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

Kander, Thomas; Brokopp, Jens; Erlinge, David; Lood, Christian; Schött, Ulf

Published in:
Scandinavian Journal of Clinical & Laboratory Investigation

DOI:
10.3109/00365513.2014.965735

2015

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

Short Title:

Temperature effects on haemostasis.

Authors:

Thomas Kander, MD ()[1,2]

Jens Brokopp, MD[1]

David Erlinge, MD, PhD[3]

Christian Lood, PhD[4]

Ulf Schött, MD, PhD[1]

[1]Department of Intensive and Perioperative Care, Skåne University Hospital Lund

[2]Corresponding Author: Thomas Kander. Department of Intensive and Perioperative Care, Skåne University Hospital Lund

221 85 Lund, Sweden.

Phone: +46 46 17 11 56

Fax: +46-46-17 60 50

Email: thomas.kander@med.lu.se or thomas.kander@skane.se

[3]Department of Cardiology, Lund University, Skåne University Hospital

[4]Department of Clinical Sciences Lund, Section of Rheumatology, Lund University, Lund, Sweden. (Current Affiliation: Division of Rheumatology, University of Washington, Seattle, U.S.A.)

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 8 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 hypothermic 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 activation 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.

Keywords: 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 Temperature 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 temperature 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 electrode 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]. Furthermore, 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 hour at temperatures that represent deep hypothermia (28°C), mild hypothermia (33°C), normothermia (37°C), or hyperthermia (39°C). Samples were incubated within 10 minutes 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 $x10^9$/L for adult women and 145–348 $x10^9$/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 seconds.

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+^{TM} 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:

a. Activated clotting time (ACT) (100–155 s): time required for the first fibrin to form. ACT corresponds to aPTT and traditional ACT tests.
b. Clot rate (CR) (9–35%/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.
c. 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].
d. 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 minutes 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 aggregation 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 minutes 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 minutes 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 analysed with flow cytometry (Accuri C6, BD) within 2 hours 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 appendix 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) (appendix and Fig 1a). CR (%/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); $P=0.01$) (appendix and Fig 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) (appendix and Fig 1a). The Sonoclot Signature in patients showed no signs of fibrinolysis.

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 changes (appendix and Fig 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 (appendix and Fig. 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 (appendix and Fig. 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 (appendix and Fig. 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 (appendix and Fig. 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 analysed the role of in vitro-applied hypothermia on haemostasis by several different methods.

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 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 33-39°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.

Acknowledgement

We would like to thank laboratory researcher Birgitta Gullstrand for the invaluable tutorial during the laboratory work and flow cytometry analysis.

Funding:

No commercial support.

Lund University, ISEX-ALF Fund (Ulf Schött).

Conflicts of interest

None declared

Tables

Table I. ***Patient Demographics***

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 ($x10^9$/L)	246±74
PT (INR)	1.1±0.3
aPTT (sec)	27±4

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

(Only available in Appendix). **Table IIa** ***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, %/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

Table IIb *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, %/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 (0.8–4.4)	*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 (10–40)	18 (7–25)	*P*=0.94

Table IIa-b. Blood analyses 1 hour after incubation at specified temperatures in patients with acute coronary syndrome (**Table IIa**) and in healthy volunteers (**Table IIb**). 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.

Figures

Figure 1a

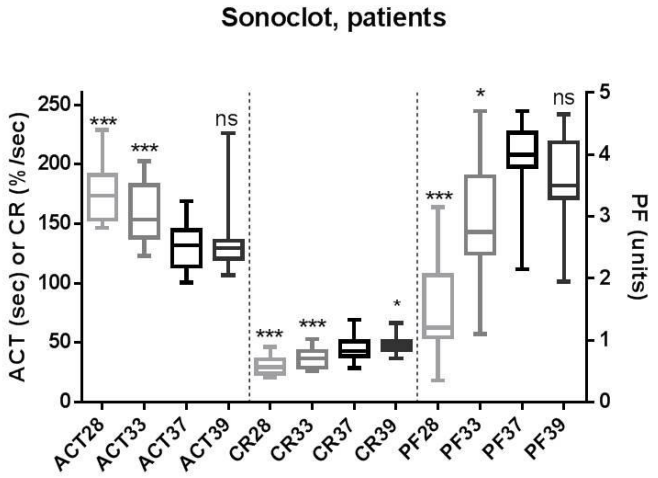

Figure 1b

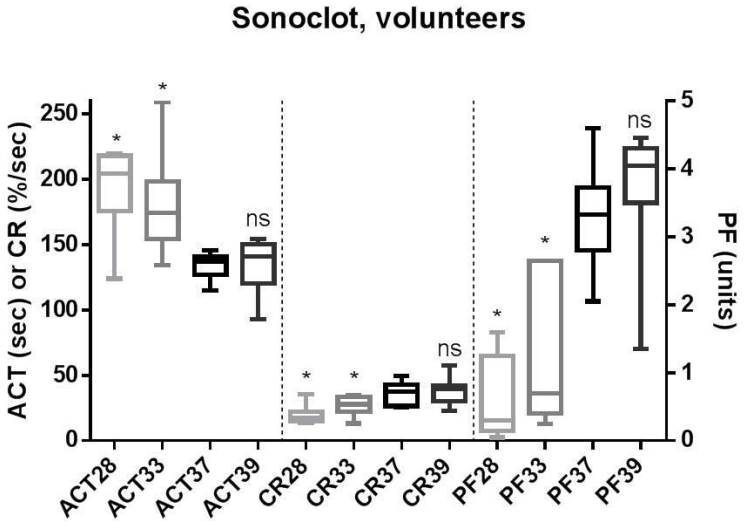

Figure 1a-b. Sonoclot analysis in blood samples from patients with acute coronary syndrome (**Figure 1a**) and in healthy volunteers (**Figure 1b**). Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C and analysed with the instrument set at the corresponding temperature as indicated by the numbers on the x axis. ACT: Activated clotting time (sec). CR: Clotting rate (%/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 2a

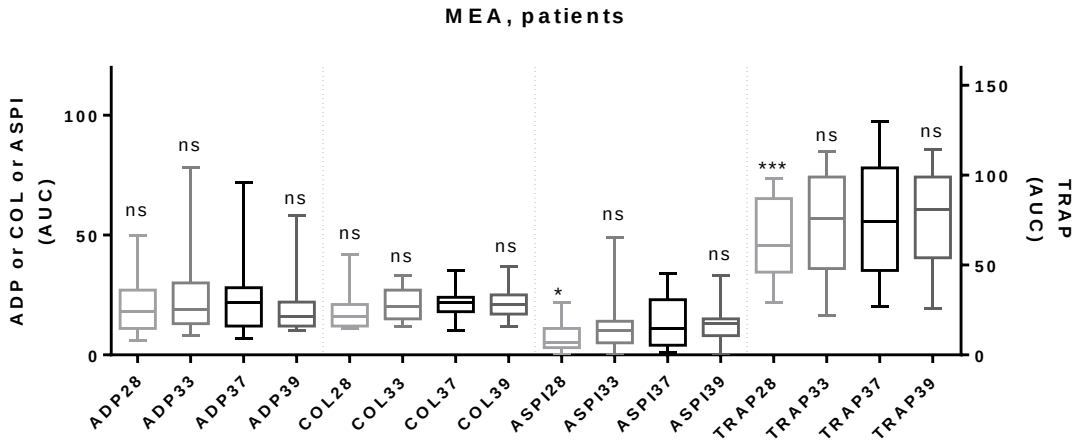

Figure 2b

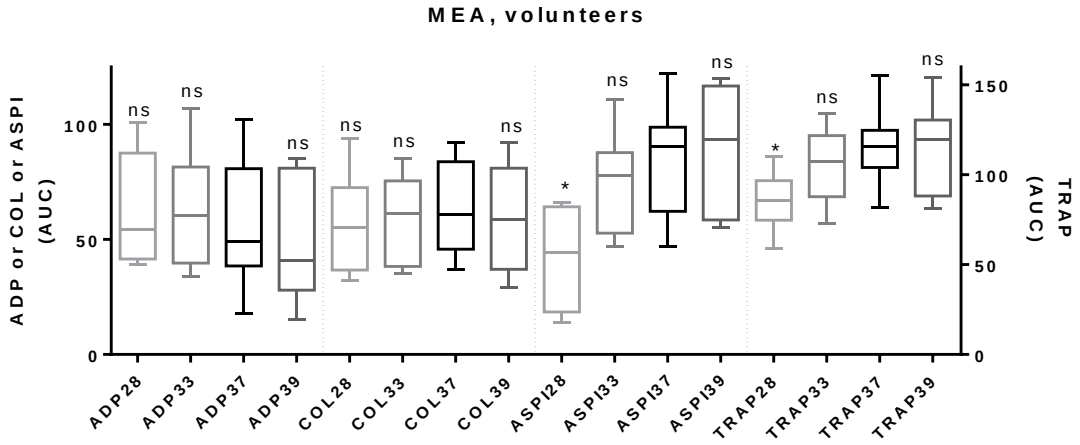

Figure 2a-b. Multiple electrode aggregometry (MEA) (Multiplate®) in blood samples from patients with acute coronary syndrome (**Figure 2a**) and in healthy volunteers (**Figure 2b**). Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C and analysed with the instrument set at the corresponding 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.

Figure 3a

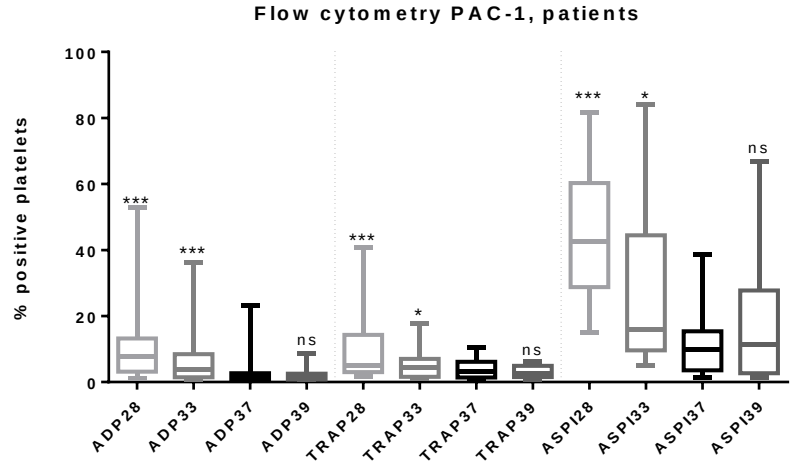

Figure 3b

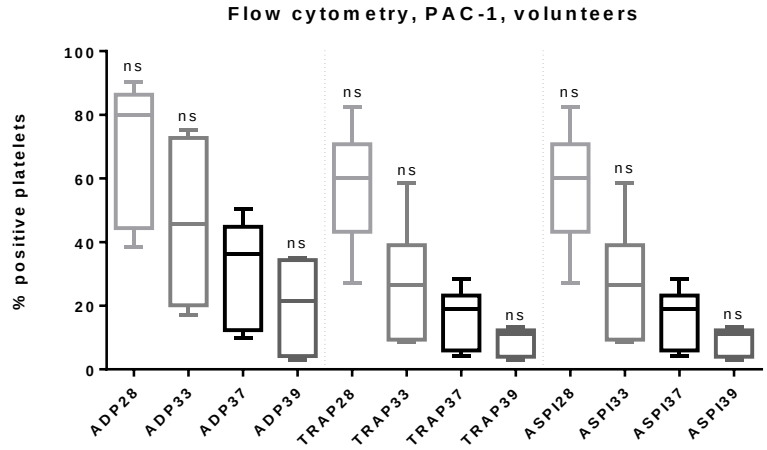

Figure 3c

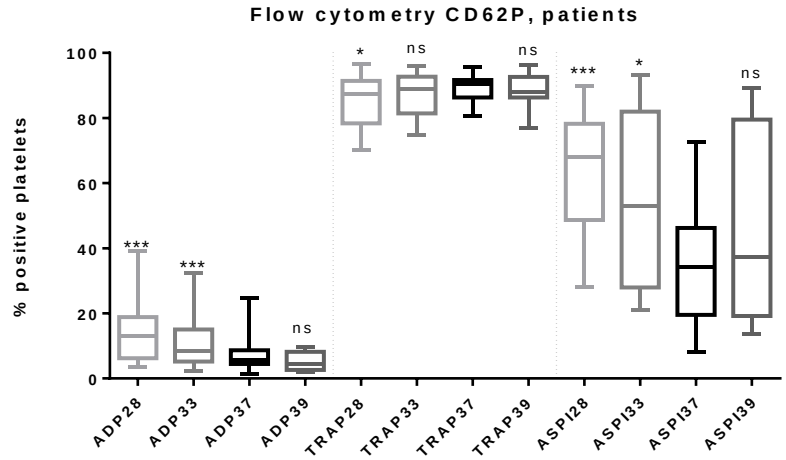

Figure 3d

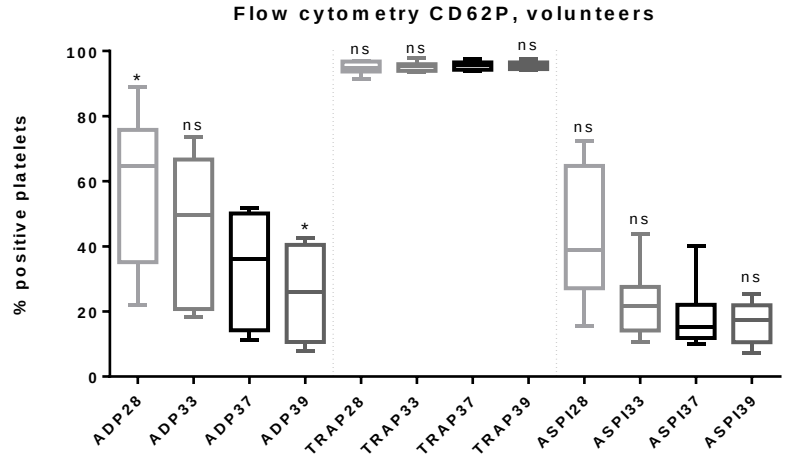

Figure 3a-d. Results from flow cytometry analysis with the platelet activation marker PAC-1 (activation of GPIIb-IIIa) or CD62P (P-selectin) in blood samples from patients with acute coronary syndrome (**Figure 3a and 3c**) and in healthy volunteers (**Figure 3b and 3d**). Blood samples were incubated 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.

Figure 4a

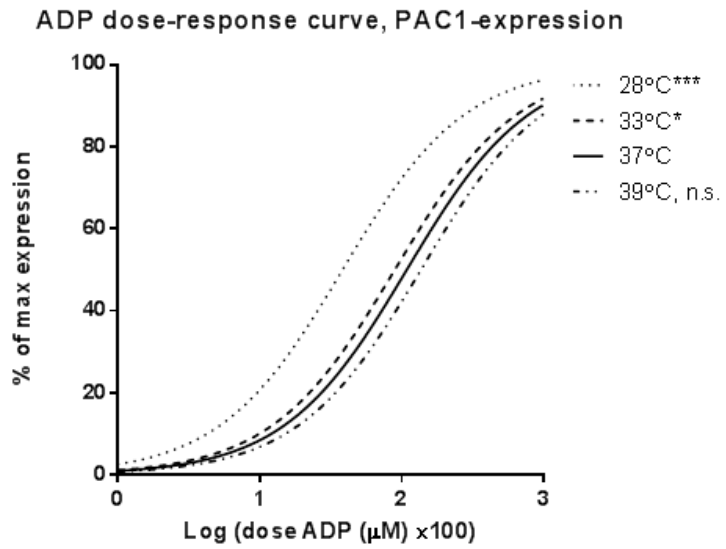

Figure 4b

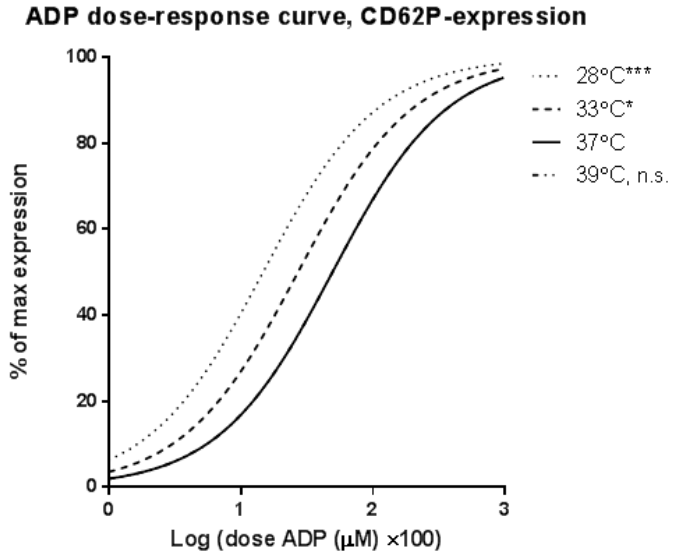

Figure 4a-b. Results from flow cytometry analyses in blood samples from patients with acute coronary syndrome. ADP dose-response curve for PAC-1 (**Figure 4a**) and CD62P (**Figure 4b**) expression. Blood samples were incubated for 1 hour at 28, 33, 37, or 39°C prior to the

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.

References

[1] Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N Engl J Med 2002;346:557-63.

[2] Holzer M, Sterz F, Darby JM, Padosch SA, Kern KB, Bottiger BW, Polderman KH, Girbes ARJ, Holzer M, Bernard SA, Buist MD, Safar P, Kochanek PM. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N Engl J Med 2002;346:549-56.

[3] Peberdy MA, Callaway CW, Neumar RW, Geocadin RG, Zimmerman JL, Donnino M, Gabrielli A, Silvers SM, Zaritsky AL, Merchant R, Vanden Hoek TL, Kronick SL. Part 9: Post-cardiac arrest care: 2010 American Heart Association Guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care. Circulation 2010;122:S768-S86.

[4] Nielsen N, Wetterslev J, Cronberg T, Erlinge D, Gasche Y, Hassager C, Horn J, Hovdenes J, Kjaergaard J, Kuiper M, Pellis T, Stammet P, Wanscher M, Wise MP, Aneman A, Al-Subaie N, Boesgaard S, Bro-Jeppesen J, Brunetti I, Bugge JF, Hingston CD, Juffermans NP, Koopmans M, Kboer L, Langorgen J, Lilja G, Mloler JE, Rundgren M, Rylander C, Smid O, Werer C, Winkel P, Friberg H. Targeted temperature management at 33(degrees)c versus 36(degrees)c after cardiac arrest. N Engl J Med 2013;369:2197-206.

[5] Dirkmann D, Hanke AA, Gorlinger K, Peters J. Hypothermia and acidosis synergistically impair coagulation in human whole blood. Anesth Analg 2008;106:1627-32.

[6] Frelinger I AL, Furman MI, Barnard MR, Krueger LA, Dae MW, Michelson AD. Combined effects of mild hypothermia and glycoprotein IIb/IIIa antagonists on platelet–platelet and leukocyte–platelet aggregation. Am J Cardiol 2003;92:1099-101.

[7] Heinius G, Wladis A, Hahn RG, Kjellstrom BT. Induced hypothermia and rewarming after hemorrhagic shock. J Surg Res 2002;108:7-13.

[8] Ivan Jr C, Vladimír S, Martin P, Pavel S, Iveta R, Václav Z. The influence of temperature adjustment on thromboelastography results: Prospective cohort study. Anesteziologie a Intenzivni Medicina 2011;22:253-9.

[9] Martini WZ, Cortez DS, Dubick MA, Park MS, Holcomb JB. Thrombelastography is better than PT, aPTT, and activated clotting time in detecting clinically relevant clotting abnormalities after hypothermia, hemorrhagic shock and resuscitation in pigs. J Trauma Inj Infect Crit Care 2008;65:535-43.

[10] Martini WZ, Pusateri AE, Uscilowicz JM, Delgado AV, Holcomb JB, Tyburski JG, Rhee PM, Schreiber MA. Independent contributions of hypothermia and acidosis to coagulopathy in swine. J Trauma Inj Infect Crit Care 2005;58:1002-10.

[11] Michelson AD, MacGregor H, Barnard MR, Kestin AS, Rohrer MJ, Valeri CR. Reversible inhibition of human platelet activation by hypothermia in vivo and in vitro. Thromb Haemost 1994;71:633-40.

[12] Rundgren M, Engström M. A thromboelastometric evaluation of the effects of hypothermia on the coagulation system. Anesth Analg 2008;107:1465-8.

[13] Ruzicka J, Stengl M, Bolek L, Benes J, Matejovic M, Krouzecky A. Hypothermic anticoagulation: Testing individual responses to graded severe hypothermia with thromboelastography. Blood Coagul Fibrinolysis 2012;23:285-9.

[14] Shimokawa M, Kitaguchi K, Kawaguchi M, Sakamoto T, Kakimoto M, Furuya H. The influence of induced hypothermia for hemostatic function on temperature-adjusted measurements in rabbits. Anesth Analg 2003;96:1209-13.

[15] Spiel AO, Kliegel A, Janata A, Uray T, Mayr FB, Laggner AN, Jilma B, Sterz F. Hemostasis in cardiac arrest patients treated with mild hypothermia initiated by cold fluids. Resuscitation 2009;80:762-5.

[16] Staikou C, Paraskeva A, Donta I, Theodossopoulos T, Anastassopoulou I, Kontos M. The effects of mild hypothermia on coagulation tests and haemodynamic variables in anaesthetized rabbits. West Indian Med J 2011;60:513-8.

[17] Winstedt D, Thomas O, Schott US. In vitro correction of hypothermic and dilutive crystalloid and colloid rotational thromboelastography-monitored coagulopathy with f brinogen and factor XIII. Crit Care 2013;17:S136.

[18] Wolberg AS, Meng ZH, Monroe DM, 3rd, Hoffman M. A systematic evaluation of the effect of temperature on coagulation enzyme activity and platelet function. J Trauma 2004;56:1221-8.

[19] Faraday N, Rosenfeld BA. In vitro hypothermia enhances platelet GPIIb-IIIa activation and P- selectin expression. Anesthesiology 1998;88:1579-85.

[20] Ferreiro JL, Sanchez-Salado JC, Gracida M, Marcano AL, Roura G, Ariza A, Gomez-Lara J, Lorente V, Romaguera R, Homs S, Sanchez-Elvira G, Teruel L, Rivera K, Sosa SG, Gomez-Hospital JA, Angiolillo DJ, Cequier A. Impact of Mild Hypothermia on Platelet Responsiveness to Aspirin and Clopidogrel: an In Vitro Pharmacodynamic Investigation. J Cardiovasc Transl Res 2014;7:39-46.

[21] Högberg C, Erlinge D, Braun OÖ. Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment. Throm J 2009;7:2.

[22] Maurer-Spurej E, Pfeiler G, Maurer N, Lindner H, Glatter O, Devine DV. Room temperature activates human blood platelets. Lab Invest 2001;81:581-92.

[23] Mohr J, Ruchholtz S, Hildebrand F, Flohe S, Frink M, Witte I, Weuster M, Frohlich M, Van Griensven M, Keibl C, Mommsen P. Induced hypothermia does not impair coagulation system in a swine multiple trauma model. J Trauma Acute Care Surg 2013;74:1014-20.

[24] Park KH, Lee KH, Kim H. Effect of hypothermia on coagulatory function and survival in Sprague-Dawley rats exposed to uncontrolled haemorrhagic shock. Injury 2013;44:91-6.
[25] Scharbert G, Kalb M, Marschalek C, Kozek-Langenecker SA. The effects of test temperature and storage temperature on platelet aggregation: A whole blood in vitro study. Anesth Analg 2006;102:1280-4.
[26] Scharbert G, Kalb ML, Essmeister R, Kozek-Langenecker SA. Mild and moderate hypothermia increases platelet aggregation induced by various agonists: A whole blood in vitro study. Platelets 2010;21:44-8.
[27] Staab DB, Sorensen VJ, Fath JJ, Raman SBK, Horst HM, Obeid FN. Coagulation defects resulting from ambient temperature-induced hypothermia. J Trauma 1994;36:634-8.
[28] Xavier RG, White AE, Fox SC, Wilcox RG, Heptinstall S. Enhanced platelet aggregation and activation under conditions of hypothermia. Thromb Haemost 2007;98:1266-75.
[29] Sibbing D, Braun S, Morath T, Mehilli J, Vogt W, Schomig A, Kastrati A, von Beckerath N. Platelet reactivity after clopidogrel treatment assessed with point-of-care analysis and early drug-eluting stent thrombosis. J Am Coll Cardiol 2009;53:849-56.
[30] Alstrom U, Granath F, Oldgren J, Stahle E, Tyden H, Siegbahn A. Platelet inhibition assessed with VerifyNow, flow cytometry and PlateletMapping in patients undergoing heart surgery. Thromb Res 2009;124:572-7.
[31] Bjelland TW, Hjertner O, Klepstad P, Kaisen K, Dale O, Haugen BO. Antiplatelet effect of clopidogrel is reduced in patients treated with therapeutic hypothermia after cardiac arrest. Resuscitation 2010;81:1627-31.
[32] Ibrahim K, Christoph M, Schmeinck S, Schmieder K, Kolschmann S, Pfluecke C, Kindler S, Schoen S, Wunderlich C, Strasser RH. Clopidogrel and prasugrel non-responder in therapeutic hypothermia after cardiac arrest. Eur Heart J 2012;33:315.

[33] Ibrahim K, Schmeink S, Kolschmann S, Steiding K, Schoen S, Wunderlich C, Pfluecke C, Christoph M, Strasser RH. Clopidogrel resistance in patients in therapeutic hypothermia after sudden cardiac death. Hamostaseologie 2011;31:A48.

[34] Penela D, Magaldi M, Fontanals J, Martin V, Regueiro A, Ortiz JT, Bosch X, Sabate M, Heras M. Hypothermia in acute coronary syndrome: brain salvage versus stent thrombosis? J Am Coll Cardiol 2013;61:686-7.

[35] Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, Horrow J, Husted S, James S, Katus H, Mahaffey KW, Scirica BM, Skene A, Steg PG, Storey RF, Harrington RA. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2009;361:1045-57.

[36] Wallentin L. P2Y12 inhibitors: Differences in properties and mechanisms of action and potential consequences for clinical use. Eur Heart J 2009;30:1964-77.

[37] Michelson AD, Barnard MR, Khuri SF, Rohrer MJ, MacGregor H, Valeri CR. The effects of aspirin and hypothermia on platelet function in vivo. Br J Haematol 1999;104:64-8.

[38] Ibrahim K, Christoph M, Schmeinck S, Schmieder K, Steiding K, Pfluecke C, Quick S, Mues C, Jellinghaus S, Wunderlich C, Strasser RH, Kolschmann S. High rates of prasugrel and ticagrelor non-responder in patients treated with therapeutic hypothermia after cardiac arrest. Resuscitation 2014;85:649-56.

[39] Nguyen NQ, Chapman MJ, Fraser RJ, Bryant LK, Burgstad C, Ching K, Bellon M, Holloway RH. The effects of sedation on gastric emptying and intra-gastric meal distribution in critical illness. Intensive Care Med 2008;34:454-60.

[40] Souckova L, Opatrilova R, Suk P, Cundrle Jr I, Pavlik M, Zvonicek V, Hlinomaz O, Sramek V. Impaired bioavailability and antiplatelet effect of high-dose clopidogrel in patients after cardiopulmonary resuscitation (CPR). Eur J Clin Pharmacol 2013;69:309-17.

[41] Ganter MT, Hofer CK. Coagulation monitoring: Current techniques and clinical use of viscoelastic point-of-care coagulation devices. Anesth Analg 2008;106:1366-75.

[42] Bindi ML, Biancofiore GD, Consani G, Cellai F, Cecconi N, Romanelli A, Filipponi F, Mosca F, Amorese G, Vagelli A. Blood coagulation monitoring during liver transplantation: Sonoclot analysis and laboratory tests. Minerva Anestesiol 2001;67:359-69.

[43] Hett DA, Walker D, Pilkington SN, Smith DC. Sonoclot analysis. Br J Anaesth 1995;75:771-6.

[44] Ortmann E, Walsh R, Klein AA, Jenkins DP, Luddington RJ, Besser MW. Point of care assessment of hypothermia induced platelet dysfunction during cardiopulmonary bypass with multiple electrode aggregometry (Multiplate®). Anaesthesia 2012;67:313.

[45] Gibbs NM. Point-of-care assessment of antiplatelet agents in the perioperative period: A review. Anaesth Intensive Care 2009;37:354-69.

[46] Schött U, Johansson PI. Bringing flow into haemostasis diagnostics. Br J Anaesth 2013;111:864-7.