

Multiple Electrode Aggregometry

Multiplate (MEA, Roche, Rotkreuz, Switzerland) was used to measure an agonist-induced platelet aggregation. Hirudin-anticoagulated whole blood was stored at room temperature before analysis within 0.5 to 3.0 hours of blood sampling. The analysis was performed in duplicate at 37°C. The extent of platelet aggregation was measured by resistance (impedance) changes between 2 electrodes and is depicted as a graph. The area under the curve (AUC) is the best measure of platelet function. Three test assays were performed: ADP test (platelet aggregation in response to adenosine-5′-diphosphate), COL test (platelet aggregation in response to collagen), and TRAP test (platelet aggregation in response to thrombin receptor agonist peptide).

Flow Cytometry Analysis

Platelet function analysis using MEA is dependent not only on platelet function but also on platelet count.[21–23] Therefore, increased platelet aggregation in response to agonists in blood from thrombocytopenic patients could be due to either improved platelet function, increased platelet count, or both. To address this, we performed flow cytometry that assessed the functional status of individual platelets independent of platelet count.

Flow cytometry analyses were performed within 2 hours after blood samples. Platelet-rich plasma (PRP) was obtained by centrifugation at 140g for 10 minutes. PRP 50 μL was incubated with 5 μL CD41a-PerCPCy5.5 (BD, cat no 333148) or 5 μL CD42b-PE (Dako, cat no R7014) for 15 minutes at room temperature. To activate the platelets, 10 μL labeled PRP was incubated with 40 μL ADP (final concentration (FC) 5 μM), CRP-XL (cross-linked collagen reactive peptide; FC 0.2 μg/mL), TRAP (2.5 μM) or HEPES-bovine serum albumin (BSA) buffer (20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 1% BSA) (resting platelets) for 10 minutes at room temperature. To detect activation, PRP labeled with CD41a-PerCPCy5.5 was subsequently incubated with 5 μL CD62P-PE (Beckman Coulter, cat no IM1759) and PRP labeled with CD42b-PE was incubated with 5 μL PAC-1-FITC (BD cat no 340507) for 15 minutes at room temperature. As control 5 μL MsIgM-FITC and 5 μL MsIgG1-PE was incubated with 10 μL of resting PRP in 40 μL HEPES-BSA buffer. The samples were subsequently diluted in 2 mL Isoflow (Beckman Coulter, Bromma, Sweden) and run directly on flow cytometer, Gallios (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). In the Gallios, the laser setting W^2 was used. Thresholds for forward scatter and side scatter were set to 2 and with live gate on CD41a-PerCPCy5.5 or CD42b-PE positive events. Forward scatter, side scatter, and fluorescence channels were set at logarithmic gain and live gate on CD41a-PerCPCy5.5 or CD42b-PE positive events were used. For MsIg-controls, no live gate was used, and platelets were defined according to their forward scatter/side scatter characteristics. The flow cytometry data were analyzed using the software Kaluza Flow Cytometry Software. Activation was expressed as a percentage of positive platelets, where a positive gate was set using the MsIg control antibodies (2% false positive).

Bleeding Complication

Local hematomas were evaluated daily by trained staff on the hematological ward and documented on the electronic charts under set headings. Bleeding was classified according to the Common Terminology Criteria for Adverse Events (Version 4.0),[24] where grade 1 bleeding is characterized by mild symptoms. Grade 2 bleeding may require minimally invasive evacuation or aspiration. In grade 3 bleeding, transfusion, radiologic, endoscopic, or elective operative intervention are indicated. Grade 4 is life-threatening bleeding, and grade 5 is death.

Statistics

A power analysis was performed using ROTEM-CFT and MCF changes from the previously published data in the same type of patients at our institution.[10] The sample size requirement was lower with the MCF-based calculation, and thus, CFT was used for the final sample size. Based on the change of CFT of EXTEM (CFT_{EXT}) from 181.5 to 123 seconds after platelet transfusion with a SD of 144 seconds, the required minimal sample size was calculated to be 35 with an α level of 5% and a β level of 50%. Differences between laboratory results before, 1, and 4 hours after transfusion were calculated using 2-tailed, Wilcoxon matched pairs signed test. Correlation coefficients were calculated using Spearman rank correlation method. All variables were considered nonparametric (Gaussian distribution not assumed), and all distributions are summarized using the median with 25th and 75th percentiles Q2 (Q1 and Q3). P values < 0.01 were considered significant.

To address the risk that the increased values seen after platelet transfusion are caused by regression to the mean, we performed 3 different tests on platelet count (for details see Supplemental Digital Content, http://links.lww.com/AA/A878).

- Regression analysis baseline versus 1 and 4 hours after transfusion.
- Modified Bland-Altman plot. Baseline versus Δplatelet count 1 and 4 hours after transfusion.
- Median split analysis.

Statistical analyses were performed using GraphPad Prism version 6.03 for Windows, GraphPad Software, La Jolla, California.

RESULTS

Demographic data of the subjects are shown in Table 1. In terms of types of transfused platelets, 2 of 39 platelet transfusions were blood pooled buffy-coat platelets, and 31 of 39 platelet transfusions were ABO-matched (Table 1).

Because gender differences did not affect the results after the preliminary analysis, all data are presented in aggregate form. All patients received a single-lumen CVC. Twenty-six CVCs were inserted in the subclavian vein and 13 in the internal jugular vein. Four grade 1 bleeding events occurred, but no grade 2 to 4 bleeding events were detected.

Conventional Hematological Tests

Platelet count increased from 24×10^9/L (18–32) at baseline to 42×10^9/L (31–50) at 1 hour after transfusion ($P < 0.0001$). The

Table 2. Blood Analyses

	Before (Q1–Q3)	1 h (Q1–Q3)	4 h (Q1–Q3)
Blood analysis; n = 39			
Hemoglobin, (g/L)	93 (82–100)		
WBC, ×10⁹/L	7.6 (0.6–47)		
Fibrinogen, g/L	3.3 (2.4–5.2)	3.3 (2.3–4.7)	3.7 (2.6–5.0)
PT, INR	1.2 (1.1–1.3)	1.2 (1.1–1.3)	1.2 (1.0–1.3)
aPTT, s	30.0 (26–37)	29.0 (26–34)	31.0 (27–36)
Platelets, ×10⁹/L	24 (18–32)	42 (31–50)	40 (29–50)
ROTEM; n = 39			
MCF_{EXTEM}, mm	38.5 (32–45)	46 (41–52)	44 (40–51)
CT_{EXTEM}, s	58.5 (50–78)	53 (45–61)	57 (52–70)
CFT_{EXTEM}, s	241 (130–518)	201 (91–292)	216 (118–280)
α-angle $_{EXTEM}$, °	69 (40–80)	70 (49–80)	68 (53–79)
MCF_{FIBTEM}, mm	17 (10–28)	16 (12–28)	17 (12–26)
MEA; n = 39			
ADP, AUC	3 (0–9)	7 (0–19)	5 (0–12)
TRAP, AUC	5 (1–18)	17 (2–37)	14 (2–30)
COL, AUC	5 (1–16)	12 (3–39)	12 (3–32)
FLOW CYTOMETRY; n = 17			
Resting-CD62P	13.0 (11.1–22.0)	18.0 (13.2–29.7)	11.9 (8.8–19.4)
Resting-PAC-1	5.5 (2.9–6.9)	3.2 (2.2–4.9)	4.0 (2.1–8.0)
ADP-CD62P	64.0 (55.7–74.6)	62.0 (55.2–72.9)	67.0 (55.5–73.0)
ADP-PAC-1	56.8 (49.6–64.5)	43.7 (34.0–55.3)	59.8 (52.2–68.6)
CRP-CD62P	78.9 (68.0–85.4)	79.0 (69.0–86.3)	77.0 (65.5–85.0)
CRP-PAC-1	36.7 (25.9–54.1)	37.1 (24.7–47.0)	37.5 (27.0–55.5)
TRAP-CD62P	62.0 (47.3–78.2)	53.0 (50.4–67.0)	58.5 (52.4–65.5)
TRAP-PAC-1	8.1 (4.1–9.2)	5.5 (3.9–6.2)	7.3 (4.0–11.0)

Results from blood analysis before, 1, and 4 hours after completion of platelet transfusion. Medians with interquartile range (Q1–Q3).
WBC = white blood cells; MCF = maximal clot firmness; ROTEM. CT = clotting time, ROTEM; CFT = clot formation time; ROTEM. MEA = multiple electrode aggregometry performed with Multiplate. ADP = adenosid diphosphate-agonist; COL = collagen-agonist; TRAP = thrombin-agonist. FLOW CYTOMETRY = The platelet agonists used were: [ADP = adenosid diphosphate; CRP = cross-linked collagen reactive peptide and TRAP = thrombin receptor agonist peptide-6]. The platelet activation markers were: [CD62P = P-selectin and PAC-1 = activation of glycoprotein IIb–IIIa].

count was $40 \times 10^9/L$ (29–50) 4 hours after transfusion ($P = 0.047$ vs 1 hour after transfusion) (Table 2 and Fig. 1). PT-INR, aPTT, and fibrinogen were unchanged after transfusion.

ROTEM
MCF_{EXT} was increased from 38 (32–45 mm) before to 46 (41–52 mm) at 1 hour after transfusion ($P < 0.0001$) and did not change 4 hours after transfusion ($P = 0.06$). The increase in platelet count after 1 hour (ΔPLC) correlated statistically with the increase in MCF_{EXT} ($r = 0.56$, $P < 0.0002$) (Table 2 and Fig. 2).

Clotting time EXTEM (CT_{EXT}) was decreased from 58.5 (50–78 s) before to 53 (45–61 s) at 1 hour after transfusion ($P = 0.0006$) and was 57 (52–70 s) 4 hours posttransfusion ($P = 0.025$, vs 1 hour after transfusion). CFT EXTEM (CFT_{EXT}) was changed in a similar manner. The α-angle$_{EXT}$ was not significantly changed at any time (both $P > 0.04$). Similar to plasma fibrinogen values, FIBTEM results were not affected by platelet transfusion (both $P > 0.74$). Fibrinogen level correlated well with MCF_{FIBTEM} (MCF_{FIB}) ($r = 0.84$, $P < 0.0001$) (Table 2 and Fig. 2).

Multiplate
All Multiplate analyses were significantly increased after 1 hour and were not diminished at 4 hours after the transfusion (Table 2 and Fig. 1).

Flow Cytometry
There was no change in the expression of CD62P in any of the samples. The binding of PAC-1 after ADP-stimulation showed a small but significant decrease 1 hour after transfusion. After stimulation with CRP, the binding of PAC-1 did not change after transfusion compared to before transfusion (Table 2 and Fig. 3).

DISCUSSION
This prospective observational study in thrombocytopenic patients with bone marrow failure evaluated the duration and mechanisms of changes in coagulation induced by prophylactic platelet transfusion. We found that platelet transfusions increased platelet count significantly by 74% at 1 hour after transfusion, and that this increase persisted for 4 hours after transfusion. When evaluating coagulation using thromboelastometry (ROTEM), we found that MCF_{EXT} increased 1 hour after transfusion, and that this increase persisted for 4 hours. Platelet transfusions also accelerated the onset of tissue factor-induced coagulation (CT_{EXT}). CT_{EXT} is a sensitive parameter to detect the initiation of the coagulation cascade. One therapeutic unit of platelet transfusion contains about 100 mL plasma, which may also accelerate CT_{EXT} onset. This effect may partially explain the transient decrease in CT_{EXT} observed 1 hour after transfusion. Platelet aggregation in response to 3 different agonists on Multiplate also demonstrated steady increases in aggregation throughout the entire time of observation. These results indicate that hemostatic effects of platelet transfusion last up to at least 4 hours and suggest that an optimal window for performing an invasive procedure in patients with bone marrow failure would be 4 hours. Although the hemostatic
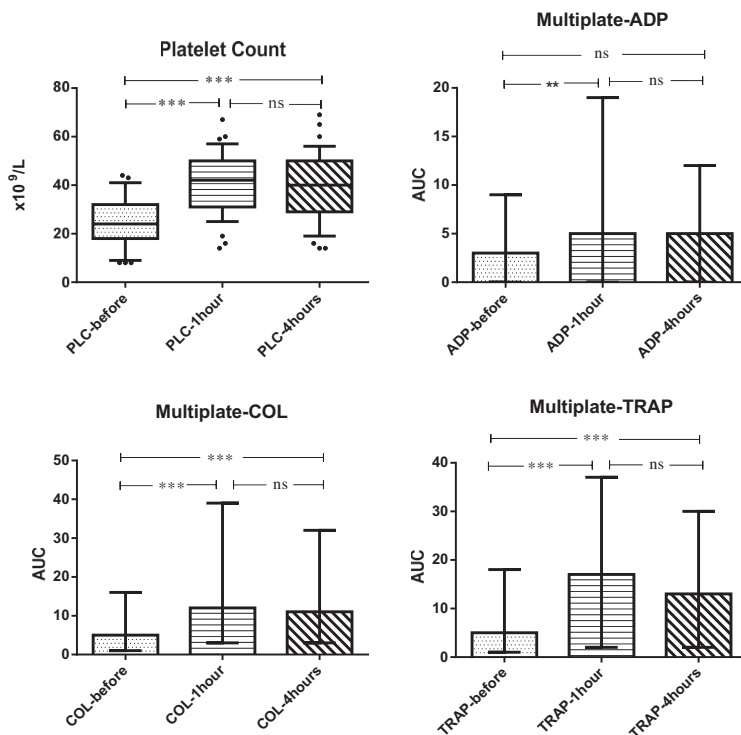
Figure 1. Platelet count and multiple electrode aggregometry performed with Multiplate. ADP = adenosid diphosphate-agonist; COL = collagen-agonist; TRAP = thrombin-agonist before, 1, and 4 hours after completion of platelet transfusion; AUC = area under curve. Boxplots with 10 to 90 percentile whiskers. Bar graphs presented as the median with interquartile range. **$P < 0.01$. ***$P < 0.001$.

improvement may last longer, we were limited by office hours and sampling volumes for further measurements.

Flow cytometry analyses showed that after platelet activation, expression of the granule protein CD62P on the surface of the platelets was unchanged after stimulation with different platelet activators (see Methods). Given that almost every other platelet in the blood samples after transfusion was a transfused platelet, this finding suggests that transfused platelets were functioning as well as the patient's own platelets.

Still, after platelet activation with ADP, we observed a slight decrease in the expression of PAC-1. Overall, the nonsignificant changes in platelet function from flow cytometry indicate that the increase in hemostasis after transfusion is not explained by better functioning platelets but rather by an increase in the platelet count.

We found no significant bleeding complications but did identify 4 cases of grade I bleeding events. These findings suggest that the carefully designed clinical practice at our institution is safe. However, our study was not sufficiently powered to make a conclusive statement on this clinical end point.

Patients in our study had fibrinogen levels in the upper part of the reference range, well correlated with MCF_{FIBTEM} (Fig. 2). Lang et al.[25] suggest that low clot firmness in

thrombocytopenic patients, measured with ROTEM, may be restored with high fibrinogen levels. Velik-Salichner et al.[26] performed a study on thrombocytopenic pigs and showed that high-dose fibrinogen administration improved clot firmness more than platelet transfusion and that fibrinogen concentrate stopped induced bleeding faster than did platelet transfusion. Whether the high fibrinogen levels noted in our study resulted from an inflammatory process, or if they were a compensatory mechanism for low platelets, is unclear. Further clinical studies are needed to verify whether platelet transfusions can be avoided in thrombocytopenia if fibrinogen levels are high. There was a statistically nonsignificant increase of plasma fibrinogen (3.7 g/L) at 4 hours compared with the baseline and 1 hour after transfusion (both, 3.3 g/L; Table 2). This increase could have been an inflammatory reaction secondary to the CVC procedure in itself or a reaction to the transfused platelets. Because the effect was more pronounced after 4 hours as compared with 1 hour after platelet transfusion, it was unlikely to have been caused by plasma contained in platelet concentrate.

A limitation with the present study is that analyses were not performed immediately after completion of the platelet
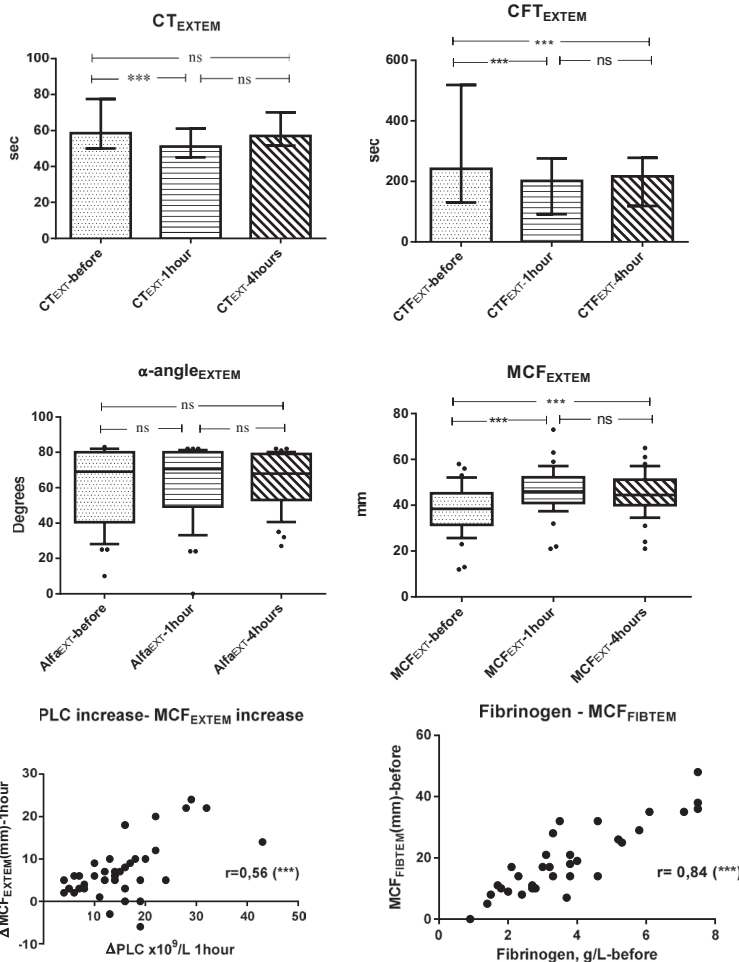
Figure 2. Viscoelastic hemostatic test, ROTEM; CT = clotting time; CFT = clot formation time, α angle, MCF = maximal clot firmness before 1 and 4 hours after completion of platelet transfusion. Boxplots show the median with the interquartile range and 10-90 percentile whiskers. PLC increase–MCF$_{EXTEM}$ increase = Increase in platelet count (PLC) and maximal clot firmness–EXTEM (MCF$_{EXTEM}$), ROTEM, 1 hour posttransfusion. Fibrinogen-MCF$_{FIBTEM}$ = fibrinogen and maximal clot firmness-FIBTEM (MCF$_{FIBTEM}$) before platelet transfusion. **P <0.01. ***P <0.001.

transfusion. The coagulation enhancement immediately after transfusion might have been even greater than the improvement that we observed 1 hour after transfusion. However, current recommendations state that if refractoriness to platelet transfusion is suspected, a new platelet count should be measured 10 to 60 minutes after completion of the transfusion.[3,16] Our study complied with this recommendation. Another limitation of this study is that platelet function assays were done under minimal shear so that platelet function under higher flow conditions, which is influenced by hematocrit and von Willebrand factor, could be assessed.[27]

Neither ABO-matching nor the age of the platelet transfusions affected the coagulation enhancement in this study. This relationship has been shown in other studies,[3,28] but our study was underpowered and not designed to detect these effects.

The present study is an important reappraisal of hemostatic effects of platelet transfusion in thrombocytopenic patients. There is a paucity of evidence for prophylactic platelet transfusion in the threshold for bleeding and the dose for hemostasis.[3–6] Furthermore, it is unclear whether prophylactic platelet transfusion before an intervention is superior to a strategy of therapeutic transfusion only.[7,8] The threshold platelet count of $\geq 50 \times 10^9$ at our institution was safe in a
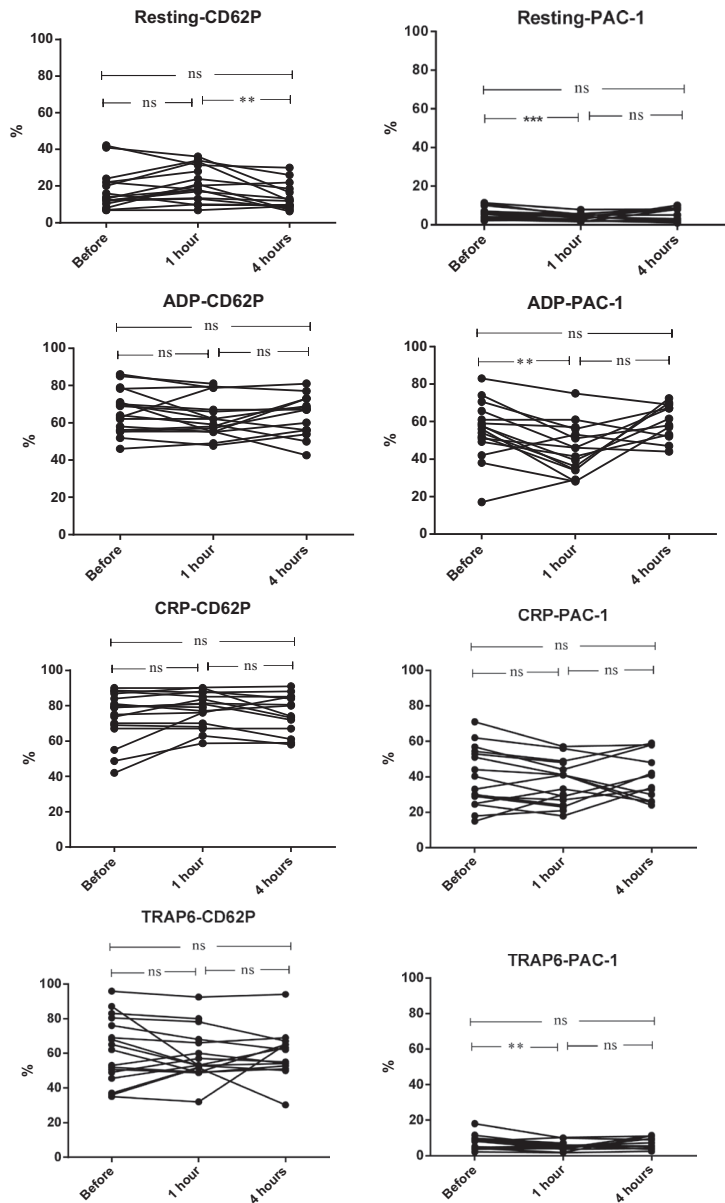
Figure 3. Results from flow cytometry, before, 1 hour, and 4 hours after completion of the platelet transfusion, Activation expressed as percentage of positive platelets. The platelet agonists used were: [ADP = adenosid diphosphate; CRP = cross-linked collagen reactive peptide, and TRAP = thrombin receptor agonist peptide-6]. The platelet activation markers were: [CD62P = P-selectin and PAC-1 = activation of glycoprotein IIb–IIIa]. **P <0.01. ***P <0.001.

recent retrospective analysis[29] but is arbitrary and not based on large randomized trials. Another retrospective study[30] showed that a platelet count as low as 20×10^9/L did not increase bleeding complications during CVC insertions in patients with acute leukemia. On the contrary, 2 independent studies, 1 prospective audit,[31] and 1 retrospective analysis[32] indicated that a platelet count <50×10^9/L increased the risk for hemorrhagic complications. Furthermore, the site of CVC insertion is important. If the bleeding site is compressible (e.g., the internal or external jugular vein), the need for

normal hemostasis is not as high as if the insertion site were noncompressible (e.g., the subclavian vein). Other factors of proven importance are the caliber of the inserted catheter, the experience of the operator, and the use of real-time ultrasound guidance.[33] Taken together, prospective studies are needed to define optimal transfusion trigger(s) for platelet concentrates in thrombocytopenic patients due for a CVC.

This study adds information about how a platelet transfusion contributes to the coagulation system, how its effect may be measured, and the duration of this measured coagulation enhancement.

CONCLUSIONS

Prophylactic platelet transfusions given to thrombocytopenic patients with bone marrow failure improve hemostatic variables by increasing the number of platelets, and not via enhancement of platelet function. Improved clotting parameters as measured by thromboelastometry, and platelet aggregation on MEA appear to persist between 1 and 4 hours after transfusion. ▪▪

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Are We Begging a Question or Begging an Answer?

Aryeh Shander, MD,*† and Terry Gernsheimer, MD‡

Timely identification of patients in whom the potential benefits of transfusions outweigh the risks remains hotly debated.[1] Despite their ubiquitous clinical use, many red blood cell (RBC) transfusion decisions are still based on arbitrary hemoglobin triggers.[2] The case of prophylactic platelet transfusion is even more enigmatic, given that clinicians must consider not only platelet number (count) but also the functional status of platelets, not yet commonly measured in the clinical arena and the state of the endothelium, still an evolving field. Platelets are one of many contributors to the complex hemostatic system, and the platelet count and function needed to control bleeding in any clinical situation remains unclear.

Severe thrombocytopenia predisposes to spontaneous bleeding and possibly an increased size of the bleed (both prominent concerns in the central nervous system [CNS]). Thrombocytopenia may also increase the risk of bleeding and adverse outcomes in adults and pediatric patients undergoing invasive procedures.[3] Recommendations for platelet transfusion typically use a threshold platelet count number as a cutoff for transfusion.[4,5] Commonly used thresholds include: platelet count $<10 \times 10^9/L$ to reduce the risk of spontaneous bleeding in general patients; platelet count $<50 \times 10^9/L$ in patients who are actively bleeding, those with qualitative platelet defects or scheduled for invasive procedures; and platelet count of $70–100 \times 10^9/L$ in patients with CNS injury or undergoing invasive CNS procedures including intrathecal and epidural catheter placement.[4,5] Platelets have also been used as part of balanced transfusion protocols in resuscitation of trauma patients.[6] Adding to the arbitrary nature of these recommendations is the clinical observation that many invasive procedures do not lead to significant bleeding despite the presence of thrombocytopenia.[7,8] It thus appears likely that many patients receive platelet transfusions with questionable clinical justification or demonstrable benefit.[9] A recent Cochrane review of 18 clinical trials has concluded that

while there was no evidence to indicate a change from the practice of using a platelet count threshold of $<10 \times 10^9/L$ for transfusion, there is little evidence that prophylactic platelet transfusion (although somewhat more efficacious than therapeutic transfusion in lower platelet counts) prevented bleeding or that the platelet dose affected the number of patients with significant bleeding.[10]

The usefulness of platelet transfusion in active bleeding due to platelet dysfunction also remains uncertain.[4,5] Despite its perceived importance, platelet function has not been well evaluated in clinical studies. In this issue of the journal, Kander et al.[11] provide intriguing in vivo data on the impact of platelet transfusion on various laboratory parameters and functional platelet and clotting assays. In their study, they took blood samples before and 1 and 4 hours after transfusion of 1 unit of platelets in 39 patients with hypoproliferative thrombocytopenia (defined as platelet count $<50 \times 10^9/L$) undergoing central venous catheter insertion. Conventional coagulation tests, thromboelastometry, multiple electrode aggregometry, and platelet flow cytometry were performed. Platelet transfusion resulted in a proportional rise in the patients' platelet counts at 1 hour that persisted during the 4-hour period and was associated with improved clotting based on thromboelastometry and multiple electrode aggregometry results. The platelet functional status was not significantly improved following transfusion, suggesting that improved clotting parameters following platelet transfusion were predominantly driven by increased platelet counts and not better function.[11]

Changes that occur in blood upon removal from the circulation and during processing and storage (the so-called "storage lesion") are well documented in RBC transfusions at various molecular, subcellular, and cellular levels, with some studies suggesting that these changes adversely affect patient outcomes.[12,13] The evidence for platelet transfusions is more limited, but studies suggest some loss of effectiveness after prolonged storage,[14] with decreased platelet transfusion increments and in vivo survival as soon as 3 days into storage.[5,15] Platelets thus have a far shorter shelf-life (5–7 days) than RBCs (42 days). In the study by Kander et al.,[11] the transfused platelet units were relatively old (median age of 6 and interquartile range 4–7 days). Hence, it is not surprising that transfused platelets offered little to improve the patients' platelet functional status overall. Unlike stored RBCs, the lost functionality is primarily related to reduced platelet viability and is thus not expected to be reversible.

Although the observations of Kander et al. shed some light on real-world changes in laboratory parameters following prophylactic platelet transfusions, they should be

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viewed in light of their limitations. The population studied had a very low bleeding tendency despite low platelet counts and possibly impaired platelet function. Even lower platelet counts (e.g., $20 \times 10^9/L$) have been associated with similar rates and grade of bleeding.[16] The power of the study is low and could undermine the reliability of any reported statistically nonsignificant P values. Although several statistically significant changes in laboratory parameters are reported, the clinical significance of those changes is undetermined. Platelet transfusions were associated with near doubling of the platelet counts of the recipients, but even the most significant changes in thromboelastometry parameters at the fourth hour were less than 15% of baseline values. Four grade-1 bleedings were reported, but with the study not powered for clinical outcomes and without a control arm, clinical conclusions are severely limited and the necessity of the transfusions remains unknown. Flow cytometry on blood samples obtained from the patients contained platelets from both the patient and transfused units, further blurring the functional contribution of the transfusion.

In their discussion, Kander et al.[11] emphasize the paucity of evidence identifying a threshold and dose of platelets transfused prophylactically for invasive procedures. We could not agree more. We need a better understanding of which patients are at risk of bleeding due to thrombocytopenia when undergoing invasive procedures. Performing controlled randomized studies of prophylactic versus no platelet transfusion prior to invasive procedures is understandably difficult (particularly if patients are not adequately advised of the risks associated with platelet transfusions), and trials have yielded mixed results due to the low frequency of bleeding. The often risk-averse medical community has generally preferred transfusing platelets rather than risking bleeding despite the marginal risk/benefit ratio. It is likely that hundreds of patients every year are transfused to prevent 1 bleed that may or may not be clinically significant (grade 1 bleeding) and may result in adverse transfusion effects, not a clinically justified or cost-effective practice.

In their study, Kander et al. approached this problem from behind rather than head-on, relying on laboratory rather than clinical endpoints, demonstrating some degrees of improvements in certain laboratory measures correlated with increased platelet counts but not with demonstrable clinical improvement. Without clear clinical implications, one must ask, "Are we begging for a question or are we begging for an answer?" The key questions remain: Who is at risk of bleeding in the presence of thrombocytopenia, how can we identify them reliably ahead of time, what dominates that risk (count, function, or both), and how can platelet transfusions help with clinical outcomes? Unfortunately, the clinician is left empty-handed despite this and several other studies, and the clinical determination of the safe platelet count for an invasive procedure remains arbitrary with little or no clear clinical evidence.

The take-home messages of the study by Kander et al. are that given a low baseline platelet count, prophylactic platelet transfusion will improve the clotting profile and that the improvements appear to be dominated by platelet counts and not functional status. However, we cannot be assured whether it changes the incidence of clinically significant bleeding. Correlating the decline in platelet number with clotting (assuming the 2 are interrelated) could shed enormous light on this very dark space. We hope this intriguing study will stimulate others to address this question head-on and where it matters most, at the bedside. ■

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Paper III

CRITICAL CARE

Platelet aggregation and clot formation in comatose survivors of cardiac arrest treated with induced hypothermia and dual platelet inhibition with aspirin and ticagrelor; a prospective observational study

Thomas Kander[*], Josef Dankiewicz, Hans Friberg and Ulf Schött

Abstract

Introduction: We conducted a prospective observational study in cardiac arrest survivors treated with mild induced hypothermia, evaluating different platelet function tests at hypo- and normothermia. We also investigated the relation between gastric emptying and vasodilator stimulated phosphoprotein (VASP).

Methods: Comatose survivors of out of hospital cardiac arrest were included and divided into two groups, depending on whether dual platelet inhibition with peroral ticagrelor and aspirin was given or not. The first blood samples (T1) were collected 12–24 hours after reaching target temperature (33°C) and were compared to blood samples collected 12–28 hours after reaching normothermia (37°C) (T2) within each group. All samples were analysed by Sonoclot viscoelasticity, flow cytometry based VASP and with multiple electrode aggregometry, Multiplate®; adenosine diphosphate (ADP), collagen (COL), thrombin receptor agonist peptide (TRAP) and arachidonic acid (ASPI). Sonoclot and Multiplate® instruments were set on in vivo temperatures. Gastric secretion from the nasogastric tube was measured to assess absorption of per orally administered antiplatelet drugs. Differences between T1 and T2 within each group were calculated using Wilcoxon matched pairs signed test. Significance levels were set at $P < 0.01$.

Results: In total, 23 patients were included. In patients with dual platelet inhibition (n =14) Multiplate®-analyses showed no changes in ADP stimulated platelets. COL, TRAP and ASPI aggregations were higher at T2 compared to T1. Sonoclot-analyses showed that activated clotting time (ACT) was unchanged but both clot rate (CR) and platelet function (PF) were higher at T2 compared to T1. VASP decreased from 53 ± 28(T1) to 24 ± 22(T2), ($P < 0.001$). The average volume of gastric secretion aspirated before T1 correlated well with VASP (T1), r =0.81 ($P < 0.001$). In patients with no platelet inhibition, (n =9) similar changes between T1 and T2 were seen as in patients with dual platelet inhibition while VASP was unchanged.

Conclusions: We have demonstrated increased platelet aggregation and strengthened clot formation over time in out of hospital cardiac arrest patients treated with hypothermia. In patients on oral dual platelet inhibition, the effect of ticagrelor was delayed, probably due to slow gastric emptying.

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BioMed Central

Introduction

Mild induced hypothermia (MIH) is indicated for comatose survivors of out-of-hospital cardiac arrest (OHCA) to improve neurological outcome [1-3]. However, a recent multicenter study - the target temperature management (TTM) trial [4] in OHCA patients found that a targeted temperature of 33°C did not confer a benefit as compared with a targeted temperature of 36°C and has in some sense challenged current guidelines.

In trauma, hypothermia increases bleeding and worsens outcome [5,6]. Therefore MIH is considered contraindicated in cardiac arrest patients with bleeding and especially intracerebral bleeding [3] and computer tomography (CT) of the brain is often performed prior to MIH. Conventional wisdom holds that hypothermia reduces coagulation, platelet function and impairs primary and secondary haemostasis. Whether this is true also during MIH is still debated [7]. A few animal studies support weakened markers of haemostasis during hypothermia [8-12] while others do not [13-15]. Several reports of studies performed using blood from healthy volunteers, which was incubated at different temperatures, have been published with contradictory results. Some studies show that hypothermia decreases haemostasis [16-21], while others show the opposite [22-26]. Studies including patients treated with MIH after OHCA are more infrequent. In two such studies [27,28] thromboelastography analyses were performed, both studies indicating decreased coagulation with prolonged clot initiation during hypothermia.

Cardiac arrest patients often undergo emergency coronary interventions with stenting, and receive dual antiplatelet therapy, including aspirin and a $P2Y_{12}$ antagonist. The effect of platelet inhibition with the $P2Y_{12}$-antagonist pro-drug clopidogrel may vary secondary to differences in intestinal absorption, variations in liver cytochrome activities, drug interactions, and platelet receptor polymorphisms [29].

Viscoelastic tests such as thromboelastography or Sonoclot do not detect aspirin or $P2Y_{12}$-antagonist effects on haemostasis [30]. With flow cytometry-based vasodilator-stimulated phosphorylated phosphoprotein (VASP) analysis, the effect of $P2Y_{12}$-antagonists can be detected and has been shown to be decreased when clopidogrel is given during MIH [31,32]. To our knowledge there are presently no studies analysing VASP in patients receiving the more potent $P2Y_{12}$-antagonist, ticagrelor, together with aspirin in the OHCA treatment setting. Additionally, cardiac arrest patients develop a systemic inflammatory response syndrome (SIRS) analogous to the changes seen in sepsis, which may be both pro- and antihaemostatic [33-36].

Multiple electrode aggregometry (Multiplate®) is a relatively new tool used to assess adequate patient response to platelet inhibitors [37] and also to evaluate platelet aggregability in sepsis [33,35,36]. The viscoelastic test, Sonoclot has been shown to be superior to thromboelastographic methods for detection of platelet inhibition in hypothermic animals, using glass bead activation [11,15]. It is unknown how haemostasis measured with Multiplate® and Sonoclot is affected after OHCA and MIH in the intensive care setting and the effect of ticagrelor on the VASP analysis is also unexplored. We conducted a prospective observational study in cardiac arrest survivors either with or without ticagrelor and aspirin treatment and assessed haemostasis using Multiplate®, Sonoclot and VASP. We also investigated the relationship between gastric emptying and VASP.

Methods

This prospective, observational, single-centre study was approved by the regional ethical review board in Lund (registration numbers 411/2004, 223/2008 and 2013/284) and included comatose survivors of OHCA of all origins at the Department of Intensive and Perioperative Care, Skåne University Hospital, Lund, Sweden from February 2012 to October 2013. Informed and written consent was obtained from the next of kin and from all survivors.

Patients were eligible if they had return of spontaneous circulation (ROSC) after non-traumatic OHCA of any origin, were comatose (Glasgow coma scale (GCS) score ≤7) upon admission, and >18 years old. Exclusion criteria were pregnancy, suspicion of intracranial haemorrhage, diagnosed terminal illness, known coagulopathy, and anticoagulant therapy (other than prophylactic dose of low-molecular-weight heparin (LMWH)). Patients were divided into two groups dependent on whether they received dual platelet inhibition or not. All analyses were performed over time within each group; no comparisons were made between the groups.

Patients were investigated with CT of the brain to rule out cerebral haemorrhage before inclusion. Coronary angiography and percutaneous coronary intervention (PCI) was performed at the discretion of the treating cardiologist. All patients were treated in accordance with a standardized protocol for MIH. Hypothermia was induced with cold saline (30 ml/kg) and maintained using a cooling device, that is, surface cooling (Arctic Sun®, Medivance®, Louisville, CO, USA) or intravascular cooling (IcyCath®, ZOLL, Sunnyvale, CA, USA) at the discretion of the responsible intensivist. Patients received hypothermia for 24 h at 33 ± 1°C and controlled rewarming at 0.5°C/h. Patients received the following standardized procedures: Foley catheters with incorporated temperature probes, arterial catheters, central venous catheters, and mechanical ventilation after intubation. Patients were sedated with propofol 2 to 4 mg/kg/h and fentanyl 1 to 3 µg/kg/h. Neuromuscular blockade was induced with rocuronium (0.5 mg/kg bolus) if needed, to treat shivering. Enteral

nutrition (Isosource® Standard, Nestlé HealthCare Nutrition, New York, NY, USA) was started on arrival at the ICU, at 10 ml/h and continued throughout the study period. Aspiration of gastric secretions from the nasogastric tube was performed every 4 h and enteral nutrition paused if the aspirate exceeded 200 ml. The average aspirated volume was registered. See Gastric secretion below, and flowchart (Figure 1).

Anticoagulation and platelet inhibition
No patients received thrombolysis. All patients received LMWH, enoxaparin, 40 mg subcutaneously once daily. The clinician decided whether patients should receive dual platelet inhibition with ticagrelor (180 mg loading dose followed by 90 mg twice daily) and aspirin (300 mg loading dose followed by 75 mg daily), normally depending on whether or not emergency PCI was performed. Administration of the dual platelet inhibition was standardized; a loading dose was given 0 to 4 h before target temperature was reached and further maintenance doses were given every 12 (ticagrelor) or 24 (aspirin) h. In the data analysis patients treated with dual platelet inhibition were treated separately from patients not receiving this treatment.

Blood sampling
Blood was drawn through an arterial catheter using a Safedraw™ PMSET 1DT, (Argon Critical Care Systems, Singapore) and collected in a vacutainer system (BD, Plymouth, UK). The first sampling occasion, time-1 (T1), was during stable hypothermia, that is, 12 to 24 h after reaching target temperature (33°C). The second sampling occasion (T2) was 12 to 28 h after reaching normothermia.

Blood samples were analysed at each time point by conventional coagulation tests (activated partial thromboplastin time (aPTT), prothrombin time, international normalized ratio (PT-INR), platelet count and fibrinogen) along with Multiplate®, Sonoclot, VASP and C-reactive

protein (CRP). Blood samples for Multiplate® analysis were collected in 3.0-ml tubes containing recombinant hirudin (Dynabyte GmbH, Munich, Germany). Blood for VASP-analysis was collected in a 4.5-ml tube containing 0.109 M citrate (BD Vacutainer systems, Plymouth, UK). The Sonoclot analysis was performed on native blood collected in a 2-ml syringe (BD, Plymouth, UK).

Conventional haematological tests: PT-INR, aPTT, fibrinogen and platelet count analyses were performed at the accredited hospital laboratory. PT-INR, aPTT and fibrinogen were measured using a Sysmex 5100 analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). The PT-INR assay was performed with the Owren PT reagent (Siemens) calibrated using international normalized ratio (INR) calibrators certified by the Swedish external quality assessment organization EQUALIS AB (Uppsala, Sweden), traceable to World Health Organisation (WHO) RBT/90 standard. The normal value for PT-INR is <1.2. For aPTT, actin FSL and for fibrinogen, Dade Thrombin reagent was used (Siemens). The reference intervals have been established locally for aPTT to 26 to 33 s and for fibrinogen to 2 to 4 g/L.

Platelet count was measured using the Sysmex XN-10 analyzer (Siemens). The locally determined reference range for platelet count is 165 to 387×10^9/L for adult women and 145 to 348×10^9/L for adult men. CRP-was measured using the Cobas 6000/8000 (Roche Diagnostics GmbH, Mannheim, Germany) and a standard procedure; the normal value is <3 mg/L.

VASP was analysed at 37°C using a dual colour flow cytometer assay (PLT VASP/$P2Y_{12}$, BioCytex, Marseille, France), which is specific for $P2Y_{12}$ inhibitors [38]. The ratios between phosphorylated and non-phosphorylated VASP were used to calculate the platelet reactivity index (PRI), which reflects the effect of ticagrelor. According to previous studies, a PRI <50% is regarded as a satisfactory effect of $P2Y_{12}$ inhibitors, a PRI ≥50% is regarded as an unsatisfactory effect of $P2Y_{12}$ inhibitors

Figure 1 Flowchart. Loading and daily doses of ticacrelor and aspirin at the clinicians descretion (n =14). Nine patients did not receive ticagrelor and aspirin. All patients received enoxaparin. T1 blood samples 12 to 24 h after reaching hypothermia. T2 blood samples 16 to 28 hours after reaching normothermia. OHCA, out-of-hospital cardiac arrest; MIH, mild induced hypothermia.
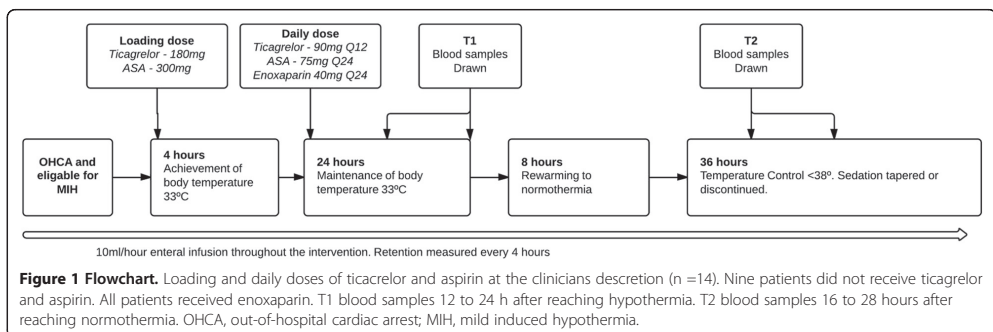
[39], and PRI ≥70% is considered a normal value for untreated patients.

Multiplate®, multiple electrode aggregometry (Roche, Rotkreuz, Switzerland) was used to measure an agonist-induced platelet aggregation. Hirudin-anticoagulated whole blood was stored at room temperature before analysis within 0.5 to 3.0 h of blood sampling. Both the samples, taken with the patient in stable hypothermia (T1) and in stable normothermia at (T2), were analysed with the Multiplate® set first at 33°C in duplicate test cells and then at 37°C in duplicate test cells. The extent of platelet aggregation is measured by resistance (impedance) changes between two electrodes, and then depicted as a graph. The area under the curve (AUC) is the best aggregometry parameter of the Multiplate® test. Four test assays were performed; ADPtest (platelet aggregation in response to adenosine-5′-diphosphate), COLtest (platelet aggregation in response to collagen), TRAPtest (platelet aggregation in response to thrombin receptor agonist peptide) and ASPItest (platelet aggregation in response to arachidonic acid).

A Sonoclot Analyzer (Sienco® Inc. Arvada, USA) with a temperature regulated heating or cooling plate was used to study temperature effects on native blood. Both the samples taken with the patient in stable hypothermia (T1) and in stable normothermia at (T2) were analysed within 2 minutes from sampling with the Sonoclot Analyzer set at 33°C and at 37°C. Analyses were performed in duplicate for both temperatures according to the manufacturer's recommendations. A glass bead test (Sienco® gbACT + ™ Kit), which has been designed to initiate coagulation in a more stable manner than previous tests, was used. The Sonoclot measures the viscoelastic drag-impedance that fibrin and platelets in a whole blood sample impose upon the oscillating Sono-probe. A time-based graph reflects the different steps in the clotting whole blood sample, called the Sonoclot signature. The measured parameters are with defined normal values in parentheses are as follows: 1) ACT (activated clotting time) (100 to 155 s) is the time taken for the first fibrin to form, and is defined as the time it takes for the signature to move 1% upscale from the start of the graph (immersion response) and corresponds to aPTT and traditional ACT tests; 2) clot rate (CR) (9 to 35 units/minute): the rate of increase in the clot impedance due to fibrin formation and polymerization (the slope of the signature after ACT) in % of full scale per minute; 3) platelet function (PF) (>1.5 units) is the point where the squeezing out of trapped serum in the contracting clot (sign of functioning platelets) exceeds the accumulation of clot bulk on the probe and can be described as the time to peak from the immersion response. PF performs better than previous peak amplitude and time to peak parameters, reflecting the gPIIb/IIIa-dependent clot retraction [40].

Gastric secretion

To estimate the degree of ventricle retention, aspiration of gastric secretions was performed every 4 h. The average aspirated volume during 0 to 24 h after reaching hypothermia (for T1 samples) and during 0 to 28 h after reaching normothermia (for the T2 samples) was registered.

Statistics

Non-parametric variables (Gaussian distribution not assumed) were summarized using the median with range (minimum to maximum). Parametric variables were summarized using mean ± SD. To reduce the risk of a Type I error due to multiple testing the significance level was set at a P-value <0.01. Differences between results were calculated using the two-tailed, Wilcoxon matched-pairs signed test. Correlation coefficients were calculated using Spearman's rank correlation method. Differences in categorical data were calculated using Fisher's exact test. All statistical analyses were performed using GraphPad Prism 5, version 5.03; GraphPad Software, La Jolla, CA, USA.

Results

Twenty-three patients were included, fourteen patients in the group with dual platelet inhibition therapy and nine in the group without platelet inhibition therapy. Demographic data of all subjects are shown in Table 1.

Results from the blood analyses are shown in Table 2. The most important findings are presented below.

Table 1 Patient demographics

	Platelet inhibition (n =14 patients)	No platelet inhibition (n =9 patients)
Age, years	66 ± 8	65 ± 13
Male sex, n (%)	10 (71)	8 (89)
Simplified acute physiology score 3	71 ± 16	75 ± 15
Estimated mortality risk, %	54 ± 27	69 ± 24
Bystander cardiopulmonary resuscitation, n (%)	10 (71)	5 (56)
Time to return of spontaneous circulation, minutes	30 (5 to 45)	25 (6 to 37)
30-day mortality, n (%)	8 (58)	5 (56)
Percutaneous coronary intervention, n (%)	12 (86)	0 (0)
Origin of cardiac arrest		
Acute myocardial infarction, n (%)	12 (86)	0 (0)
Primary arrhythmia, n (%)	2 (14)	5 (56)
Hypoxia, not hanging (%)	0 (0)	3 (33)
Hanging, n (%)	0 (0)	1 (11)

Results presented as mean ± SD, n (%) or medians (range (minimum to maximum)).

Table 2 Blood analyses

	With platelet inhibition (n =14 patients)			With no platelet inhibition (n =9 patients)		
	T1	T2	*P*-value	T1	T2	*P*-value
Platelets, 10^9/L	189 ± 59	179 ± 64	0.26	206 ± 61	191 ± 68	0.42
Prothrombin time, international normalized ratio	1.1 ± 0.1	1.2 ± 0.2	0.03	1.2 ± 0.1	1.4 ± 0.4	0.14
Activated partial thromboplastin time, s	34 ± 8	36 ± 11	0.63	34 ± 10	40 ± 17	0.08
Fibrinogen, g/L	3.2 ± 0.9	4.9 ± 1.3**	0.002	3.4 ± 1.8	5.1 ± 1.6	0.02
C-reactive protein, mg/L	52 ± 38	147 ± 72***	<0.001	73 ± 68	141 ± 28	0.02
VASP						
VASP, PRI,%	53 ± 28	24 ± 22***	<0.001	83(75 to 85)	76(50 to 84)	0.38
Samples with adequate effect (PRI <50%), n (%)	7 (50)	12 (86)	0.10	1 (11)	1 (11)	
Samples below normal value (PRI <70%), n (%)	9 (64)	13 (93)	0.16	1 (11)	1 (11)	
Multiple electrode aggregometry						
Adenosine diphosphate-agonist, AUC	22 (0 to 79)	20 (12 to 43)	0.61	65 (30 to 114)	46 (22 to 145)	0.67
Opposite-analysis temperature	21 (4 to 58)	18 (11 to 52)		62 (24 to 140)	61 (40 to 120)	
Collagen agonist, AUC	41 (26 to 62)	56 (25 to 117)***	<0.001	64 (42 to 104)	72 (52 to 147)**	0.0039
Opposite-analysis temperature	42 (27 to 94)	49 (27 to 75)		68 (40 to 123)	74 (60 to 122)	
Thrombin receptor agonist peptide, AUC	90 (8 to 116)	99 (82 to 151)**	0.006	83 (40 to 153)	98 (91 to 183)**	0.008
Opposite-analysis temperature	86 (7 to 143)	83 (50 to 107)		89 (44 to 154)	102 (83 to 161)	
Arachidonic acid-agonist, AUC	11 (2 to 39)	20 (11 to 62)**	0.003	46 (7 to 130)	66 (18 to 163)**	0.0039
Opposite-analysis temperature	14 (3 to 129)	12 (3 to 28)[a]		50 (9 to 180)	72 (10 to 148)	
Sonoclot						
Activated clotting time, s	144 (72 to 253)	120 (88 to 191)	0.19	133 (95 to 244)	153 (76 to 213)	0.98
Opposite-analysis temperature	121 (74 to 192)	126 (102 to 226)		120 (79-238)[a]	166 (81 to 223)	
Clotting rate, units/minute	25 (15 to 49)	37 (23 to 47)***	<0.001	28 (18 to 64)	41 (24 to 63)	0.44
Opposite-analysis temperature	33 (18 to 50)	32 (15 to 44)[a]		39 (22 to 74)[a]	35 (17 to 46)	
Platelet function, units	2.9 (0.4 to 4.4)	3.5 (2.8 to 5.0)***	<0.001	2.9 (0.9 to 4.9)	4.1 (1.8 to 5.2)**	0.0039
Opposite-analysis temperature	4.2 (0.45 to 4.7)[b]	3.2 (1.3 to 4.2)[a]		3.9 (1.9 to 5.0)	3.4 (2.7 to 5.1)	

Results presented as mean ± SD, median (range (minimum to maximum), or number (%). T1, blood sampling 12 to 24 h after reaching 33°C; T2 blood sampling 16 to 28 h after reaching normothermia. Multiple electrode aggregometry, (Multiplate®) and Sonoclot analyses set on the patient's body temperature at the sampling occasion and at Opposite-analysis temperature (that is, 37°C if the patient's body temperature was 33°C and vice versa). Differences between laboratory results for T2 versus T1 (*P*-values) were calculated using two-tailed, paired *t*-test for means, two-tailed paired Wilcoxon matched pairs signed test for medians and Fisher's exact test for categorical variables. **P <0.01. ***P <0.001. [a]P <0.01 compared to the other analysed temperature. [b]P <0.001 compared to the other analysed temperature on the same sampling occasion. VASP, vasodilator-stimulated phosphorylated phosphoprotein; PRI, platelet reactivity index; AUC: area under the curve.

Patients with dual platelet inhibition: platelet count, PT-INR and aPTT were all unchanged between T1 and T2. Fibrinogen increased from 3.2 ± 0.9 (T1) to 4.9 ± 1.3 (T2), (*P* =0.002) and CRP increased from 52 ± 38 (T1) to 147 ± 72 (T2) (*P* <0.001). VASP decreased from 53 ± 28 (T1) to 24 ± 22 (T2), (*P* <0.001). Multiplate®-analyses, with the analyses temperature set on the *in vivo* temperature, showed no changes in ADP-stimulated platelets. COL, TRAP and ASPI tests were all increased at T2 compared to T1 (Table 2 and Figure 2). In the Sonoclot-analyses ACT was unchanged but both CR and PF was increased at T2 compared to T1 (Table 2 and Figure 2).

Patients with no platelet inhibition: platelet count, PT-INR, aPTT, and VASP did not change significantly. Multiplate®-analyses were performed with the analyses temperature set on the patient's body temperature. ADP

and TRAP tests were not changed significantly. COL and ASPI tests increased at T2 compared to T1 (Table 2 and Figure 2). In the Sonoclot®-analyses ACT and CR were unchanged but PF was increased in T2 compared to T1 (Table 2 and Figure 2).

Correlation aspirated gastric secretion - VASP: the median volume of gastric secretion aspirated in patients with dual platelet inhibition was 105 (10 to 200) ml during T1 and 65 (10 to 200) ml during T2 (not significant). The volume of gastric secretion aspirated during T1 correlated well with VASP (T1), *r* =0.81 (*P* <0.001) (Figure 3). This correlation was not detected at T2.

Discussion

In this prospective observational study on OHCA patients treated with MIH we have demonstrated an increase in

Figure 2 Results from blood analyses for individual patients. Multiplate® and Sonoclot instruments set on the *in-vivo* temperature. PI, patients with dual platelet inhibition (n =14); nPI, patients with no platelet inhibition (n =9); T1, blood sampling 12 to 24 h after reaching 33°C body temperature; T2, blood sampling 16 to 28 h after reaching normothermia. Multiple electrode aggregometry, Multiplate®: ADP: adenosine diphosphate-agonist; COL, collagen-agonist; TRAP, thrombin-agonist; ASPI, arachidonic-acid agonist. Sonoclot analyses: ACT, activated clotting time; CR, clotting rate; PF, platelet function. AUC, area under curve; **P <0.01; ***P <0.001.

Multiplate®-assays COL, TRAP, ASPI and Sonoclot assays CR and PF between the stable hypothermic and the stable normothermic state, indicating increased platelet aggregation and strengthened clot formation.

This observational study did not include a normothermic control group, thus the cause for the increased platelet aggregability and viscoelastic clot formation between stable hypothermia (T1) and stable normothermia

Figure 3 Correlation between aspirated gastric secretion and vasodilator-stimulated phosphorylated phosphoprotein (VASP). Patients with dual platelet inhibition (n =14). Gastric secretion T1, median volume of gastric secretion aspirated from nasogastric tube, 0 to 24 h after reaching 33°C body temperature; VASP T1, 12 to 24 h after reaching 33°C body temperature; PRI, platelet reactivity index.

(T2) is not known but two possible explanations will be discussed. Hypothermia has been shown to decrease haemostasis measured with viscoelastic methods [11,18,19,28] and with platelet function testing [16,17,21]. Thus, the lower temperature at T1 could be responsible for the decreased values as compared to T2. In addition, post-resuscitation stress responses after cardiac arrest mimic the immunologic and coagulation disorders observed in severe sepsis [41]. Presumably the low-flow state during cardiac arrest, followed by a reperfusion injury after return of spontaneous circulation is responsible for a SIRS reaction that may cause an activation of the coagulation system [36,42]. This can be compared to studies by Adamzik *et al*. [33] and Brenner *et al*. [35] that showed reduced platelet aggregation, measured with Multiplate®, in patients with severe sepsis. In our study CRP and fibrinogen increased between the sampling occasions (Table 2) in all patients indicating a significant SIRS with an acute phase reaction 2 to 3 days after OHCA, although this did not reach statistical significance in the group without platelet inhibition.

The effect of ticagrelor depends on intestinal absorption to the systemic circulation. It is well-known that hypothermia, opioids and acute critical illness reduce gastrointestinal motility [43]. In the present study 50% of the patients with dual platelet inhibition did not reach the target VASP PRI <50% at the first sampling occasion (T1), thus placing them at risk for thromboembolic events. This is in agreement with previous findings of impaired bioavailability of clopidogrel in critically ill patients [44] and comparable with the results from Bjelland *et al*. [31] and Ibrahim *et al*. [32], which demonstrated high rates of non-responders in clopidogrel-treated patients

(100% and 83%, respectively) as well as in ticagrelor-treated patients (61%) [45] in MIH after cardiac arrest. In the present study we also demonstrated significant correlation between gastric emptying and VASP during hypothermia (Figure 3), indicating that gastric emptying is responsible for the limited ticagrelor effect observed at T1. These findings underline the importance of stimulating motility of the gastro-intestinal tract as soon as possible in the cardiac arrest patient who is dependent on absorption of oral $P2Y_{12}$ inhibitors to limit the risk of stent thrombosis. This is also demonstrated in a recent observational study by Joffe *et al*. [46], including patients with acute myocardial infarction treated with coronary stents. In the study by Joffe *et al*. cardiac arrest patients did not receive $P2Y_{12}$ inhibitors and had a much higher incidence of stent thrombosis (10.9%) than patients in the control group (2.0%), who were treated with $P2Y_{12}$ inhibitors but had not had previous cardiac arrests.

VASP analysis is specifically designed to monitor only the $P2Y_{12}$ platelet inhibitory effect on platelets [38] and as expected, only patients on $P2Y_{12}$ platelet inhibitory medication actually exhibited VASP changes. Multiplate® and Sonoclot tests on the other hand, are not as specific as VASP analysis and are sensitive to many pro- and anti-haemostatic variables [24,25,33,35,47], not detectable with VASP. Furthermore, Multiplate®-assays (except ADPassay) and Sonoclot assays cannot detect moderate $P2Y_{12}$ inhibition [30]. These characteristics of the different analyses explain why Multiplate®-assays, COL, TRAP, ASPI and Sonoclot assays, CR and PF, in our study show strengthened platelet function and coagulation over time, and on the other hand, $P2Y_{12}$-sensitive VASP analysis show weakened platelet function in ticagrelor treated patients.

We performed all Multiplate® and Sonoclot analyses with the instruments set both at the patient's actual body temperature (the *in vivo* temperature) and at the opposite temperature, that is, 33°C or 37°C. This was not performed to determine whether the rise in body temperature or the SIRS is responsible for the increased values in normothermia, but rather to identify how the temperature of the instrument affects the results. We found that ASPItest in the Multiplate® analyses and multiple assays in the Sonoclot analyses indicated weaker values when normothermic samples were cooled and stronger values when hypothermic samples were warmed (Table 2). This is in agreement with results from Shimokawa *et al*. [11] that showed the importance of performing hemostatic measurements with the Sonoclot and thromboelastography at the actual *in vivo* (hypothermic) temperature and not only at the traditional 37°C setting. The PF platelet parameter used in the present study better reflects clot contraction than the parameters used by Shimokawa [40].

Other limitations of the present study include that platelet function assay was done under minimal shear, so

that platelet function under flow, which is influenced by haematocrit and von Willebrand Factor, could not be assessed [48]. Furthermore, this study did not explore the causes for the increased platelet aggregability and the viscoelastic clot formation. We welcome a randomized controlled trial to explore whether hypothermia, the SIRS reaction, an unknown factor or a combination of factors are responsible for the observed changes.

Conclusions

We have demonstrated increased platelet aggregation and strengthened clot formation over time in out-of-hospital cardiac arrest patients treated with hypothermia. In patients on oral dual platelet inhibition, the effect of ticagrelor was delayed, probably due to slow gastric emptying.

Key messages

- Platelet aggregation and clot formation demonstrated with Multiplate® and Sonoclot are strengthened over time in out-of-hospital cardiac arrest patients treated with hypothermia
- Fifty percent of the patients on oral ticagrelor did not reach the target VASP PRI <50% at the first sampling occasion 12 to 24 hours after reaching hypothermia, thus placing them at risk for thromboembolic events
- The effect of ticagrelor was delayed in survivors of cardiac arrest, probably due to slow gastric emptying

Abbreviations

ACT: activated clotting time; ADP: adenosine-5′-diphosphate agonist; aPTT: activated partial thromboplastin time; ASPI: arachidonic acid agonist; COL: collagen agonist; CR: clot rate; CRP: C-reactive protein; GCS: Glasgow coma scale; LMWH: low-molecular-weight heparin; MIH: mild induced hypothermia; nPI: non-platelet inhibition; OHCA: out-of-hospital cardiac arrest; PCI: percutaneous coronary intervention; PF: platelet function; PI: platelet inhibition; PRI: platelet reactivity index; PT-INR: prothrombin time international normalized ratio; ROSC: return of spontaneous circulation; SAPS3: simplified acute physiology score 3; SIRS: systemic inflammatory response syndrome; TRAP: thrombin receptor agonist peptide; VASP: vasodilator-stimulated phosphoprotein.

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Paper IV

Wide Temperature Range Testing
with ROTEM Coagulation Analyses

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Mild induced hypothermia is used for neuroprotection in patients successfully resuscitated after cardiac arrest. Temperature-dependent effects on rotational thromboelastometry (ROTEM®) assays with EXTEM®, FIBTEM®, or APTEM® in cardiac arrest patients have not previously been studied. Ten patients with out-of-hospital cardiac arrest who underwent induced hypothermia were studied during stable hypothermia at 33°C. ROTEM temperature effects on EXTEM, FIBTEM, and APTEM assays were studied at temperatures set between 30°C and 42°C. Citrated whole blood test tubes were incubated in temperature-adjusted heating blocks and then investigated at respective temperature in the temperature-adjusted ROTEM. The following variables were determined: clotting time (CT), clot formation time (CFT), α-angle, and maximum clot firmness (MCF). The results from hypo- and hyperthermia samples were compared with the samples incubated at 37°C using the Wilcoxon matched-pairs signed-rank test. A p-value of <0.05 was considered significant. CT-EXTEM® and CT-APTEM® were prolonged by hypothermia at 30°C ($p < 0.01$ for both) and 33°C ($p < 0.05$ for both). Hyperthermia at 42°C shortened CT-EXTEM ($p < 0.05$) and CT-APTEM ($p < 0.01$). CFT-EXTEM® and CFT-APTEM® were markedly prolonged by hypothermia at 30°C, 33°C, and 35°C ($p < 0.01$ for all except CFT-EXTEM, 35°C [$p < 0.05$]). The α-angle-EXTEM was markedly decreased at 30°C, 33°C, and 35°C ($p < 0.01$) but increased at 40°C ($p < 0.05$) and 42°C ($p < 0.01$); α-angle-APTEM showed similar results. MCF was unchanged at different temperatures for all tests. ROTEM (EXTEM, FIBTEM, and APTEM assays) revealed a hypocoagulative response to *in vitro*-applied hypothermia in the blood of cardiac arrest patients reflected in the prolonged clot initiation and decreased clot propagation. Hyperthermia showed the opposite effects. Clot firmness was not affected by temperature.

Introduction

HYPOTHERMIA IS FREQUENTLY used as a therapeutic option to protect against neurological deficits in comatose survivors after cardiac arrest (Bernard *et al.*, 2002; Holzer *et al.*, 2002; Peberdy *et al.*, 2010) and in cardiac surgery (Luehr *et al.*, 2014). In trauma, the combination, hypothermia, acidosis, and coagulopathy, is commonly referred to as the lethal triad and affects mortality (Mikhail, 1999). Deep hypothermia has been shown to affect both coagulation and platelet function (Wolberg *et al.*, 2004; Ortmann *et al.*, 2012). Whether mild induced hypothermia, 33°C–36°C, has the same effect is still debated (Varon and Acosta, 2009; Polderman, 2012).

The effect of hypothermia on thromboelastography (TEG®) (Heinius *et al.*, 2002; Shimokawa *et al.*, 2003; Martini *et al.*, 2008; Ivan *et al.*, 2011) and rotational thromboelastometry (ROTEM®, Pentapharm GmbH, Munich, Germany), using INTEM® or HEPTEM® assays (Dirkmann *et al.*, 2008; Rundgren and Engström, 2008; Spiel *et al.*, 2009), has pre-

viously been investigated. Two of these studies were performed on cardiac arrest patients (Spiel *et al.*, 2009; Ivan *et al.*, 2011) and the others on healthy volunteers (Dirkmann *et al.*, 2008; Rundgren and Engström, 2008) or animals (Heinius *et al.*, 2002; Shimokawa *et al.*, 2003; Martini *et al.*, 2008). These studies show that during hypothermia (30°C–35°C), the time of clot initiation and the speed of clot propagation are increased, but maximal clot strength and firmness are essentially unchanged compared with normothermia. EXTEM®, FIBTEM®, and APTEM® are the other ROTEM® assays (Table 1) with different activators that have emerged and are extensively used in trauma patients (Schochl *et al.*, 2010). To the best of our knowledge, the effect of different temperatures on these assays in cardiac arrest patients has not been investigated. The aim of the present study was to investigate the temperature-dependent effects on ROTEM (EXTEM, FIBTEM, and APTEM assays) using whole blood isolated from patients who suffered an out-of-hospital cardiac arrest (OHCA) and were treated with hypothermia.

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TABLE 1. OVERVIEW OF ROTEM ASSAYS

Assay	Activator	Additives	Information
EXTEM®	Tissue factor		Global test. Plasmatic coagulation factors, fibrin polymerization, platelet function and count.
FIBTEM®	Tissue factor	Platelet inhibition by cytochalasin D	Fibrin status. Identification of polymerization disorders or deficiency.
APTEM®	Tissue factor	Fibrinolysis inhibition by aprotinin	Detection or exclusion of hyperfibrinolysis.
INTEM®	Contact activator		Global test. Plasmatic coagulation factors, fibrin polymerization, platelet function and count.
HEPTEM®	Contact activator	Heparin inactivation by heparinase	Screening test in the presence of heparinase.

Materials and Methods

This prospective observational study was approved by the regional ethical review board in Lund (registration numbers 411/2004, 223/2008, and 2013/284) and included comatose survivors of OHCA of all origins at the Department of Intensive and Perioperative Care, Skåne University Hospital, Lund, Sweden. Informed and written consent was obtained from all survivors. Patients were eligible if they had return of spontaneous circulation after nontraumatic OHCA of all origins, were comatose (GCS ≤ 7) upon admission, and were > 18 years old. Patients were treated according to the standard of care after cardiac arrest with body temperature at 33°C for 24 hours.

During stable hypothermia, blood was drawn through an arterial catheter using the Safedraw™ PMSET 1DT (Argon Critical Care Systems, Singapore, Singapore) and collected in tubes (BD Vacutainer Systems, Plymouth, United Kingdom). Blood samples were analyzed by conventional coagulation tests, prothrombin time (PT)/international normalized ratio (INR), activated partial thromboplastin time (aPTT), platelet count, fibrinogen, and C-reactive protein (CRP), along with ROTEM.

Conventional laboratory analyses were performed at the accredited hospital laboratory according to the standard procedure. The reference range for platelets is $165–387 \times 10^9$/L for adult women and $145–348 \times 10^9$/L for adult men. The reference range for PT/INR is 0.9–1.2, for aPTT is 22–44 seconds, and for fibrinogen is 2–4 g/L. Normal value for CRP is <3 mg/L.

ROTEM

Blood was collected in 4.5-mL tubes containing 0.109 M citrate (BD Vacutainer Systems). The tubes were incubated for 30–60 minutes at specified temperatures of 30°C, 33°C, 35°C, 37°C, 40°C, and 42°C in heating blocks. Temperatures were ensured with a temperature probe in reference sample tubes stored in the heating blocks. Analyses were performed with the ROTEM instrument set on the incubation temperatures. Tissue factor was used to trigger coagulation for all assays (EXTEM, FIBTEM, and APTEM). Thrombin-mediated platelet activation and fibrin polymerization are reflected on EXTEM, whereas fibrin polymerization is selectively shown on FIBTEM by inhibiting platelet–fibrin interactions using cytochalasin D. In APTEM, aprotinin is added to eliminate any fibrinolytic component in the sample. A summary of the various tests is shown in Table 1.

The following parameters were analyzed and digitally recorded:

Clotting time (CT): time from the start of measurement until the first signs of clotting— reflects *initiation of clotting* with thrombin and fibrin formation and then the initial start of clot polymerization (on ROTEM defined as the time until a 2 mm amplitude is detected on the thromboelastogram).

Clot formation time (CFT): time after CT until a clot firmness of 20 mm is detected on the thromboelastogram— reflects clot formation dynamics (*clot propagation*) and depends on fibrin polymerization, stabilization of the clot with platelets, and F XIII.

The α-angle: also reflects clot formation dynamics (*clot propagation*) and is defined by the angle between the center horizontal line and a tangent to the curve through the 2 mm amplitude point.

Maximum clot firmness (MCF): firmness of the clot— increasing stabilization of the clot by the polymerized fibrin, platelets, as well as F XIII.

TABLE 2. PATIENT DEMOGRAPHICS AND LABORATORY RESULTS (N = 10)

Age (years)	63 ± 9
Male (%)	5 (50)
SAPS3	68 ± 18
EMR (%)	51 ± 29
Bystander CPR (%)	8 (80)
Time to ROSC (min)	27 (5–45)
30-Day mortality (%)	5 (50)
PCI (%)	6 (60)
Dual platelet inhibition[a]	6 (60)
Origin of cardiac arrest	
Acute myocardial infarction (%)	7 (70)
Primary arrhythmia (%)	2 (20)
Hypoxia (%)	1 (10)
Laboratory results[b]	
Platelets, 10^9/L	176 ± 61
PT/INR	1.1 ± 0.1
aPTT, seconds	36 ± 9
Fibrinogen, g/L	3.6 ± 1.1
CRP, mg/L	68 ± 34

Mean ± SD or medians with range (min–max).
[a]Ticagrelor and aspirin.
[b]At stable hypothermia, 33°C.
SAPS3, simplified acute physiology score 3; EMR, estimated mortality risk; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation; PCI, percutaneous coronary intervention; PT/INR, prothrombin time/international normalized ratio; aPTT, activated partial thromboplastin time; CRP, C-reactive protein.

Statistics

Variables were considered nonparametric (Gaussian distribution not assumed) and were summarized using the median with 25th and 75th percentiles Q2 (Q1 and Q3). Results for 30°C, 33°C, 35°C, 40°C, and 42°C samples were compared with results from the normothermic blood samples (37°C) using two-tailed, Wilcoxon matched-pairs signed-rank test. p-Values <0.05 were considered significant. All statistical analyses were performed using the GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Results

Whole blood from 10 cardiac arrest patients was included. For patient demographics, see Table 2.

Results from the conventional blood analyses are shown in Table 2. Results from ROTEM analyses are shown in Table 3 and Figures 1 and 2. The most important findings are presented below. All p-values represent a comparison with the results obtained at normothermia (37°C).

CT-EXTEM® and CT-APTEM® were prolonged by hypothermia at 30°C ($p < 0.01$ for both) and 33°C ($p < 0.05$ for both). Hyperthermia at 42°C shortened CT-EXTEM ($p < 0.05$) and CT-APTEM ($p < 0.01$) (Table 3 and Fig 1).

CFT-EXTEM® and CFT-APTEM® were prolonged by hypothermia at 30°C, 33°C, and 35°C ($p < 0.01$ for all except CFT-EXTEM, 35°C [$p < 0.05$]) (Table 3 and Fig. 1).

The α-angle-EXTEM® was decreased at 30°C, 33°C, and 35°C ($p < 0.01$) but increased at 40°C ($p < 0.05$) and 42°C ($p < 0.01$). The α-angle-APTEM® showed similar results (Table 3 and Fig. 2).

No temperature-dependent differences were observed for MCF-EXTEM®, MCF-FIBTEM®, and MCF-APTEM® (Table 3 and Fig. 2).

Discussion

In this prospective observational study in OHCA patients treated with mild induced hypothermia, we have demonstrated that *in vitro*-applied hypothermia prolonged the clot initiation and decreased the clot propagation as measured with ROTEM (EXTEM and APTEM assays). Hyperthermia had the opposite effects. Clot strength (MCF) was not affected by temperature in this study. Our results are in agreement with previous studies, which were performed with TEG or other ROTEM assays with nontissue factor-activated agonists (Heinius *et al.*, 2002; Shimokawa *et al.*, 2003; Martini *et al.*, 2008; Rundgren and Engström, 2008; Spiel *et al.*, 2009; Ivan *et al.*, 2011).

Viscoelastic instruments such as TEG and thromboelastometry (ROTEM) reflect both the initiation of the coagulation cascade and its propagation and the final clot structure, revealing dynamic interactions of fibrin polymerization, platelet function, and fibrinolysis at different temperatures, not detected by routine coagulation tests (Ganter and Hofer, 2008). However, in hypo- and hyperthermia, there are many factors affecting hemostasis that are not measurable with TEG or ROTEM. OHCA patients often develop post-resuscitation stress responses after cardiac arrest, presumably due to the low-flow state during cardiac arrest, followed by a reperfusion injury, which causes the procoagulative systemic inflammatory response syndrome (Adrie *et al.*, 2005; Koch *et al.*, 2013). Furthermore, several studies have shown increased platelet activity in conjunction with mild induced hypothermia (Xavier *et al.*, 2007; Högberg *et al.*, 2009; Scharbert *et al.*, 2010; Ortmann *et al.*, 2012). These factors that strengthen hemostasis in OHCA patients during hypothermia are offset by delayed and slower clot formation demonstrated with thromboelastometry in this and previous studies (Heinius *et al.*, 2002; Shimokawa *et al.*, 2003; Martini *et al.*, 2008; Rundgren and Engström, 2008; Spiel *et al.*, 2009; Ivan *et al.*, 2011). In patients who underwent percutaneous coronary intervention, the procoagulative effects described above are also offset by dual platelet inhibition medication. Gibbs (2009) confirmed that the effect of these powerful drugs is not detectable with thromboelastometry, which warrants the treatment of patients with and without dual platelet inhibition in one coherent group when evaluating ROTEM results, as was performed in the present study by us.

In this and other studies (Dirkmann *et al.*, 2008; Rundgren and Engström, 2008; Meyer *et al.*, 2013), *in vitro*-applied hyperthermia (39°C–42°C) strengthened coagulation as demonstrated by decreasing ROTEM CT, CFT, and an increasing α-angle (Table 3 and Figs. 1 and 2). This indicates

TABLE 3. ROTEM ANALYSES (*N* = 10)

	30°C	33°C	35°C	37°C	40°C	42°C
CT, seconds						
EXTEM	87 (79–113)[a]	76 (69–89)[b]	70 (66–76)	72 (63–84)	66 (53–79)	56 (46–68)[b]
APTEM	98 (75–123)[a]	76 (62–128)[b]	74 (68–87)	74 (64–85)	64 (60–85)	58 (50–66)[a]
CFT, seconds						
EXTEM	152 118–192)[a]	112 (92–148)[a]	108 (80–121)[b]	90 (63–100)	73 (58–82)[a]	73 (52–78)[a]
APTEM	162 (128–189)[a]	119 (85–145)[a]	106 (81–122)[a]	92 (65–103)	74 (59–86)[a]	66 (47–89)[a]
α-Angle						
EXTEM	66 (59–70)[a]	70 (66–75)[a]	72 (69–74)[a]	76 (72–77)	77 (75–78)[b]	79 (78–80)[a]
APTEM	64 (75–68)[a]	69 (65–73)[b]	71 (67–74)[b]	73 (70–77)	76 (74–78)[a]	78 (75–80)[a]
MCF, mm						
EXTEM	58 (52–71)	60 (56–70)	62 (57–68)	64 (59–69)	64 (59–67)	61 (58–68)
FIBTEM	18 (13–22)	20 (13–24)	20 (15–25)	22 (16–24)	22 (13–24)	22 (14–25)
APTEM	58 (52–69)	62 (57–71)	60 (56–68)	62 (57–66)	62 (58–72)	59 (57–67)

[a]$p < 0.01$ and [b]$p < 0.05$ indicates significance level when compared to 37°C.
CFT, clot formation time; CT, clotting time; MCF, maximum clot firmness.

FIG. 1. ROTEM® (EXTEM® and APTEM® assays) on whole blood from cardiac arrest patients in stable hypothermia. The blood was incubated at specified temperatures of 30°C, 33°C, 35°C, 37°C, 40°C, and 42°C before analysis. Analyses were performed with the ROTEM instrument set on the incubation temperatures. Clotting time (CT). Clotting formation time (CFT). Box plots presented as the median with interquartile range and min–max whiskers. *$p < 0.05$. **$p < 0.001$.

FIG. 2. ROTEM (EXTEM, FIBTEM, and APTEM assays) on whole blood from cardiac arrest patients in stable hypothermia. The blood was incubated at specified temperatures of 30°C, 33°C, 35°C, 37°C, 40°C, and 42°C before analysis. Analyses were performed with the ROTEM instrument set on the incubation temperatures. Maximum clot firmness (MCF). Box plots presented as the median with interquartile range and min–max whiskers. *$p < 0.05$. **$p < 0.001$.

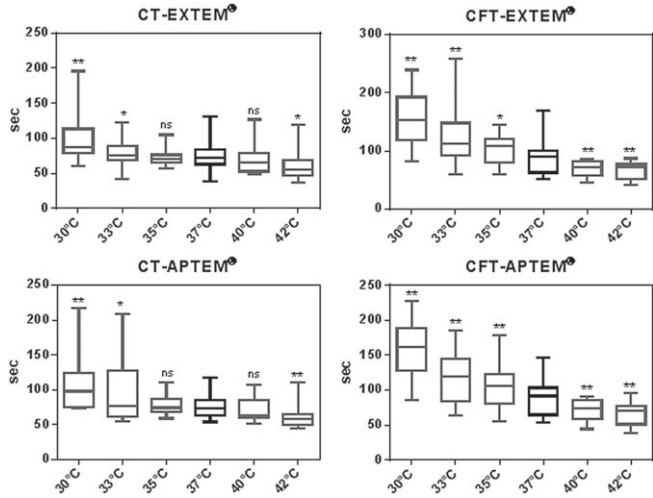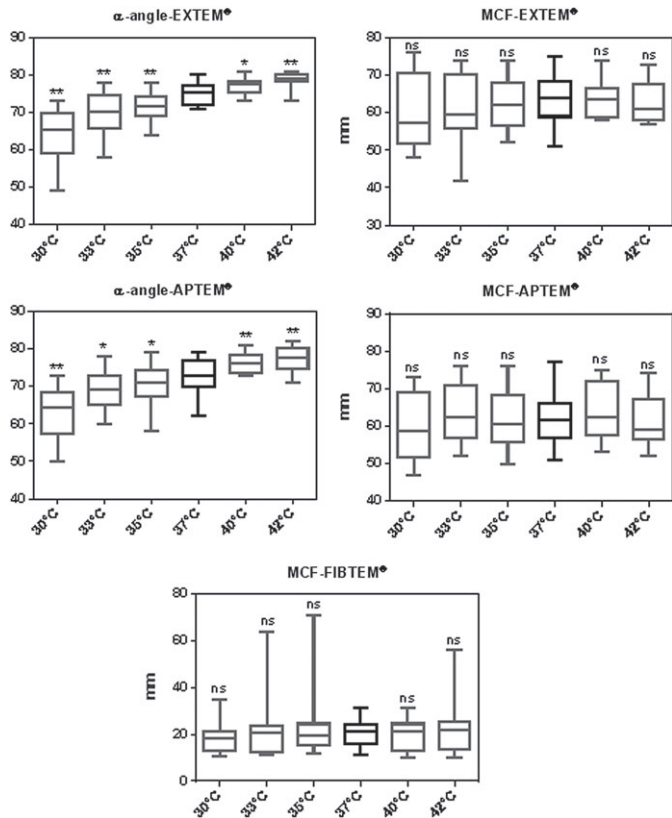that clot initiation and propagation is faster in the febrile patient, thus inducing a possible hypercoagulable state.

The current investigation was performed on OHCA patients to ascertain temperature-dependent effects on the ROTEM analysis. This population was chosen since healthy volunteers and animals in an experimental model probably do not have the same coagulation profile as OHCA patients. However, this may also be a limitation of this study since OHCA patients are a divergent cohort. We also acknowledge the inherent limitations of this investigation due to its *in vitro* design.

Measuring hemostasis is very complex. Multiple pathways for platelet activation coagulation exist, and some are flow and shear stress dependent (Schött and Johansson, 2013). Different *in vitro* tests reflect limited characteristics of hemostasis. Our study broadens the understanding of how temperature affects ROTEM, using EXTEM, FIB-TEM, and APTEM assays, and the demonstrated delayed coagulation start may be part of the explanation for the bleeding diathesis seen in hypothermic patients (Schmied *et al.*, 1996).

Conclusion

ROTEM (EXTEM, FIBTEM, and APTEM assays) revealed a hypocoagulative response to *in vitro*-applied hypothermia in the blood of cardiac arrest patients reflected in the prolonged clot initiation and decreased clot propagation. Hyperthermia showed the opposite effects. Clot firmness was not affected by temperature.

Author Disclosure Statement

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Paper V

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ORIGINAL ARTICLE

Temperature effects on haemostasis in whole blood from ticagrelor- and aspirin-treated patients with acute coronary syndrome

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Abstract

Background. Comatose survivors after cardiac arrest are treated with mild induced hypothermia and potent platelet-inhibiting drugs after coronary stenting. Previous studies have shown an increased incidence of stent thrombosis during clopidogrel and aspirin treatment in conjunction with induced hypothermia. The aim of this study was to investigate the *in vitro* effect of induced hypo- and hyperthermia on blood from patients undergoing ticagrelor- and aspirin-mediated platelet inhibition. *Methods.* Whole blood from 15 patients with acute coronary syndrome who were treated with ticagrelor and aspirin and from eight healthy volunteers was incubated for 1 hour at 28, 33, 37, and 39°C. *Results.* In blood from patients with acute coronary syndrome, the activated clotting time (Sonoclot) was prolonged in mild hypothermic (33°C) compared to normothermic (37°C) samples. Sonoclot, clotting rate and platelet function were decreased in hypo-thermic compared to normothermic samples. Platelet-induced activation and aggregation (Multiplate®) was unchanged in mild hypothermic compared to normothermic samples. In contrast, mild hypothermia supported increased platelet activa-tion as measured with flow cytometry with up-regulation of PAC-1 and P-selectin on the platelet surface. *Conclusion.* In acute coronary syndrome patients treated with ticagrelor and aspirin, in vitro hypothermia to 33°C markedly increased platelet activity measured with flow cytometry, whereas viscoelastic coagulation test (Sonoclot) revealed a hypocoagulative response. Prospective clinical trials studying platelet inhibition at different temperatures and correlating changes in platelet function to bleeding or stent occlusion are needed.

Key Words: *Induced hypothermia, platelet aggregation, platelet function tests, blood coagulation, antiplatelet drugs, flow cytometry*

Introduction

Mild induced hypothermia (MIH) is indicated for comatose survivors of out-of-hospital cardiac arrest to improve neurological outcomes [1–3]. However, in a recent multicenter study (the Target Tempera-ture Management trial) [4] of out-of-hospital cardiac arrest patients, a targeted temperature of 33°C did not confer a benefit compared with a targeted tem-perature of 36°C, somewhat challenging current guidelines.

Hypothermia is generally considered to reduce coagulation and platelet function. However, studies performed in animals, healthy volunteers, and MIH patients have shown conflicting results. Some studies show that mild hypothermia decreases haemostasis [5–18], whereas others show the opposite [19–28]. The varying results may be explained by the many different methods used to study the different aspects of haemostasis. Commonly used methods to study the response to platelet inhibitors are multiple elec-trode aggregometry (MEA) [29] and flow cytometry [30]. A viscoelastic test, Sonoclot, has been shown to be superior to thromboelastographic methods for detecting inhibition of haemostasis in hypothermic animals using glass bead activation [14,27]. Further-more, separating the effects on platelet function from the effects on coagulation is necessary. Studies that focus on evaluating coagulation in general tend to

show weakened coagulation ability, whereas studies evaluating platelet function in mild hypothermia are more likely to demonstrate the opposite [5–28].

Cardiac arrest patients often undergo emergency coronary inventions with stenting and receive dual antiplatelet therapy including aspirin (ASA) and a $P2Y_{12}$ antagonist before inducing hypothermia. Several studies have demonstrated decreased clopidogrel-generated platelet inhibition during mild hypothermia [20,31–33], and two investigations in patients treated with clopidogrel and ASA during MIH have reported a high incidence of stent thrombosis after percutaneous coronary intervention (PCI) [33,34]. However, at many hospitals, clopidogrel has been replaced by ticagrelor because the latter drug shows better survival rates [35], probably due to the stronger platelet inhibiting effect of ticagrelor [36].

To our knowledge, only one previous investigation concerning pharmacodynamic changes with ASA during mild hypothermia has been reported, and this study showed that ASA does not augment hypothermia-induced platelet dysfunction [37]. Regarding ticagrelor and hypothermia, a recent study [38] demonstrated a high rate of ticagrelor non-responders among patients treated with MIH. However, this effect may be related to insufficient intestinal absorption of the orally administered drug in comatose survivors and not an effect of hypothermia [39,40]. To exclude the impact of the possibility of poor intestinal absorption, we conducted an in vitro study on blood from stable acute coronary syndrome patients on dual platelet inhibition with ticagrelor and ASA and compared the results to blood from healthy volunteers. Whole blood was incubated at different temperatures before assessing haemostasis using several methods that measure different parameters of haemostasis: MEA, Sonoclot, and flow cytometry. The aim of this study was to investigate the effect of in vitro-applied hypo- and hyperthermia on ticagrelor- and ASA-mediated platelet inhibition.

Material and methods

The Regional Ethical Review Board (registration number 2010/482) approved this study. Signed and informed consent was received from all patients and volunteers. Fifteen patients treated with a minimum of three doses of ASA and ticagrelor, excluding the loading doses, were recruited from the cardiac care unit at Skåne University Hospital, Lund, Sweden, September–October, 2013. Patients were considered to be in a steady-state of ticagrelor- and ASA-induced platelet inhibition. Healthy volunteers were included to evaluate how different incubation temperatures affect whole blood in the absence of ticagrelor and ASA. Healthy volunteers were non-smokers between 31 and 55 years of age who had not taken

medications during the previous 14 days. Four women and four men were included.

Blood sampling

Venous blood was drawn from an antecubital vein using a vacutainer system (BD, Plymouth, UK). Blood samples for MEA and flow cytometry analyses were collected in 3.0-ml tubes containing recombinant hirudin (Dynabyte GmbH, Munich, Germany). Blood for Sonoclot analysis was collected in a 4.5-ml tube containing 0.109 M citrate (BD Vacutainer Systems). Prior to all analyses, all sample tubes were incubated in water baths for 1 h at temperatures that represent deep hypothermia (28°C), mild hypothermia (33°C), normothermia (37°C), or hyperthermia (39°C). Samples were incubated within 10 min of sampling. The temperatures in the water baths were controlled regularly during incubation and were maintained at ± 0.2°C.

Conventional hematological tests

Platelet count (PT/INR, aPTT) analyses were performed at the accredited hospital laboratory. Platelet count was measured using the Sysmex XE 5000 cell counter (Sysmex Corp., Kobe, Japan). The locally determined reference range for platelets is 165–387×10^9/L for adult women and 145–348×10^9/L for adult men.

PT/INR was performed using a combined thromboplastin reagent (Stago prothrombin complex assay, SPA+, Stago). The Owren PT assay was calibrated using International Normalized Ratio (INR) calibrators certified by the Swedish external quality assessment organization Sysmex 5100 (Equalis, Uppsala, Sweden). The reference range for PT/INR is 0.9–1.2.

APTT was analyzed with an aPTT reagent from Actin FSL (Siemens Healthcare Diagnostics). Plasma fibrinogen concentration was measured using the Dade Thrombin reagent (Siemens Healthcare Diagnostics. CS-5100). The reference range for aPTT has been established locally to 26–33 sec.

Sonoclot analysis

A Sonoclot Analyzer (Sienco Inc. Arvada, CO, USA) with a temperature-regulated heating or cooling plate was used to study viscoelastic coagulation changes. Before analysis, the citrated blood was recalcified in accordance with the manufacturer's specifications. All Sonoclot analyses were performed with the instrument set at the corresponding incubation temperature. A glass bead test (Sienco® gbACT+™ Kit), designed to initiate coagulation in a stable manner, was used. The Sonoclot measures the viscoelastic drag impedance that fibrin and platelets in a whole blood sample impose upon the oscillating

Sono-probe. The time-based graph that is generated reflects the different steps in the clotting of the whole blood sample, called the Sonoclot Signature. Test variability of the Sonoclot analyses was determined to be 6–10%. [41]. The following parameters were measured, with the defined normal values in parentheses:

(1) Activated clotting time (ACT) (100–155 s): time required for the first fibrin to form. ACT corresponds to aPTT and traditional ACT tests.
(2) Clot rate (CR) (9–35% units/min): the rate of increase in the clot impedance due to fibrin formation and polymerisation (the slope of the signature after ACT) in % of full scale per minute.
(3) Platelet function (PF) (> 1.5 units) is the point where the squeezing out of trapped serum in the contracting clot-clot retraction (sign of functioning platelets) exceeds the accumulation of clot bulk on the probe. PF performs better than previous peak amplitude and time-to-peak parameters, reflecting the GPIIb/IIIa-dependent clot retraction [41].
(4) The occurrence of fibrinolysis was determined by manual examination of the Sonoclot Signatures [41–43].

MEA

MEA was performed with Multiplate® (Roche, Rotkreuz, Switzerland), which measures agonist-induced platelet aggregation. The extent of platelet aggregation is measured by resistance (impedance) changes between two electrodes and is depicted as a graph. The area under the curve (AUC) is the best measure of platelet function. The instrument was set at the corresponding incubation temperature. Test assays were performed in a five-channel heat-regulated block in four sessions starting at 28°C and then raising the temperature to 33°C, 37°C, and 39°C. The instrument was allowed to rest 10 min after the target temperature was reached, allowing complete temperature calibration before the next session was started. Activators used were: ADPtest (platelet aggregation in response to adenosine-5′-diphosphate, 6.5 μM), COLtest (platelet aggregation in response to collagen, 3.2 μg/ml), TRAPtest (platelet aggregation in response to thrombin receptor agonist peptide, 32 μM), and ASPItest (platelet aggregation in response to arachidonic acid, 0.5 mM). Test variability of the Multiplate instrument throughout the experiment was 2–8% between the two set of electrodes in each cuvette.

A previous study [21] has demonstrated an increased potency for ADP at 33°C compared to normothermia. To further evaluate how different ADP concentrations affect ticagrelor-induced platelet inhibition at different temperatures, we created a dose-response curve for ADP-induced platelet aggre-gation and activation in blood from acute coronary syndrome patients, using ADP concentrations of 0.01, 0.1, 1.0, 6.5, and 10 μM in both MEA and flow cytometry analyses. These concentrations were tested in pilot experiments in which 10 μM ADP gave the maximum response.

Flow cytometry

Antibodies were all from BD Biosciences (San Jose, CA, USA) and included APC-conjugated anti-human CD41a, fluorescence isothiocyanate (FITC)-conjugated anti-human PAC-1, phycoerythrin (PE)-conjugated anti-human CD62P (P-selectin) as well as the two control antibodies; PE-conjugated mouse anti-human IgG and FITC-conjugated mouse anti-human IgM. ADPtest, TRAPtest, and ASPItest with the same final concentrations, including dose-response analysis of the ADPtest, as in the MEA assays were used. Isotype controls were run at all temperatures to ensure that no false representative activation from other cells besides platelets was detected by the flow cytometer. Values below 1% activation were considered accurate, and all assessments passed this criterion. Repeated measurements in our laboratory of the P-selectin expression using the same blood sample showed a variance of 2.5% (0–6.9).

After temperature incubation, whole blood was added to pre-warmed phosphate-buffered saline (PBS) containing agonists and antibodies and incubated for another 20 min at specified temperatures. To stop platelet activation, 400 μL ice-cold 0.2% paraformaldehyde was added to each sample and incubated at 4°C for 30–45 min to allow red blood cells to sediment. After settling of the red blood cells, 100 μL of the top layer was pipetted into a new tube containing 200 μL PBS and analyzed with flow cytometry (Accuri C6, BD) within 2 h after completion. Platelets were characterized according to CD41a expression as well as forward and side scatter properties. The platelet count for each test was set to 25,000. Activation was expressed as a percentage of positive platelets. Test data were extracted using Cflow® Plus software (BD).

Statistics

The primary endpoint of this study was the comparison of ticagrelor- and ASA-induced platelet inhibition between measurements of samples incubated at 33°C versus 37°C that were stimulated with ADP agonists in flow cytometry and MEA. When calculating sample size using data from previous studies [21,26,28], flow cytometry with an ADP agonist was shown to be the variable generating the largest sample size. With a standard deviation of 31 (% positive cells), we would be able to detect a difference between activation at 33°C and 37°C of 30 (% positive cells) with 12 patients, with 80% power and a two-tailed

alpha value less than 0.05 for a paired data comparison. Secondary endpoints included comparison of samples incubated at 28, 33, and 39°C versus 37°C measured with Sonoclot, MEA, and flow cytometry in both patients with acute coronary syndrome treated with dual platelet inhibition and in healthy volunteers.

Variables were considered non-parametric (Gaussian distribution not assumed) and were summarized using the median with range (min-max) as distribution measurement. Results for 28, 33, and 39°C samples were compared to results from the normothermic blood samples (37°C) using the two-tailed Wilcoxon matched pairs signed test. To reduce the risk of a type I error due to multiple testing, $p < 0.017$ was considered significant. This p-value was calculated in accordance with Bonferroni by dividing 0.05 by the number of different *in vitro* temperatures tested against normothermia for each variable, i.e. 3 ($0.05/3 = 0.017$). All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Demographics

Fifteen patients were studied along with the eight healthy volunteers. Baseline characteristics for the patients are shown in Table I.

Blood analyses

Results from the blood analyses are shown in the Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/00365513.2014.965735 and Figures 1–4. The most important findings are described below.

Sonoclot in patients with acute coronary syndrome

ACT (sec) was increased at 28°C (174 [146–228]; $p < 0.001$) and 33°C (154 [123–202]; $p < 0.001$) compared to 37°C (132 [101–169]), whereas ACT was not changed with hyperthermia (39°C) (Supplementary Appendix and Figure 1a). CR (% units/min) was decreased at 28°C (30 [21–46]; $p < 0.001$) and 33°C (37 [26–53]; $p < 0.001$) compared to 37°C (43 [29–69]), whereas CR was increased with hyperthermia (39°C; 48 [37–66]; $p = 0.01$) (Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/00365513.2014.965735 and Figure 1a). PF (units) was decreased at 28°C (1.2 [0.4–3.1]; $p < 0.001$) and 33°C (2.8 [1.1–4.7]; $p < 0.01$) compared to 37°C (4.0 [2.2–4.7]), whereas PF was not changed with hyperthermia (39°C) (Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/00365513.2014.965735 and Figure 1a). The Sonoclot Signature in patients showed no signs of fibrinolysis.

Table I. Patient demographics.

Patient demographics	($n = 15$)
Age (years)	71 ± 14[a]
Male sex (%)	12 (80)
Body temperature at blood sampling	36.5 ± 0.5[a]
AMI (%)	11 (73)
STEMI (%)	5 (33)
Non-STEMI (%)	6 (40)
Unstable angina (%)	4 (27)
Coronary angiography (%)	15 (100)
PCI with stent (%)	13 (87)
Conventional haematological tests	
Platelet count ($\times 10^9$/L)	246 ± 74
PT (INR)	1.1 0.3
aPTT (sec)	27 ± 4

[a]Mean ± SD. AMI, Acute myocardial infarction; STEMI, ST-elevated myocardial infarction; PCI, Percutaneous coronary intervention.

Sonoclot in healthy volunteers

At 37°C, ACT was 137 (115–170), CR was 38 (26–50), and PF was 3.3 (2.1–4.6). The overall trends in hypo- and hyperthermic samples were similar to those of patients but with fewer significant

Figure 1. (a–b) Sonoclot analysis in blood samples from patients with acute coronary syndrome (a) and in healthy volunteers (b). Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C and analyzed with the instrument set at the corresponding temperature as indicated by the numbers on the x axis. ACT, Activated clotting time (sec); CR, Clotting rate (% units/min); PF, Platelet function (units). Results from the 28, 33, and 39°C samples were compared to results from the normothermic blood sample (37°C) using the two-tailed paired Wilcoxon matched pairs signed test. Boxplots show the median with the interquartile range and min–max whiskers. ns, not significant. *$p < 0.017$. ***$p < 0.001$.

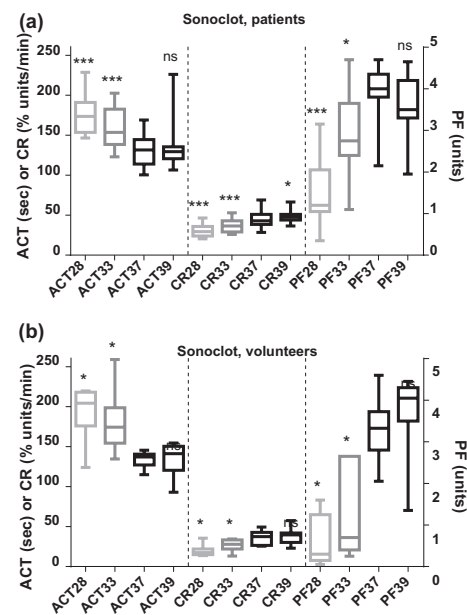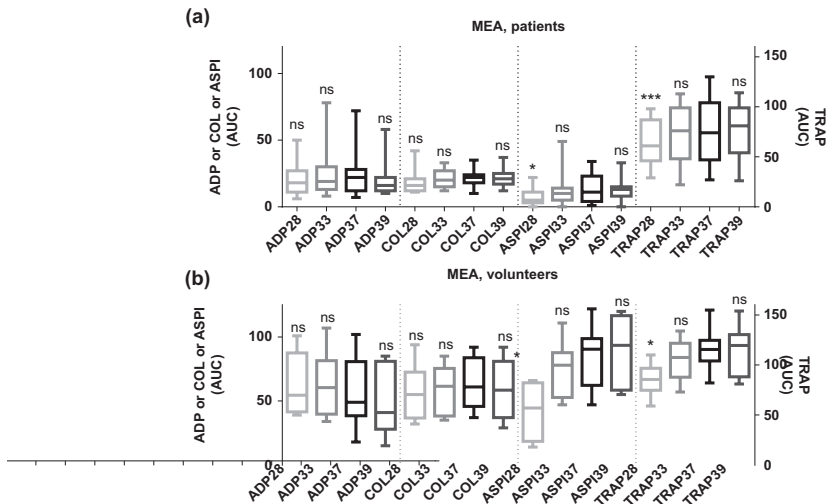Figure 2. (a–b) Multiple electrode aggregometry (MEA) (Multiplate®) in blood samples from patients with acute coronary syndrome (a) and in healthy volunteers (b). Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C and analyzed with the instrument set at the corresponding temperature as indicated by the numbers on the x axis. ADP, Adenosine diphosphate, agonist. COL, Collagen, agonist; TRAP, Thrombin, agonist; ASPI, Arachidonic acid, agonist; AUC, Area under curve. Results from the 28, 33, and 39°C samples were compared to results from the normothermic blood sample (37°C) using the two-tailed paired Wilcoxon matched pairs signed test. Boxplots show the median with the interquartile range and min-max whiskers. ns, not significant. *$p < 0.017$. ***$p < 0.001$.

changes (Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/00 365513.2014.965735 and Figure 1b). The Sonoclot Signature in healthy volunteers showed no signs of fibrinolysis.

MEA in patients with acute coronary syndrome and healthy volunteers

In blood from patients, the ASPItest (AUC) was decreased at 28°C (5 [0–22]; $p = 0.002$) compared to 37°C (11 [1–34]), and the TRAPtest (AUC) was decreased at 28°C (61 [29–98]; $p < 0.001$) compared to 37°C (74 [27–130]). The other MEA assays, including the EC50 for the ADP concentration, were unchanged. Similar results were found in healthy volunteers (Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/0036 5513.2014.965735 and Figure 2).

Flow cytometry in patients with acute coronary syndrome

The platelet activation markers PAC-1 and CD62P were increased at 28 and 33°C compared to 37°C when samples were stimulated with ADP or ASPI. Samples stimulated with TRAP showed an increase in PAC-1 but not CD62P at 28 and 33°C compared with 37°C (Supplementary Appendix available online at http://informahealthcare.com/doi/

abs/10.3109/00365513.2014.965735 and Figures 3a, 3c). Dose-response curves for ADP concentrations showed a significant leftward shift with a concordant decrease in EC50 for both PAC-1 and CD62P (Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/ 00365513.2014.965735 and Figures 4a–b).

Flow cytometry in healthy volunteers

PAC-1 was not changed. CD62P was increased at 28°C and decreased at 39°C compared to 37°C in samples stimulated with ADP but not with TRAP or ASPI (Supplementary Appendix available online at http://informahealthcare.com/doi/abs/10.3109/ 00365513.2014.965735 and Figures 3b, 3d).

Discussion

Mild induced hypothermia, in conjunction with potent platelet-inhibiting drugs, is used to treat comatose survivors after cardiac arrest [1–4]. In previous studies, using clopidogrel and aspirin, mild induced hypothermia was associated with increased incidence of stent thrombosis [33,34]. In this investigation, using blood from patients with acute coronary syndrome treated with ticagrelor and ASA as well as healthy unmedicated volunteers, we have analyzed the role of *in vitro*-applied hypothermia on haemostasis by several different methods.
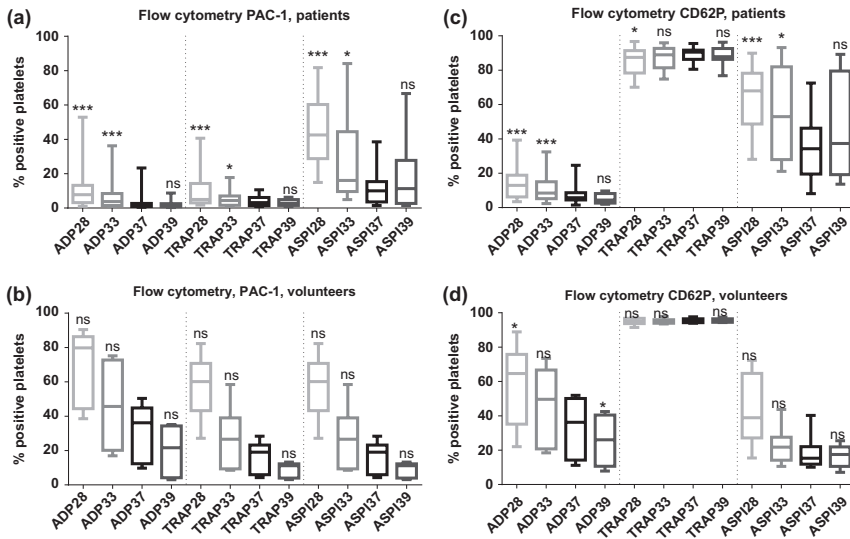
Figure 3. (a–d) Results from flow cytometry analysis with the platelet activation marker PAC-1 (activation of GPIIb-IIIa) or CD62P (P-selectin) in blood samples from patients with acute coronary syndrome (a and c) and in healthy volunteers (b and d). Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C prior to the analysis as indicated by the numbers on the x axis. ADP, Adenosine diphosphate, agonist; TRAP, Thrombin, agonist; ASPI, Arachidonic acid, agonist. Results from the 28, 33, and 39°C samples were compared to results from the normothermic blood sample (37°C) using the two-tailed paired Wilcoxon matched pairs signed test. Boxplots show the median with the interquartile range and min–max whiskers. ns, non-significant. $*p < 0.017$. $***p < 0.001$.

Using flow cytometry, in vitro hypothermic conditions were found to markedly increase ADP-TRAP- and ASPI-mediated platelet activation in blood from patients on ticagrelor and ASA, consistent with previous reports on patients treated with clopidogrel [20,21]. The flow cytometry-derived data on increased platelet reactivity during hypothermia found by us and others could explain the previously observed increased risk of stent thrombosis during therapeutic hypothermia [33,34].

Although *in vitro*-applied hypothermia increased platelet reactivity as determined by flow cytometry, other measures of haemostasis, including viscoelasticity, were impaired. Ganter and Hofer [41] have described that by using a glass bead test and the calculated parameter platelet function (PF), Sonoclot has increased the possibility to detect decreased coagulation ability and inhibited platelet function. We observed that hypothermia prolonged the time until clot initiation (Sonoclot ACT), and decreased clot amplification and propagation (Sonoclot CR) as well as decreased clot retraction (Sonoclot PF). This hypocoagulative impact of hypothermia on initial coagulation has previously been shown with thromboelastometry [12,15].

For the Multiplate aggregometry analyses no significant effects on temperature were seen in patients and volunteers with ADP or collagen. However, at 28°C, but not at 33°C, decreased aggregation was

seen in samples stimulated by TRAP and arachidonic acid. This is in agreement with Ortmann et al. [44] who described hypothermia below 32°C to decrease TRAP-induced platelet aggregation. On the other hand, in a previous in vitro study with citrated whole blood from healthy volunteers, Scharbert et al. [25] showed increased aggregation in response to ADP, collagen and TRAP at temperatures ranging between 30 and 34°C.

In all, results from the flow cytometry analyses indicated that in vitro-applied hypothermia induced increased platelet activation. On the other hand, the Sonoclot results indicated decreased coagulation, clot structure, and platelet-dependent clot retraction. The explanation for this discrepancy may be related to the fact that Sonoclot and thromboelastography fail to detect changes in platelet activation, whereas flow cytometry is designed to do this. The powerful platelet inhibition, exerted by ASA and $P2Y_{12}$ blockade, not detectable by Sonoclot, is probably more important for prevention of coronary reocclusion than the delayed ACT, decreased CR, and PF as measured with Sonoclot [45,46].

We acknowledge the inherent limitations of this investigation due to its *in vitro* design. Several parameters may be affected by temperature regulation *in vivo* that are not demonstrable when temperature is regulated *in vitro*. Furthermore, our test systems have no natural flow, and the blood is stagnant. Many

(a) ADP dose-response curve, PAC1-expression

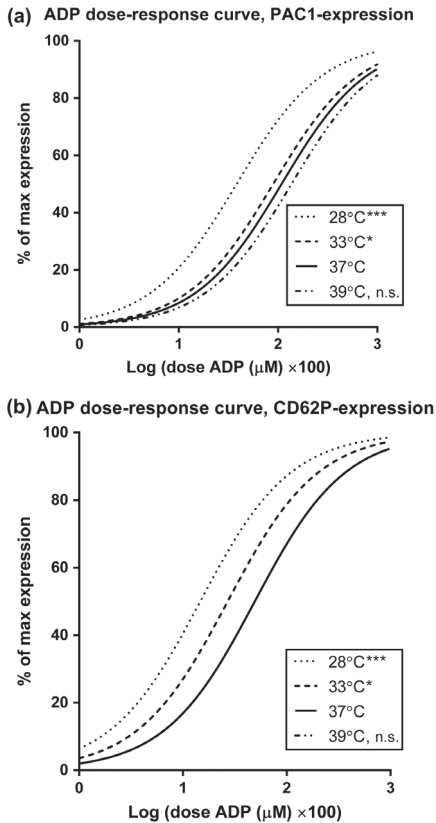## (b) ADP dose-response curve, CD62P-expression

Figure 4. (a–b) Results from flow cytometry analyses in blood samples from patients with acute coronary syndrome. ADP dose-response curve for PAC-1 (a) and CD62P (b) expression. Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C prior to the analyses as indicated by the legends in the upper right corner. ADP, Adenosine diphosphate, agonist. ADP concentrations used were: 0.1, 1, 6.5, and 10 μM. EC50 (half maximal effective concentration) medians from the 28, 33, and 39°C samples were compared to EC50 medians from 37°C using the two-tailed paired Wilcoxon matched pairs signed test. ns, non-significant. $*p < 0.017$. $***p < 0.001$.

platelet receptors depend on higher flow as in arterioles for activation [46]. New automated flow chamber microchip technics are currently introduced in clinical research.

Our results demonstrate that even in patients treated with ASA and ticagrelor, hypothermia may induce increased platelet reactivity. Even though ticagrelor was unable to fully inhibit the hypothermia-mediated increased platelet reactivity, the ADP inhibitory effects of ticagrelor were markedly better than those of clopidogrel in previous studies [21,31–33]. Thus, platelet inhibition with ticagrelor during hypothermia may still be protective against stent thrombosis despite the increased platelet activity

demonstrated with flow cytometry, but the importance of these observations requires investigation in clinical trials. With Multiplate, a device designed for quantification of ASA and ADP platelet inhibition in cardiology there were no effects of in vitro temperature changes in between 33 and 39°C

Conclusion

In acute coronary syndrome patients treated with ticagrelor and aspirin, *in vitro* hypothermia to 33°C markedly increased platelet activity measured with flow cytometry, had no effects on Multiplate aggregometry, whereas a viscoelastic coagulation test (Sonoclot) revealed a hypocoagulative response to hypothermia. Increased understanding of the clinical significance of these findings requires further investigation and prospective clinical trials to determine if ticagrelor and aspirin treatment can prevent stent thrombosis during mild induced hypothermia.

Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Appendix.

Supplementary material for Kander T, et al. Temperature effects on haemostasis in whole blood from ticagrelor- and aspirin-treated patients with acute coronary syndrome. *Scand J Clin Lab Invest,* 2014; DOI: 10.3109/00365513.2014.965735.

Supplementary Appendix

	Blood analyses in patients with acute coronary syndrome ($n = 15$)						
	28°C	*P*-value	33°C	*P*-value	37°C	39°C	*P*-value
Sonoclot							
ACT, sec	174 (146–228)***	$P < 0.001$	154 (123–202)***	$P < 0.001$	132 (101–169)	130 (107–226)	$P = 0.75$
CR, % units/min	30 (21–46)***	$P < 0.001$	37 (26–53)***	$P < 0.001$	43 (29–69)	48 (37–66)*	$P = 0.02$
PF, units	1.2 (0.4–3.1)***	$P < 0.001$	2.8 (1.1–4.7)*	$P = 0.009$	4.0 (2.2–4.7)	3.5 (2.0–4.6)	$P = 0.07$
MEA							
ADP (6.5 μM), AUC	18 (6–50)	$P = 0.09$	19 (8–78)	$P = 0.70$	22 (7–72)	16 (10–58)	$P = 0.05$
ADP, EC50 (μM)	1.0 (0.1–2.6)	$P = 0.68$	0.9 (0.1–2.2)	$P = 0.49$	0.6 (0.1–4.1)	0.7 (0.1–1.8)	$P = 0.64$
COL, AUC	16 (11–42)	$P = 0.05$	20 (12–33)	$P = 0.88$	22 (10–35)	21 (12–37)	$P = 0.92$
TRAP, AUC	61 (29–98)***	$P < 0.001$	76 (22–113)	$P = 0.03$	74 (27–130)	81 (26–114)	$P = 0.98$
ASPI, AUC	5 (0–22)*	$P = 0.002$	10 (0–49)	$P = 0.05$	11 (1–34)	13 (0–33)	$P > 0.99$
Flow cytometry, PAC-1							
Resting (%)	0.2 (0.1–0.5)	$P = 0.98$	0.2 (0.04–0.4)	$P = 0.80$	0.2 (0.04–0.4)	0.1 (0–0.34)	$P = 0.80$
ADP (6.5 μM (%))	7.8 (1.1–52.9)***	$P < 0.001$	3.8 (0.8–36.2)***	$P < 0.001$	1.6 (0.5–23.3)	1.5 (0.3–8.7)	$P = 0.09$
ADP, EC50 (μM)	0.69 (0.17–1.98)***	$P < 0.001$	1.38 (0.39–2.24)*	$P = 0.01$	1.63 (0.52–3.22)	1.85 (3.22–3.12)	$P = 0.80$
TRAP, (%)	5.0 (1.7–40.6)***	$P < 0.001$	4.3 (1.0–17.7)*	$P = 0.004$	3.2 (0.8–10.6)	2.7 (0.8–6.2)	$P = 0.54$
ASPI, (%)	43 (15–82)***	$P < 0.001$	16 (5–84)*	$P = 0.002$	10 (2–39)	11 (2–67)	$P = 0.21$
Flow cytometry, CD62P							
Resting (%)	1.1 (0.1–4.5)	$P = 0.98$	0.9 (0.4–4.4)	$P = 0.80$	1.0 (0.2–2.6)	0.9 (0.2–2.9)	$P = 0.80$
ADP (6.5 μM (%))	12.9 (3.4–39.3)***	$P < 0.001$	8.5 (2.3–32.4)***	$P < 0.001$	5.7 (1.5–24.6)	4.4 (1.9–9.7)	$P = 0.09$
ADP, EC50 (μM)	0.19 (0.11–0.46)***	$P < 0.001$	0.21 (0.12–0.26)*	$P = 0.005$	0.30 (0.15–0.58)	0.33 (0.82–1.29)	$P = 0.98$
TRAP, (%)	87 (70–97)*	$P = 0.002$	89 (75–96)	$P = 0.10$	90 (81–96)	88 (77–92)	$P = 0.86$
ASPI, (%)	68 (28–90)***	$P < 0.001$	53 (21–93)*	$P = 0.002$	34 (8–73)	37 (14–89)	$P = 0.10$

	Blood analyses in healthy volunteers ($n = 8$)						
	28°C	*P*-value	33°C	*P*-value	37°C	39°C	*P*-value
Sonoclot							
ACT, sec	211 (124–220)*	$P = 0.02$	177 (135–259)*	$P = 0.002$	137 (115–170)	141 (93–154)	$P > 0.99$
CR, % units/min	18 (14–35)*	$P = 0.02$	28 (13–34)*	$P = 0.02$	38 (26–50)	40 (23–58)	$P = 0.4$
PF, units	0.3 (0.1–1.6)*	$P = 0.02$	0.7 (0.3–2.6)*	$P = 0.02$	3.3 (2.1–4.6)	4.1 (1.4–4.4)	$P = 0.5$
MEA							
ADP (6.5 μM), AUC	55 (39–101)	$P = 0.64$	61 (34–107)	$P = 0.63$	49 (18–102)	41 (1585)	$P = 0.16$
COL, AUC	55 (32–94)	$P = 0.46$	62 (35–85)	$P = 0.15$	61 (37–95)	59 (29–92)	$P = 0.06$
TRAP, AUC	86 (59–110)*	$P = 0.008$	108 (73–134)	$P = 0.20$	116 (82–155)	120 (81–154)	$P > 0.99$
ASPI, AUC	45 (14–66)*	$P = 0.008$	78 (47–111)	$P = 0.12$	91 (47–122)	94 (55–120)	$P = 0.38$
Flow cytometry, PAC-1							
Resting (%)	0.2 (0.1–0.4)	$P = 0.80$	0.2 (0.1–0.3)	$P = 0.80$	0.1 (0.1–0.2)	0.1 (0.1–0.2)	$P = 0.98$
ADP (6.5 μM (%))	80 (38–90)	$P = 0.02$	46 (17–75)	$P = 0.06$	36 (10–50)	22 (3–35)	$P = 0.02$
TRAP, (%)	60 (27–82)	$P = 0.02$	27 (9–58)	$P = 0.02$	19 (4–28)	11 (3–13)	$P = 0.02$
ASPI, (%)	24 (2–56)	$P = 0.02$	3.7 (1.4–39)	$P = 0.02$	2.3 (0.7–11)	2.4 (0.8–1.4)	$P = 0.02$
Flow cytometry CD62P							
Resting (%)	1.1 (0–6.6)	$P = 0.98$	1.7 (0.4–2.8)	$P = 0.80$	1.1 (0.3–3.5)	1.4 (0.4–4.5)	$P = 0.80$
ADP (6.5 μM (%))	64 (22–89)*	$P = 0.008$	42 (0–73)	$P = 0.02$	36 (12–52)	26 (8–42)*	$P = 0.008$
TRAP, (%)	95 (92–97)	$P = 0.15$	95 (94–98)	$P = 0.25$	96 (94–98)	95 (94–97)	$P = 0.74$
ASPI, (%)	39 (15–72)	$P = 0.02$	22 (11–44)	$P = 0.64$	15 (10–10)	18 (7–25)	$P = 0.94$

Blood analyses 1 hour after incubation at specified temperatures in patients with acute coronary syndrome and in healthy volunteers. MEA: Multiple electrode aggregometry, (Multiplate®) and Sonoclot instruments set at the incubation temperature. ACT: Activated clotting time. CR: Clotting rate. PF: Platelet function. Platelet agonists: [ADP: Adenosine diphosphate, agonist. COL: Collagen, agonist. TRAP: Thrombin receptor, agonist peptide. ASPI: Arachidonic acid, agonist]. AUC: Area under curve. Flow cytometry: Expressed as percentage activated platelets. The platelet activation markers were: [PAC-1: activation of glycoprotein IIb-IIIa and CD62P: P-selectin]. ADP, EC50 (half maximal effective concentration) from dose-response experiments. Results are presented as medians with range (min-max). Results from 28, 33, and 39°C samples were compared to results from the normothermic blood sample (37°C) using the two-tailed paired Wilcoxon matched pairs signed test. *$P < 0.017$. ***$P < 0.001$.

Erratum Paper V

The unit for the Sonoclot parameter clot rate (CR) should be units/minute. The unit is incorrectly named in the Material and method section, in the Result section and in Figure 1a and 1b.