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The Functionality of Cold-stored Platelets with Apoptosis Inhibition

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For those who supported me

Contents

0 Abbreviation

Abbreviation is not used in titles and at the first mentioning in the text, in the legend, and in the summary section. All abbreviations in text are listed below.

0.1 Abbreviation for the English part

Table 1. English abbreviation

abbreviation	**terms**
ADP	adenosine diphosphate
APC	apheresis-derived platelet concentrate
CI	confidential interval
CS	cold storage
CSL	cold storage lesion
CSP	cold-stored platelet
DMSO	dimethyl sulfoxide
FI	fold increase
GP	glycoprotein
GPIIb/IIIa	glycoprotein IIb/IIIa
MFI	mean fluorescence intensity
PAS	platelet additive solution
PBS	phosphate-buffered saline
PC	platelet concentrate
PLT	platelet
pos.	positive
PRP	platelet rich plasma
PS	phosphatidyl serine
PSL	platelet storage lesion
RCT	randomized controlled clinical trial
RSP	room temperature-stored platelet
RT	room temperature
SEM	standard error of mean
STR	septic transfusion reaction
TRAP	thrombin receptor-activating peptide
TTBI	transfusion-transmitted bacterial infection
vs.	versus
VWF	Von-Willebrandt-Factor

0.2 Abkürzung für den deutschen Anteil

Die Abkürzung wird nicht in Überschriften sowie bei der ersten Erwähnung im Text verwendet. Alle Abkürzungen sind hier aufgelistet.

Table 2. German abbreviation

Abkürzung	**Begriffe**
ADP	Adenosindiphosphat
ATK	Apheresethrombozytenkonzentrate
GPIIb/IIIa	Glykoprotein IIb/IIIa
KL	Kaltlagerung
KT	kaltgelagerte Thrombozyten
RT	Raumtemperatur
RTT	Raumtemperatur gelagerte Thrombozyten
TF	Thrombozytenfunktion
TRAP	Thrombinrezeptor aktivierendes Peptid

1 Introduction

1.1 Platelet concentrate and its clinical need

The transfusion of platelet concentrates (PCs) is an essential intervention for the prophylactic and therapeutic treatment of bleeding and dysfunction of primary hemostasis (Humbrecht et al., 2018). It is known that low platelet (PLT) count is correlated with reduced survival of patients with massive blood loss (Perkins et al., 2009; Pidcoke et al., 2012; Spinella et al., 2012; White et al., 2017). Currently, the supply of PLTs is still challenging because of their short shelf life. The PCs have limited storage time of 4-7 days depending on the guidelines of each country (Brecher et al., 2013a; 2013b; Caram-Deelder et al., 2016; Kreuger et al., 2017). For example, in Germany, the PCs can be stored only for four days after their production or to five days if additional screening for bacterial contamination or pathogen inactivation methods are applied (Sireis et al., 2011; Vollmer et al., 2011; de Korte and Marcelis, 2014; Bundesärztekammer, 2020). The limited shelf life of PCs has three main causes: their short life span of PLTs compared to other blood cells, the platelet storage lesion (PSL), and the risk of bacterial contamination.

1.1.1 Short living cells, platelets

PLTs are one of the smallest human cells (3.6 × 0.7 μm) acting in hemostasis, inflammation, wound healing and hematogenic metastasis (Jurk and Kehrel, 2005). They are enucleated and have a discoid shape with an origin from nucleate megakaryocytes in the bone marrow (Jurk and Kehrel, 2005; Ng et al., 2018). They live 7-10 days in the circulation before the clearance by macrophages in the liver or the spleen (Hanson and Slichter, 1985).

1.1.2 Platelet storage lesion and platelet elimination

PSL is commonly used for description of structural, biochemical and functional change that occurs from blood collection to transfusion (Vollmer et al., 2011). In a meta-analyze study, it was confirmed that old PLTs are inferior to fresh PLTs for all PLT measurements and for all storage time (Caram-Deelder et al., 2016). According to the activation analysis, old PLTs are already pre-activated and show storage lesions; they have more CD62 (also known as P-selectin), activated glycoprotein IIb/IIIa (GPIIb/IIIa,

previous known as $\alpha_{IIb}\beta_3$), as well as phosphatidylserine (PS) on their surface (Currie et al., 1997; Dasgupta et al., 2010).

However, PS is not only associated with activation but also with apoptosis. In fact, Schoenwaelder et al. (2009) reported that two different pathways can regulate PS exposure. One is the activation-associated, calcium-dependent, but caspase-independent pathway which can be induced by physiologic agonists. The second is a Bak/Bax-caspase-mediated, but activation-independent pathway. Dasgupta et al. suggested that PS-exposure after storage is not due to activation but due to the induction of the mitochondrial apoptotic pathways (Dasgupta et al., 2010). In particular, the release of cytochrome c from mitochondria and the activation of the caspase-3 and ROCK1 might lead to PS-exposure on the PLT surface (Dasgupta et al., 2010).

At the same time, sialic moieties of glycoproteins (GPs) expressed on the surface of aged PLTs are cleaved (desialylated) during storage and aging (Hoffmeister et al., 2003a). Especially, the GPIbα on the PLT surface seems to play a role in the PLT clearance. Quach et al. (2018a) and Getz et al. (2019) report both in their reviews that the conformational changes in GPIbα, a component of the GPIb-IX-V receptor complex, results in PLT clearance (Hoffmeister et al., 2003b; 2003c; Rumjantseva et al., 2009; Chen et al., 2016; Quach et al., 2018b).

Furthermore, the functionality changes during the storage time: 4-day-old room temperature stored platelets (RSPs) show less aggregation and dense-granule release after activation with collagen and ristocetin (Rosenfeld et al., 1995). The adhesion ability is also reduced after 7-day-storage at room temperature (RT) (Marini et al. 2019) Also in clinical situation, PSLs are visible: old PCs survive shorter in the circulation. Beside the increased number of needed PLT transfusions, they are associated with increased risk of transfusion reaction and bleeding, the severest complications of a transfusion (Caram-Deelder et al., 2016). Prolonged storage beyond 5 days decreases the recovery (Cauwenberghs et al., 2007). However, it has been showed that some interventions such as constant agitation, the addition of new generation of platelet additive solutions (PASs) or use of oxygen permeable transfusion bags allow to maintain aerobic-dominant glycolysis improving the quality of PLTs and minimizing the PSL (Dumont et al., 2007; Ohto and Nollet, 2011; Capocelli and Dumont, 2014).

1.1.3 The risk of bacterial contamination

The risk of bacterial growth in case of bacterial contamination in PCs is higher than other blood products, such as frozen plasma or erythrocyte concentrates, which is due to the storage condition like the use of oxygen permeable bags at RT (Hong et al., 2016). The contamination leads to major causes of morbidity and mortality following PLT transfusion like transfusion-transmitted bacterial infection (TTBI) and septic transfusion reactions (STRs). In particular, from 1:1000 to 1:2500 PC units are contaminated according to the reports with the acknowledge that TTBIs and STRs historically have been underreported (Pearce et al., 2011; Hong et al., 2016; Levy et al., 2018).

Giving the clinical and economic relevance of these products, different strategies have been investigated to reduce the bacteria-induced complications. One strategy is the questionnaires for every donor to prevent the collection of blood with bacteremia which is not always reliable and does not exclude asymptomatic cases (de Korte and Marcelis, 2014). The primary contamination source is the bacteria from skin flora. To reduce this risk, the initial blood volume (10-20mL) should be not be collected into the collection bag and used for other purposes than transfusion, for instance for the diagnostics or research (de Korte and Marcelis, 2014). There are also several testing methods to verify the quality of the products, either culture based or through target component detection, both including time, cost and sensitivity challenges. Some countries in Europe such as France, Belgium, and Switzerland introduced the pathogen inactivation in their PC storage program. Major systems are INTERCEPT™ Blood System for PLTs, Mirasol® Pathogen Reduction Technology System and THERAFLEX®UV-Platelets. However, besides the cost problem, the disadvantage of this intervention are the potential cellular damages including the decrease in pH, the increased surface expression of CD62 and PS, the enhanced PLT degranulation, and the decreased agonist-induced aggregation (Apelseth et al., 2006; Johnson et al., 2011; 2013; Ostrowski et al., 2011; Abonnenc et al., 2015; Sandgren and Diedrich, 2015; Ignatova et al., 2016).

1.2 Platelet activation in vivo

To execute hemostatic function, the PLT must be activated. The activation of PLTs is a complex process involving numerous participants. The three-stage-model has been

proposed to structure the interaction and simplify the mechanism (Scharf, 2018a). It is to notice that *in vivo*, it is a fluent process, and the schematic stages partly overlap.

1.2.1 Initiation

When the vessel is damaged, negatively charged substances of the subendothelium such as collagen is exposed to the inner side of vessel (Scharf, 2018a). The reflexive vasoconstriction provides high shear stress activating the PLTs (Jurk and Kehrel, 2005). The plasma GP Von-Willebrandt-Factor (VWF) released initially from endothelial cells, later from α-granule of PLTs is an ultra large multimer and binds to GPIb-IX-V in PLT with high affinity, a necessary process for the PLT adhesion (Ruggeri and Mendolicchio, 2015; Scharf, 2018). That results to a limited, instable binding which enable to catch and recruit the PLTs from the slowed blood flow through and PLTs "roll" and capture at the injury place (Kim et al., 2010; Blenner et al., 2014; Ruggeri and Mendolicchio, 2015; Saad and Schoenberger, 2020). Furthermore, the PLT can be captured and activated in seconds through the receptor binding of GPIb-IX-V to subendothelial VWF-collagen complexes (Jurk and Kehrel, 2005). At the same time, the collagen initiates the outside-in signaling of the PLT activation. Collagen binds to two adhesion receptors, the integrin GPVI and α2β1 (GPIa/IIa) which can only propagate the aggregation in presence of the interaction between GPIb-IX-V and VWF bound to collagen (Savage and Ruggeri, 1991; Savage et al., 1996; Watson 1999; Jurk and Kehrel, 2005). Additionally, resting integrin GPIIb/IIIa shows a high affinity to immobilized fibrinogen (Factor I) (Savage and Ruggeri, 1991; Savage et al., 1996).

1.2.2 Propagation/Extension (activation and aggregation)

The most important response of PLT is the functional change followed by the structural change of the integrated membrane protein GPIIb/IIIa (Godyna et al., 1996). The conformation of the integrin changes to the activated form with spread the heterodimers (Plow, 1999). The activated GPIIb/IIIa can bind with a higher affinity to fibrinogen, fibrin and VWF (Savage and Ruggeri, 1991; Savage et al., 1996; Plow, 1999). Importantly, these bridges connect PLTs with each other (Scharf, 2018).

The PLT activation (primary hemostasis) and the plasma coagulation (second hemostasis) are related and influencing each other. The inner negatively charged

phospholipid in the cellular membrane “flips” to the outer layer. The negative charge especially through the PS, acting as an efficient catalyst is essential for the α-thrombin (Factor IIa) generation and for the coagulation cascade (Bouchard et al., 1997; Mann, 1999; 2011; Scharf, 2018).

The irreversible PLT adhesion requires additional stimuli with rapid PLT responses such as α-thrombin and other soluble weak agonists including Thromboxane A2 (TXA2), adenosine diphosphate (ADP), epinephrine, and serotonin (Duerschmied and Bode, 2009; Gremmel et al., 2016). These mediators activate PLTs by amplifying and initiating the activation of circulating PLTs into a thrombus (Scharf, 2018). Furthermore, The PLT undergoes a shape change with secreting α- and dense-granules which contain numerous agonists and activators (Jurk and Kehrel, 2005). The TXA2 and platelet activating factor (PAF) are released unrelated from the granules.

In fact, the aggregation is the redundant initiation and activation of PLTs.

1.2.3 Stabilization

The aggregate consolidates in insoluble fibrin meshwork to stable thrombus (Scharf, 2018). The stability is of the clot retraction has a high relevance for the outcome of in clinical settings: it determines the hemostatic function and embolization (Scharf, 2018).

1.3 Cold storage

1.3.1 A new old strategy

To improve the quality and prolog the shelf life of PLTs without increasing the risk for bacterial contamination, one of the most common strategies is the cold storage (CS).

Storing the PCs at 4 ˚C was performed until the 70s without agitation (Waters et al., 2018) before four studies in the late 1960s and early 1970s showed shortened circulation times and decreased survival of cold-stored platelets (CSPs) than RSPs (Murphy and Gardner, 1969; Becker et al., 1973; Valeri, 1974a; 1974b; Shea et al., 2019). At that time, the main use of the PCs was for the prophylactic treatment of patients with dysplastic thrombocytopenia (Reddoch-Cardenas et al., 2019). Therefore, it was important that the PLTs have a longer circulation time in the blood stream of the patients to prevent spontaneous bleeding. In 1969, Murphy and Gardner radiolabeled PLTs stored at RT or refrigerated (2-6˚C) in order to measure the recovery and survival

which was defined as PLT viability. They reported a remarkably shorter survival of CSP after transfusion [t1/2 (the half-life of radiolabeled PLTs) = 1–2days] compared to RSP (t1/2 = 7–9 days).

In one of the three studies, Becker and colleagues (1973) compared the hemostatic effectiveness of PLTs with different storage temperature in a randomized controlled clinical trial (RCT). During this study, thrombocytopenic patients and normal aspirin-treated volunteers received PCs derived from fresh whole blood (FWB). The PCs were stored at 22 °C or at 4 °C with agitation for up to 72 h in first generation plastic storage containers with poor gas exchange. They evaluated the effectiveness of transfusion by PLT counts and bleeding times. As expected, the survival of CSPs was shortened to two or three days. However, the PLTs stored at 4 ˚C for 24 to 72 h demonstrated a significantly superior effect on hemostasis and reduced bleeding time. They were also better preserved in terms of pH stability, aggregability, and cellular structure. Therefore, they recommended to store PLTs in the cold for up to 72 h in order to improve hemostasis in bleeding patients. Despite of their recommendation, blood banks introduced only RSP to minimize the cost and challenges of a dual PLT maintenance (Reddoch-Cardenas et al., 2019; Shea et al., 2019). Thus, in last 50 years, the RSP became a part of a standard treatment for patients with hypoproliferative thrombocytopenia (Blajchman et al., 2008; Reddoch-Cardenas et al., 2019; Shea et al., 2019) Of note, it has to be acknowledged that the immune thrombocytopenia, thrombotic thrombocytopenic purpura, heparin-induced thrombocytopenia etc. should not be treated with allogenic PLT transfusion because of the significant risks of pathogen contamination and thrombus formation (Blajchman et al., 2008; Yuan and Goldfinger, 2020).

Recently, the need of PLT transfusion has shifted. As mentioned, the PCs are not only needed for prophylactic reasons but also for therapeutic use (Humbrecht et al., 2018). Most authors use the term "therapeutic transfusion" referring to both applications: transfusion to treat active bleeding and transfusion in preparation for an intervention with bleeding risk. They use the term "prophylactic transfusion" to refer to PLT transfusion given to prevent spontaneous bleeding without any active bleeding or invasive procedure which could cause bleeding. In fact, transfusion of PLTs is applied

to treat active bleeding as well as in preparation for an invasive procedure that could cause bleeding. In the new German Guideline for hemotherapy in 2020, the combined transfusion of erythrocytes concentrates and PCs for actively bleeding patients with massive and critical blood loss is strongly recommended, changed from weak recommendation (Bundesärztekammer, 2020). The prophylactic use is only highly recommended for patients with acute leukemia under 10,000/μL PLT concentration (Bundesärztekammer, 2020). For prophylactic use, standard practice has evolved to transfusion of PLTs at a threshold PLT count of 10,000 to 20,000/μL for most patients with severe hypoproliferative thrombocytopenia due to hematologic malignancies, cytotoxic chemotherapy, and hematopoietic cell transplant (HCT) (Estcourt et al., 2013).

Two RCTs with more than 1,000 patients (Stanworth et al., 2010; 2012; 2013; Wandt et al., 2012) evaluated the outcome upon PC transfusion for bleeding versus (vs.) routine prophylactic transfusion. In both studies the no-prophylaxis patients received fewer PLT transfusion [no prophylaxis group (176/300, 59 %) vs. prophylaxis group (266/298, 89 %)] and were associated with higher incidence of major bleeding (50 vs. 43 %) and a shorter time to first bleed (1.2 vs. 1.7 days) (Stanworth et al., 2010; 2012; 2013; Wandt et al., 2012). While there were two fatal intracranial hemorrhages in the one study (Wandt et al., 2012), other reported no differences in the duration of hospitalization, and no deaths due to bleeding (Stanworth et al., 2010; 2012; 2013).

In these cases, a better efficacy and functionality of PLTs and not only their inner survival are necessary to maintain hemostasis after massive bleeding or surgical intervention. Therefore, CSP is getting interest especially for patients with active bleeding (Cap and Spinella, 2017; Shea et al., 2019).

1.3.2 Cold storage and cold storage lesion

Waters et al. (2018) analyzed more than 10 studies comparing the reported effect upon CS and RT for the same time period; It has been observed that CSP has less active metabolism followed by decreased glycolysis and unchanged pH (Sandgren et al., 2006; Reddoch et al., 2014; Johnson et al., 2016), increased aggregation induced by ADP and collagen, as well as decreased bacterial proliferation (Currie et al., 1997; Vostal and Mondoro, 1997; Montgomery et al., 2013; Reddoch et al., 2014; Johnson et al., 2016).

However, the shelf life could be extended at least to two weeks (Sandgren et al., 2006; 2007; Wood et al., 2016).

On the other hand, Waters et al. (2018) also reported of cold storage lesions (CSLs), the negative effects due to chilling in his review. The cooling procedure led to higher level of intracellular calcium, which could be the initiator for many described changes during the CS (Oliver et al., 1999). For example, the actin fragmentation requires calcium, and it is known that CSPs undergo morphological changes through the involvement of the major cytoskeleton networks involving at least microtubules and actin filaments (Winokur and Hartwig, 1995; Wood et al., 2016; Getz, 2019). The chilling leads PLTs to a spherical form with multiple filopodia, compared to the normal discoid form, requiring calcium for actin fragmentation (Zucker and Borrelli, 1954; White and Krivit, 1967; Winokur and Hartwig, 1995).

Furthermore, the intracellular calcium leak might be also responsible for the enhanced activation. In fact, it is known that after CS the number of activated GPIIb/IIIa is increased (Wood et al., 2016; Waters et al., 2018). Moreover, the surface expression of CD62 was unchanged or increased, the PS exposure was also enhanced (Sandgren et al., 2006; Waters et al., 2018). Interestingly, the findings about the PLT desialylation are still controversy. The clustering of desialylated VWF receptor, GPIb in CSP was reported (Hoffmeister et al., 2003a). While other research groups reported minimal and not significant increase of the desialylation in CSP (Chen et al., 2017; Marini et al., 2019). After the CS, the apoptosis is likely enhanced with highly expressed PS (Marini et al., 2019). The CS leads to enhanced clearance *in vivo* (Hoffmeister et al., 2003a).

1.3.3 Possible mechanisms involved in the enhanced clearance of cold-stored platelets

The exact mechanism inducing the faster clearance of CSPs is not clarified so far. Several hypotheses have been proposed in the last years. In one study, it has been observed that after chilling, many GPIbα molecules organize as clusters over the PLT membrane deformed by internal actin rearrangements, which could lead to PLTs clearance (Hoffmeister et al., 2003a). Hoffmeister et al. (2003b) proposed the desialylation of GPIb as the mechanism for the clearance of CSPs through the hepatic macrophages with $\alpha_M\beta_2$ integrin. To verify this hypothesis, they tried to interrupt the interaction between them by galactosialylation. However, the group could not show a

longer survival of CSPs upon galactosialylation in a phase I clinical trial (Wandall et al., 2008). In 2020, Deppermann et al. proposed the mediation of the clearance of CSPs and desialylated PLTs by Ashwell-Morell receptor on the hepatocyte and Macrophage galactose lectin of the lever macrophage as mechanism responsible for the enhanced PLT clearance.

There are evidence suggesting that another mechanism than the desialylation could influence the clearance of CSPs. Recently, Chen et al. proposed that not the desialylation but the existence of VWF and the PLT-binding through GPIb induce the rapid clearance (Chen et al., 2017). While Marini et al. (2019) reported that the apoptosis is more associated with the short survival of the transfused CSP than the desialylation. So, they propose the apoptotic changes as the cause for the accelerated elimination.

1.3.4 Clinical investigation has restarted

In any case, no *in vitro* data can replace clinical results. Although there have not been clinical studies about CSP for decades, some interesting pilot studies have started recently, yet without promising results: Since 2015, Mayo Clinic has been utilizing 3-day CSP without agitation from the American Association of Blood Banks (AABB) and U.S. Food and Drug Administration (FDA) for trauma patients. At time of August 2016, only 21 of 119 CSPs could be transfused. Major cause was a high discard rate of 80.9 % (Strubbs et al., 2017). Other suggest that pathogen reduced PAS could improve this problem and make the CSP more practicable (Reddoch-Cardenas et al., 2019). Vostal et al. (2018) transfused 7-day-stored PLTs to autologous healthy donors and reported a better *in vivo* circulation kinetics of temperature cycling storage than CS but still inferior to RT storage. In December 2020, Strandenes et al., a group from Norway published a promising pilot trial at treating postoperative bleeding after cardiac surgery with 7-day old CSPs or RSPs in the double-arm stage I (n = 25) and with CSPs of 8-14 days in the single-arm stage II (n = 15). They provided no significant difference between RSPs and CSPs for hemostatic function including the clinical outcome, suggesting the possible feasibility of CS.

1.4 Project: a study for the improvement of the cold storage

1.4.1 Apoptosis inhibition for longer survival

We proposed the apoptosis as the cause of the enhanced clearance of cold-stored PLTs. In order to prolong the survival of PLTs, we treated the cold-stored apheresis-derived platelet concentrate (APC) with three different apoptosis inhibitors. The selected inhibitors, G04, forskolin, and z-LEHD-fmk block the apoptosis at different stages of the apoptotic signal cascade (Figure 1 B).

1.4.2 Main question: the platelet function

Not only the survival, but the hemostatic function is necessary for an efficient outcome after a PLT transfusion. Therefore, our group investigated both the survival and the functionality of APC-PLTs *in vitro.* While the parallel work about the survival and the effect of apoptosis inhibition of the inhibitors was done by Chiara Maettler (MD student), we focused on the evaluation of the functionality of the PLTs in this study. The aim of this project was to examine the APC-PLT function under RT storage, CS and apoptosis inhibition.

1.4.2.1 Functionality assay

In our study, we induced the hemostasis *in vitro*. To examine the function, we used the aggregometry and flow cytometry assays with different agonists (Figure 2) as described. Ristocetin, originally introduced as an antibiotic in the 60ies, causes *in vitro* agglutination by inducing the bound between VWF and GPIb (Gangarosa et al., 1958; 1960). Thrombin receptor activator peptide (TRAP) is a modified *in vitro* analogue of α-thrombin (Factor IIa), the strongest PLT activator. This synthetic hexapeptide is able to activate the thrombin receptor protease-activated receptor (PAR) directly independent of fibrin formation (Gresele et al., 2007)**.** ADP is an endogen molecule mostly released from the dense-granule which activates the PLT via receptor P_2Y_1 and P_2Y_{12} (Burnstock, 2004). Collagen, a protein in the extra cellular matrix, initiates the activation when exposed to the PLTs (Jurk and Kehrel, 2005).

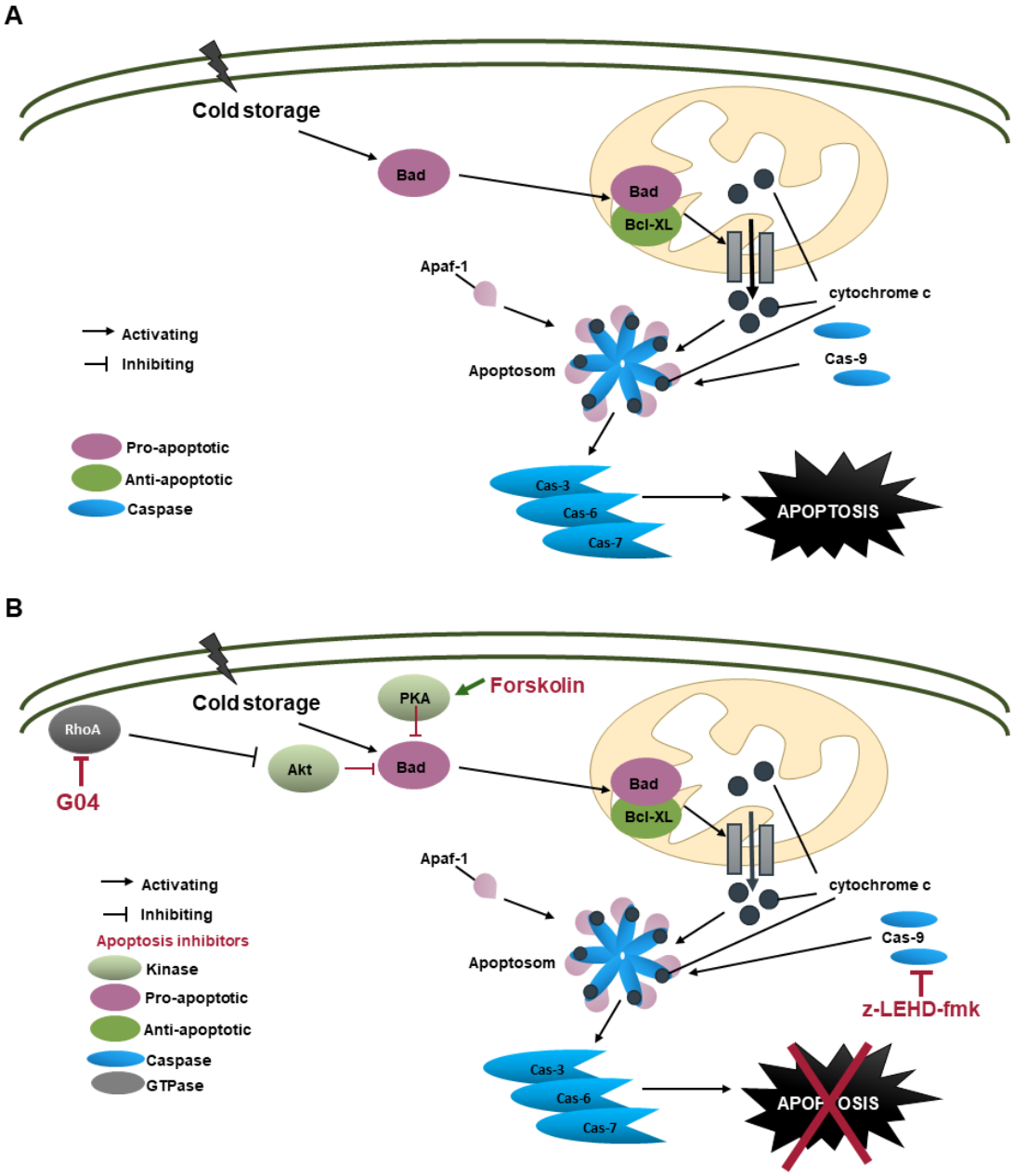

Figure 1. ***Possible Mechanism of apoptosis induction by cold storage and its inhibition.*** *(**A**) Possible mechanism of intrinsic pathway of apoptosis during the cold storage. The proteins of Bcl-family regulate the intrinsic apoptotic pathway by controlling the mitochondrial permeability. After the refrigeration, the pro-apoptotic Bcl-2 protein Bad is activated. Bad translocates itself to mitochondria and forms a pro-apoptotic complex with Bcl-xL. Bcl-xL is no more able to inhibit the mitochondrial cytochrome c release. Upon release from mitochondria, cytochrome c binds to Apaf-1 and forms an apoptosom, an activation complex with caspase-9. Once activated, the caspases cleave and activate downstream effector caspase. So does caspase-9 and it cleaves caspase-3, -6, and -7 which execute apoptosis. (**B**) Apoptosis inhibition on cold-stored platelet. In this project, three different points of the apoptotic pathway were modified to inhibit the apoptosis.*

Function testing: Aggregometry

This method, developed in the 1960s, was a revolution in the function testing history and is the gold standard today. In this technique, centrifuged platelet rich plasma (PRP) is stirred in a cuvette at 37 ºC, so the PLTs undergo a shear stress. Upon addition of an agonist, the PLTs start to aggregate. In the determined time period, the increase of light transmission due to the clot building is detected and recorded as PLT function (Budde, 2002).

Activation testing: Flow cytometry

This antibody-based method enables to observe the molecular changes on the PLT outer membrane. By using characteristic markers of PLT (Figure 3), the flow cytometry enables the quantification of PLT activation.

Besides VWF, platelet factor IV, and chemokine ligand 4 (CXCL4), CD62 is a common marker for the release of α-granules (Gremmel et al., 2016). Alpha-granules are granules of 200-500 nm containing integral membrane proteins such as GPIIb/IIIa, GPIb-IX-V, GPVI, CD62 and soluble proteins: coagulants, anticoagulants and fibrinolytic proteins (Factor V, IX, XIII, antithrombin, protein S, tissue factor pathway inhibitor, plasminogen, α2-macroglobulin), adhesion proteins (VWF, fibrinogen, thrombospondin), chemokines, growth factors, angiogenic factors and inhibitors, immune mediators and microbicidal proteins; one PLT contains more than 50 of those granules (Koseoglu and Flaumenhaft, 2013; Gremmel et al., 2016).

The membrane associated protein CD63 is a marker for the lysosome-related dense-granule. This organelle has a smaller diameter than the α-granules and exist only 3-8 per PLT. Their contents are cations (Ca^{2+}, Mg^{2+}, K^{+}), phosphates (polyphosphate, pyrophosphate) bioactive amines (serotonin, histamine) and nucleotides (ADP, ATP, UTP, GTP). (Koseoglu and Flaumenhaft, 2013; Gremmel et al., 2016).

PAC-1 antibody is a pentameric immune globulin (IgM) molecule which recognize the epitope of the activated GPIIb/IIIa after conformal change (Shattil et al., 1987; Abrams et al., 1990).

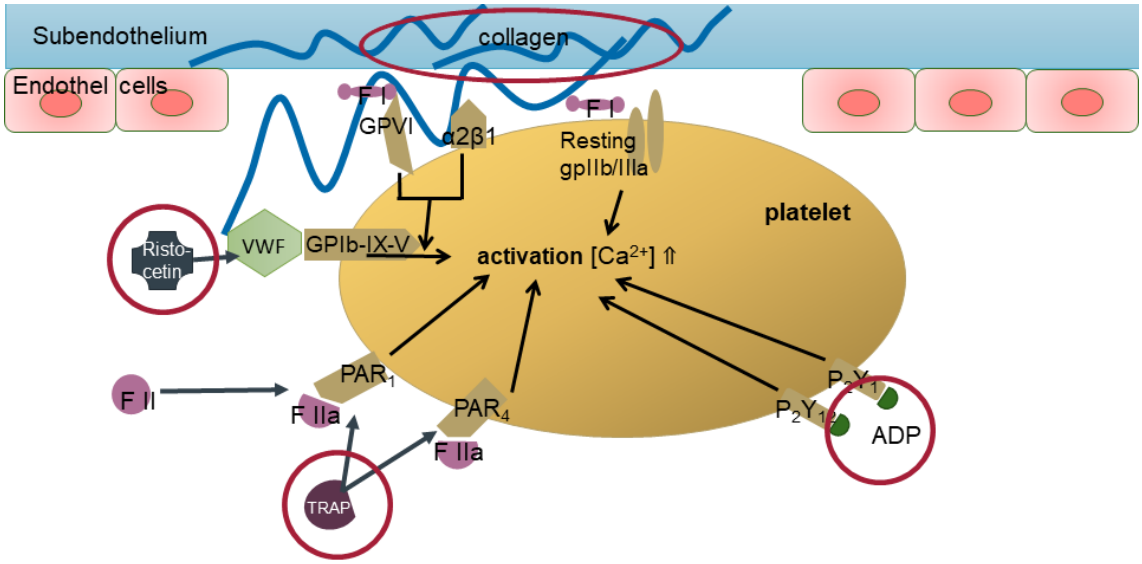

Figure 2. ***The platelet activation agonists.*** *Different agonists induce the platelet aggregation. Von-Willebrandt-Factor (VWF) binds either alone or together as a VWF-collagen-complex to GPIb-IX-V in PLT which is enhanced by Ristocetin. In presence of this interaction, collagen binds to two further adhesion receptors, the integrin glycoproteins GPVI and α2β1 (GPIIa/IIa) The immobilized fibrinogen (Factor I) and its activated form fibrin (Factor Ia), captured to collagen, bind to resting integrin GPIIb/IIIa (αIIbβ3). Thrombin receptor-activating peptide (TRAP) is an in-vitro analogue of thrombin (F IIa), the activated form of prothrombin (F II). Bound to protease-activated receptor (PAR1 and PAR4), TRAP activates the platelet. ADP induces via P_2Y_1 and P_2Y_{12} receptors the platelet aggregation. The inner cellular increase of calcium concentration leads to PLT activation. The circled agonists are used in this study.*

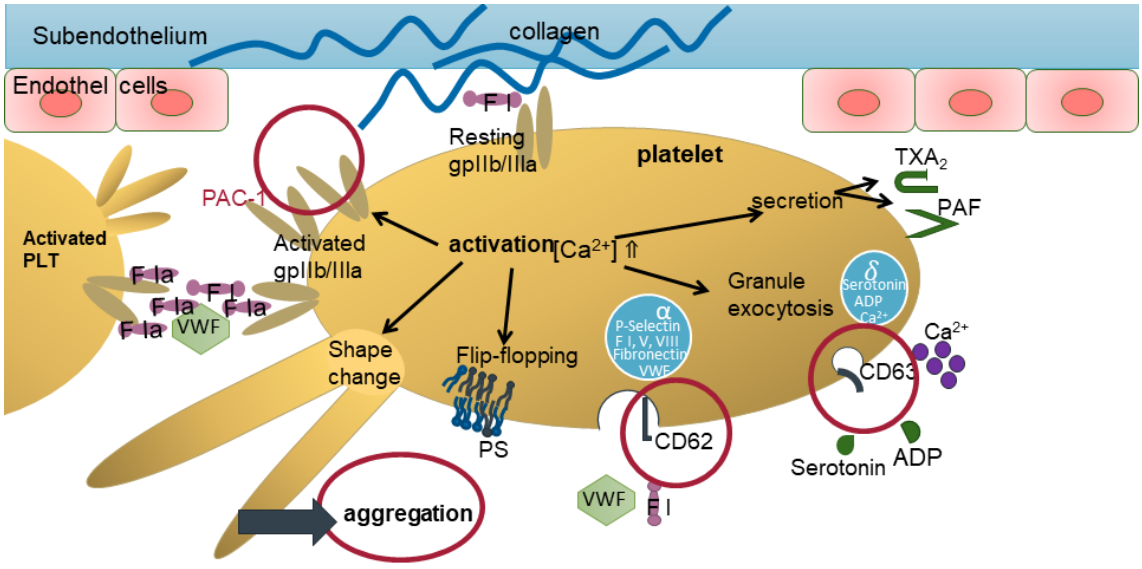

Figure 3. ***The platelet activation and its markers.*** *Upon initiation, the elevated calcium concentration activates the platelet. The PLT itself undergoes a shape change. The glycoprotein GPIIb/IIIa changes its conformation to the activated form, recognized by PAC-1. The activated GPIIb/IIIa binds with a higher affinity to fibrinogen, fibrin and VWF and furthermore, forms a platelet-to-platelet bound. Beside the direct secretion, the platelet releases α- and dense-granules (δ) by exocytosis which contain numerous agonists and activators. After the release, the membrane-associated CD62 (P-selectin) is exposed in the outer cell membrane. After the dense-granule releases, the PLT is marked with the integral protein CD63. The inner negatively charged phospholipid in the cellular membrane "flips" to the outer layer. The activation leads to aggregation. In this project, we used the circled characteristics to quantify the platelet functionality.*

1.4.2.2 Three concrete questions for the study

Since the expire date of RT-stored APC is 4-7 days after production, we focused the functional analysis not only on this period but until 10 days, by extending the storage time. By the agonist-induced aggregometry and the activation flow cytometry, we investigated the PLT functionality depending on the storage time, storage temperature and apoptosis inhibition. We defined three main questions; 1st question: *Does the APC production process impact the PLT functionality?* 2nd question: *How does the storage temperature impact the PLT functionality?* 3rd question: *Does apoptosis inhibition impact the CSP function?*

2 Methods

2.1 Materials

All materials are listed as tables after different categories.

2.1.1 Devices

Table 3. Required devices.

Device	**Company**
Agitator	noctua GmbH, Mössingen, Germany
APACT 4004	LABiTec, LAbor BioMedical Technologies, Ahrensburg, Germany
Cell-Dyn Ruby hematological	Abbott, Abbott Park, Illinois, U.S.A.
Flow-cytometer Navios	Beckman Coulter, USA
Freezer (-80 °C)	ilshin Europe, Ede, Netherlands
Heraeus 37 °C incubator	Thermo Scientific, Waltham, USA
Heraeus Cell bench, HeraSafe	Kendro Laboratory Products GmbH, Langenselbold, Germany
Refrigerator (4 °C)	Siemens, Germany
Research Plus adjustable volume	Eppendorf AG, Hamburg, Germany
Research Plus adjustable volume pipets 10; 20; 100; 200; 1000 µl	Eppendorf AG, Hamburg, Germany
Rotina 46 R Centrifuge	Hettich, Tuttlingen, Germany
SevenCompact pH meter S210	Mettler-Toledo, Greifensee, Germany
Test Tube Thermostat Model TCR100	Carl Roth, Karlsruhe, Germany
Trima Accel	Terumo BCT, Lakewood, CO USA
TubeOne microcentrifuge	Starlab, Hamburg, Germany
Vortexer, Reax-Top	Heidolph, Schwabach, Germany

2.1.2 Chemicals

Table 4. Required chemicals.

Chemical	**Company**
Adenosine diphosphate (ADP)	HART Biologicals, Hartlepool, United Kingdom
Calcein, AM	Life technologies Corporation, Thermo Fisher Scientific, Waltham, MA USA
Calcium chloride	Abcam, Cambridge, United Kingdome

Carbonyl cyanide 4-(trifluoromethoxy) Phenylhydrazone (FCCP)	Fresenius Kabi, Bad Homburg, Germany
Collagen	BioTop Medical, Haverton, PA USA
Dimethyl sulfoxide (DMSO)	Merck KGaA, Darmstadt, Germany
Distilled water	Biochrom, Berlin, Germany
Dulbecco's phosphate buffered saline (PBS)	Merck, USA
Forskolin	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
G04 Rho Inhibitor, RHOSIN	emd Millipore Corp., USA, affiliate of Merck KGaA, Darmstadt, Germany
Ionomycin	Abcam, Cambridge, UK
Ionophor	Sigma
PerFix-non centrifugation assay Kit	Thermo Fisher Scientific, Waltham, USA
Storage solution for Platelets (SSP+)	Macopharma, Mouvaux, France
Ristocetin 15 mg/ml	HART Biologicals, Hartlepool, United Kingdom
Sodium-chloride 0.9 % (NaCl)	Merck, Darmstadt, Germany
Thrombin receptor-activating peptide (TRAP)	HART Biologicals, Hartlepool, United Kingdom
Z-LEHD-fmk, Caspase-9 Inhibitor	BD Biosciences, San Jose, USA

2.1.3 Antibodies

Table 5. Required antibodies.

Antibody	**Company**
(FITC)-conjugated recombinant chicken Annexin V (AxV)	ImmunoTools, Friesoythe, Germany
FITC labelled Mouse Anti-Human CD62P, Clone CLBThromb/6	Beckman coulter, Marseille, France
FITC labelled Mouse Anti-Human CD63, Clone CLBGran/12	Beckman coulter, Marseille, France
FITC labelled Mouse Anti-Human PAC-1, Clone PAC-1	BD Biosciences, San Jose, USA
IgG1-FITC/IgG1-PE/IgG1-PC5 Antibody Cocktail, OptiClone	Beckman coulter, Marseille, France
PC5-labelled mouse anti-human CD41, Clone P2	Beckman coulter, Marseille, France
PE-labelled Tetramethylrhodamine,	abcam, United Kingdom

ethyl ester (TMRE)

2.1.4 Laboratory materials

Table 6. Required laboratory materials.

Laboratory materials	**Company**
100 Sterican 19G	Braun, Melsungen, Germany
50ml tubes	Greiner bio-one, Frickenhausen, Germany
Adapter	Fresenius Kabi AG, Bad Homburg, Germany
BD Vacutainer Na3Citrate 6.0ml	BD-Plymouth, UK
Cuvette FL with Mixer in Dispo System	LABiTec, LAbor BioMedical Technologies, Ahrensburg, Germany
Eppendorf-Cups 1.5; 2 ml	Eppendorf, Hamburg, Germany
Flow cytometer tubes	Sarstedt, Nümbrecht, Germany
Nitril gloves	Paul Hartmann, Heidenheim, Germany
oxygen peameable bag Compoflex	Fresenius Kabi AG, Bad Homburg, Germany
Pasteur pipet 5 ml	Carl Roth, Karlsruhe, Germany
S-Monovette EDTA 7.5 ml	Sarstedt, Nümbrecht, Germany
Sampling site coupler with Needle injection site	Fresenius Kabi AG, Bad Homburg, Germany
syringes for single use 2 ml	Disomed Witt oHG, Gelnhausen Germany

2.2 Methods

2.2.1 Blood samples

For this study, blood products were collected from healthy donors after obtaining written consensus. PCs and whole blood were collected according to the German guidelines for hemotherapy.

2.2.2 Study design

2.2.2.1 Apoptosis inhibition of cold-stored platelet concentrates

We investigated the effect of the apoptosis inhibitors on the CSP. The Figure 4 A illustrates the study design as schema. In this study, we used APCs collected from healthy volunteers. On the day of the production, defined as day 0, the APCs were either treated with: three different apoptosis inhibitors diluted in dimethyl sulfoxide

DMSO, the vehicle DMSO without the inhibitors, or incubated without any treatment (buffer).

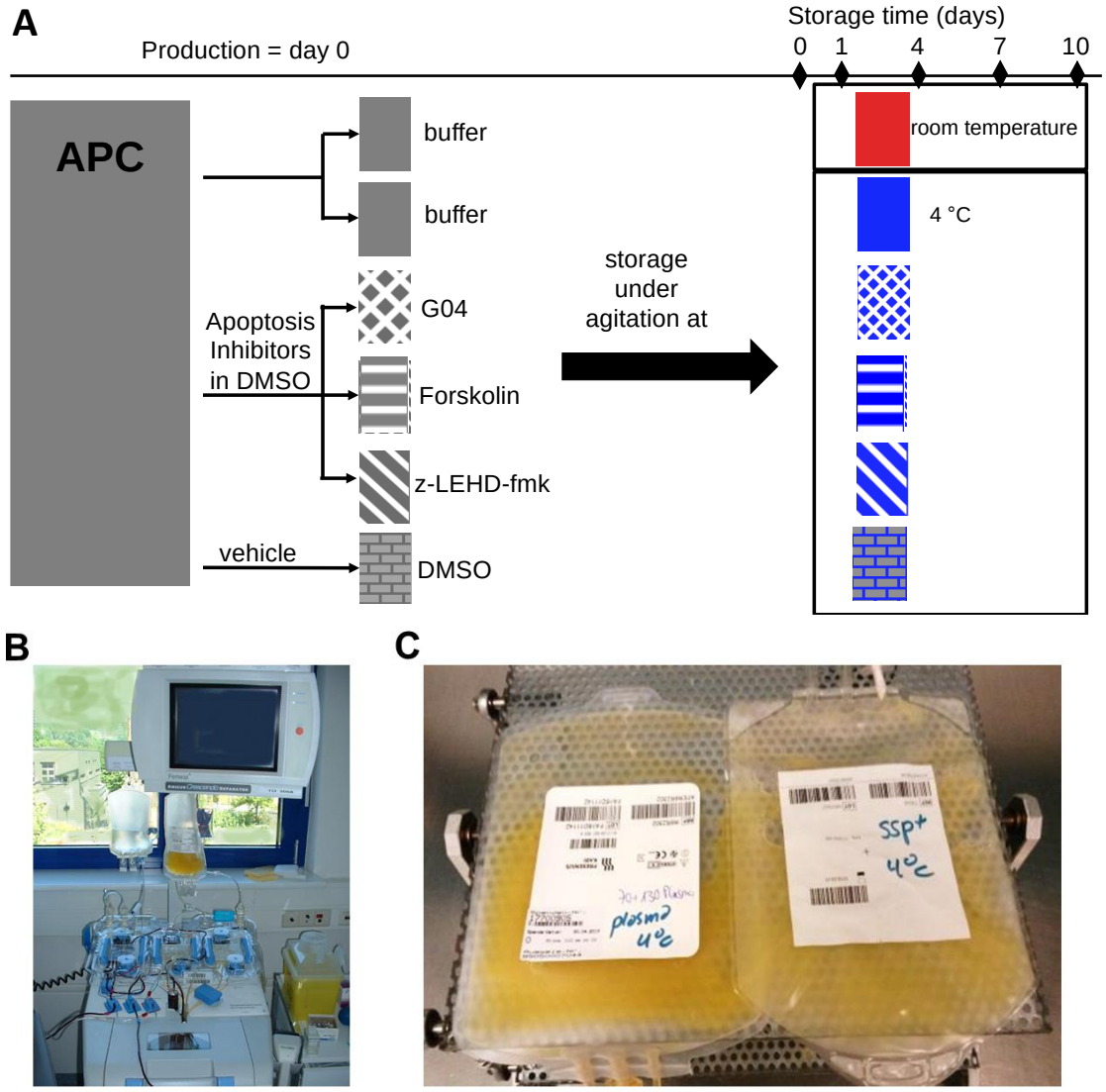

*Figure 4. **Study design**. (**A**) Schematic representation of the different treatments used for the apheresis-derived platelet concentrate (APC) and the corresponding storage conditions. After the collection with the apheresis machine Trima Accel (**B**), the samples were stored in sterile, oxygen permeable bag on a shaker (**C**).*

On day 0, we investigated the function of APC compared to PRP in order to verify the functional status of the PLTs immediately after the production. Next, the APCs were stored under constantly agitation at 4 °C except for the RT which was stored at RT. At the storage time points: 1, 4, 7, and 10 days the PLT functionality was examined. We investigated the effect of temperature (RT buffer vs. 4 ºC buffer), storage time (for each reagents) and CSP treatment with apoptosis inhibitors (4 ºC buffer vs. apoptosis inhibitors). To maximize the external validity, we compared the apoptosis-inhibitor-

treated PLTs stored with the non-vehicle-treated 4 ºC control, fulfilling the condition for transfusion except of the storage temperature. To exclude a possible impact of the vehicle DMSO, we investigated the effect of the vehicle in parallel (4 ºC buffer vs. DMSO).

2.2.3 Protocols

2.2.3.1 Platelet counts and preparation of platelet rich plasma

PLT and blood cell counts were performed with CellDyn (Abbott, Illinois, USA). The whole blood of healthy volunteers (5 ml) was taken in internationally normed tubes with citrate as a reversible anticoagulant. PRP was collected from citrate blood after centrifugation at 120 g for 15 minutes without brake. The PLTs were adjusted between 2.5 and 3×10^8/ml cells with phosphate-buffered saline (PBS) or with plasma from the same citrate-blood.

2.2.3.2 Preparation of platelet poor plasma

The platelet poor plasma (PPP) was collected from the supernatant of citrate blood upon centrifugation at 2000 g for 20 minutes without brake to separate plasma from PLTs and other cellular components.

2.2.3.3 Apheresis-derived platelet concentrates

Concerning the risk of bacterial contamination, we used APC having 4-times less infection risk than Pooled PC (Heuft et al., 2008). APC was collected Trima Accel (Terumo BCT, Lakewood, USA) by adding the PAS SSP+ (Macopharma, Mouvaux, France) to 35 % residual plasma with approximate volume 100-150ml and PLT content $1\text{-}2 \times 10^{11}$/PC.

2.2.3.4 Treatment with apoptosis inhibitors

Directly after the collection, the APCs were first stored for an hour without agitation. Then, the samples were split in different bags under sterile condition. They were treated with 150-300-600 µM G04, 0.75 µM forskolin or 40 µM z-LEHD-fmk, all diluted in DMSO. The rest of APC stayed either without treatment or the 600 µM vehicle DMSO was added. Every type of APCs was stored in sterile, oxygen permeable bag (Fresenius Kabi AG, Bad Homburg, Germany). After 2 hours of incubation at RT, the samples

excluding the control at RT were stored at 4 °C, all under constant agitation on an agitator.

For the titration, PRP was treated with different concentrations of forskolin (0.5-0.75-1.0-1.5-2.0 µM) or with 2.0 µM DMSO at RT for 2 hours.

2.2.3.5 The aggregation assays

The assay was performed using the APACT (LABiTec, LAbor BioMedical Technologies, Ahrensburg, Germany), a 4-channel-aggregometer which analyzes PLT function. PLTs from APC (4.5×10^7) were diluted in PBS and activated with 20 µM TRAP, 1.0 mg/ml ristocetin, 0.5 µg/ml collagen and 10 µM ADP. The maximal aggregation was registered during 6 min of measurement at 37 ºC. Fresh PLTs from PRP were used as positive (pos.) control.

2.2.3.6 Flow cytometry assays

PLTs (1.25×10^6) from APC were diluted in PBS with 1 mM Calcium ($CaCl_2$) and stained with IgG1-FITC/IgG1-PE/IgG1-PC5 Isotype-antibody, CD62P-FITC, CD63-FITC, or PAC-1 FITC for 20 minutes at RT. The samples were directly measured and analyzed by flow cytometry. For the activation assay, 20 µM TRAP or 10 µM ADP was added to the samples as activated control. The mean fluorescence intensity (MFI) of bound antibodies was registered. Fresh PLTs from PRP were used as pos. control.

2.2.4 Statistical analysis

Data are all expressed as mean ± standard error of mean (SEM). Numeric data were analyzed using 1-way (for single variant) or 2-way (for multiple variants) ANOVA. The 2-tailed Student's *t*-test was used to compare two groups. The Pearson correlation coefficient (r) was used to measure the strength of a linear association between two variables. Statistical analyses were performed using GraphPad Prism 8 (La Jolla, USA). $P < 0.05$ was considered statistically significant. Investigators were not blinded to the assessments.

2.2.4.1 Flow cytometry analysis

For standardization, the relative (rel.) MFI was calculated. The MFI of the sample with antibody x was divided by the MFI of isotype from the same APC.

$$rel.MFI\ [antibody\ x] = \frac{MFI\ [antibody\ x]}{MFI\ [isotype]}$$

For the presentation of inducibility of activation, we used the fold increase (FI). It represents the rate of the surface marker increases through the activator. The ratio is the division of PLT's MFI after incubation with the activator by the MFI of the baseline without activator but with buffer.

$$FI\ [antibody\ x] = \frac{MFI\ [antibody\ x\ with\ activator\ y]}{baseline\ MFI\ [antibody\ x\ without\ activator\ y]}$$

Thus, FI indicates the specific part of activation induced by the agonist. A FI of 1 means that the sample exposed the ligand in the same intensity with and without agonist: in this case, the activator did not induce activation. In this study, the PLTs were considered as activatable if the mean of FI was > 1.0.

For the quantitative analysis of the GPIIb/IIIa activation, we calculated the relative percentage of PAC-1 pos. cells considering as baseline the 1 % isotype sample.

The PLTs revealed a second peek of PLT population over the storage. Those were defined as shrunk PLTs (forward scatter < $10^{0.1}$) that are a peculiarity of the PSL (Figure 5).

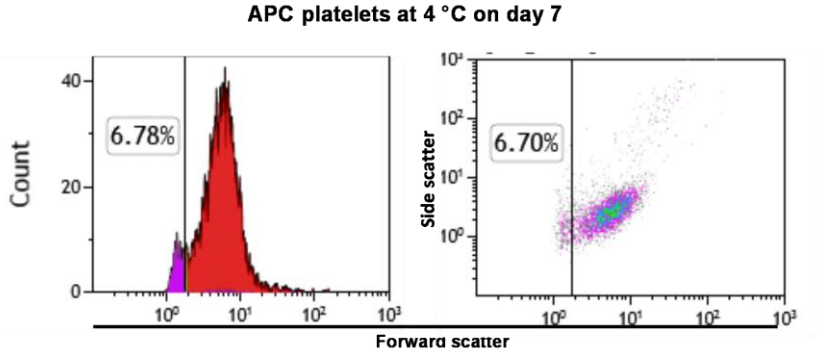

Figure 5. ***An example of flow cytometric Figures for the platelet shrinkage analysis.***

2.2.5 Study approval, Ethics

Studies involving human elements were approved by the ethics committees of the University Hospital of Tübingen respecting the declaration of Helsinki. The plan for the

raise of biobank was already advised by the ethic committee (507/2017BO1).

3 Results

3.1 Characteristics of apheresis-derived platelet concentrates

3.1.1 Fresh apheresis-derived platelet concentrates

Before investigating the stored PLTs, we examined the functionality of the APCs immediately after the production (day 0), compared to PRP.

3.1.1.1 Agonist-induced activation

3.1.1.1.1 Aggregation

Four different reagents were tested for the aggregation of the APCs: 1.0 mg/ml ristocetin, 20 µM TRAP, 0.5 µg/ml collagen, and 10 µM ADP.

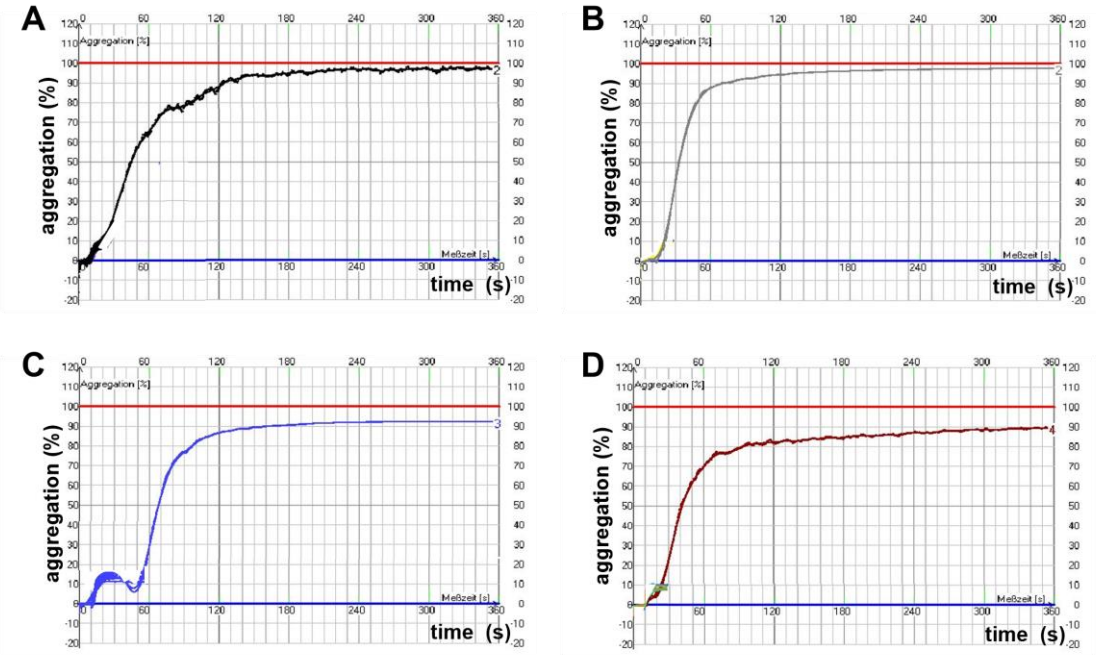

Figure 6. ***Fully aggregated platelets****. (**A**-**D**) Representative aggregometry figures of platelet rich plasma response to 10 mg/ml ristocetin (**A**), 20 µM thrombin receptor-activating peptide (TRAP) (**B**), 0.5 µg/ml collagen (**C**), and 10 µM adenosine-diphosphate (ADP) (**D**).*

APC showed the highest activation level of over 90 % in the presence of both activators ristocetin and TRAP; no significant difference was observed compared to PRP control [max. aggregation (mean ± SEM) of PRP vs. APC: ristocetin: 94.3 ± 1.8 % vs. 95.2 ± 1.9 %, P = 0.6701; n = 4; Figure 7 A; TRAP: 84.4 ± 5.0 % vs 92.4 ± 3.0 %, P = 0.0553; n = 4; Figure 7 B].

Of note, upon incubation of PLTs with the activators, collagen and ADP, reduced aggregation in APC was observed. The APC reacted moderately to collagen (PRP vs. APC: 93.9 ± 1.0 % vs. 49.0 ± 18.5 %, *P* = 0.07; n = 4; Figure 7 C). In contrast to the weak activation in fresh APC, the PLTs in PRP, as expected because used as pos. control, showed a strong aggregation, over 90 %, in the presence of ADP (PRP vs. APC: 95.3 ± 0.6 % vs. 17.8 ± 6.8 %, P < 0.001; n = 3; Figure 7 D).

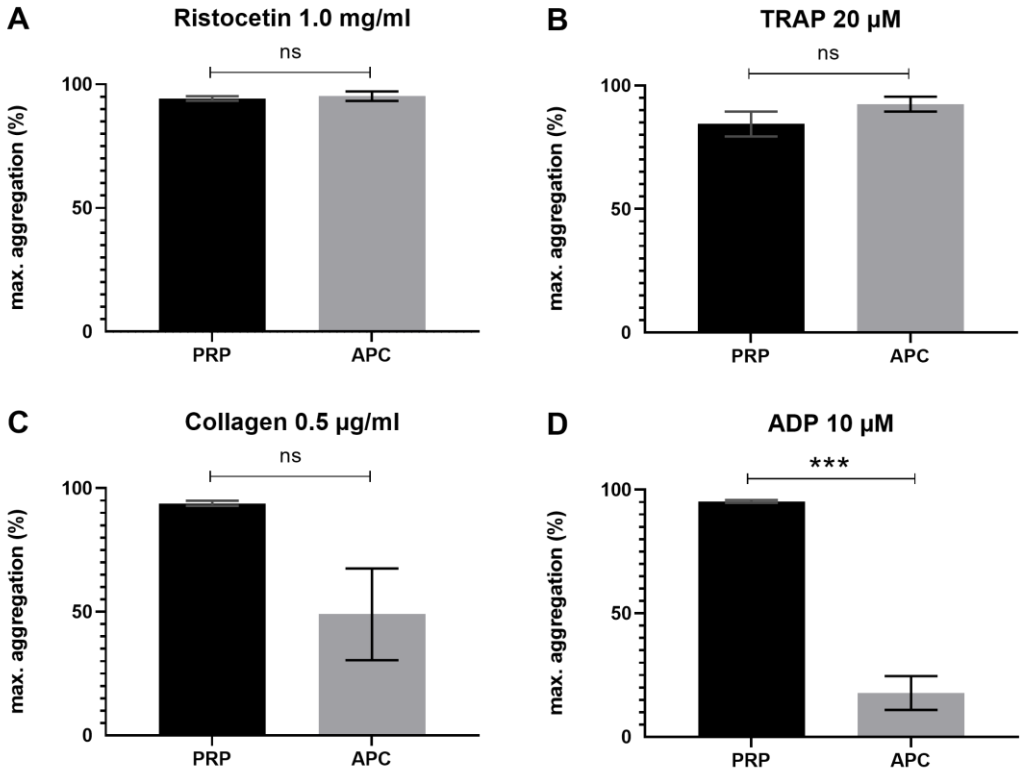

Figure 7. ***Fresh apheresis-derived platelet concentrate (APC) shows high aggregation to ristocetin and thrombin receptor-activating peptide (TRAP).*** *(**A**-**D**) Maximal (max.) aggregation (mean ± standard error mean) of PRP and fresh (day 0) APC) incubated with 1.0 mg/ml ristocetin (n = 4) (**A**), 20 µM TRAP (n = 4) (**B**), 0.5 µg/ml collagen (n = 4) (**C**), and 10 µM adenosine diphosphate (ADP) (n = 3) (**D**). ***P < 0.001 compared to PRP. ns = not significant.*

3.1.1.1.2 Granule releases and glycoprotein activation

On the production day (day 0), the APC was activated by two different agonists: 20µM TRAP and 10 µM ADP.

TRAP caused a strong activation of the PLTs as indicated by the release of α- and delta-granules markers [rel. MFI (mean ± SEM) of APC without vs. with TRAP-activation: CD62: 2.5 ± 0.6 vs. 17.9 ± 2.3, *P* = 0.008; CD63: 3.9 ± 0.7 vs. 18.9 ± 1.7, *P* = 0.0142; PAC-1: 2.0 ± 0.9 vs. 1.1 ± 0.2 vs. 13.0 ± 2.8, *P* = 0.0441; n = 5; Figure 8 B-

D] and in PRP (PRP without vs. with TRAP-activation: CD62: 1.8 ± 0.7 vs. 15.0 ± 3.8, *P* = 0.007; CD63: 2.7 ± 0.6 vs. 9.2 ± 0.6, *P* = 0.0194; PAC-1: 2.0 ± 0.9 vs. 29.3 ± 10.8, *P* = 0.008; n = 3; Figure 8 E-G).

Similarly to the aggregation result, ADP did not cause the PLT activation in fresh APC (APC without vs. with ADP-activation: CD62: 2.5 ± 0.6 vs. 2.5 ± 0.4, *P* = 0.997; CD63: 3.9 ± 0.7 vs. 3.3 ± 0.2, *P* = 0.4934; PAC-1: 1.1 ± 0.2 vs. 0.8 ± 0.4, *P* = 0.6345; n = 3; Figure 8 B-D). In PRP, ADP induced significant elevation of CD62 and PAC-1 expression (PRP without vs. with ADP-activation: CD62: 1.8 ± 0.7 vs. 3.1 ± 0.1, *P* = 0.0222; CD63: 2.7 ± 0.6 vs. 2.5 ± 0.6, *P* = 0.9256; PAC-1: 2.0 ± 0.9 vs. 17.5 ± 6.4, *P* = 0.0269; n = 3; Figure 8 E-G).

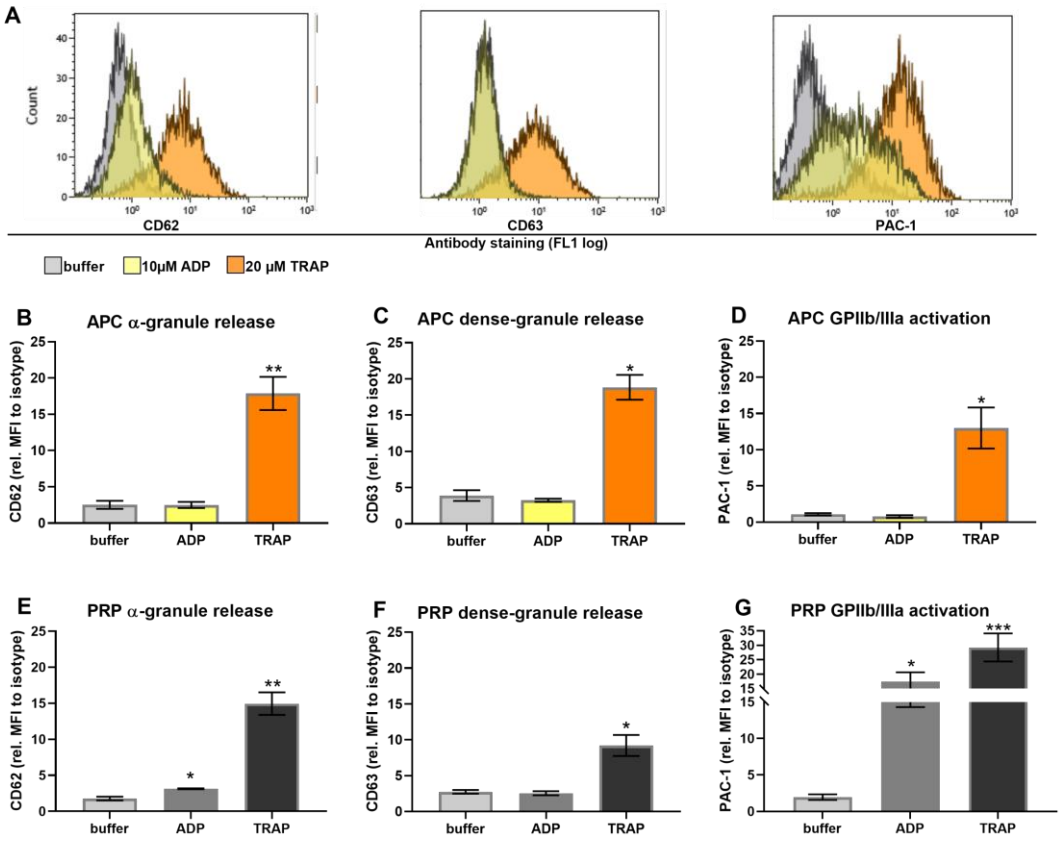

Figure 8. ***Thrombin receptor activating peptide (TRAP) induces strong platelet activation of fresh apheresis-derived platelet concentrate (APC).*** *Representative flow cytometric histograms (**A**). Quantification of α-granule release (**B** and **E**), dense-granule release (**C** and **F**) and activated glycoprotein IIb/IIIa (**D** and **G**) of APC (**A-D**; n = 3) or PRP (**E-F**; n = 5) on day 0 with buffer, 20 µM thrombin receptor-activating peptide (TRAP), or 10 µM adenosine diphosphate (ADP) (mean ± standard error of mean). Rel. MFI = relative mean fluorescence intensity. *P < 0.05; **P < 0.01; ***P < 0.001 compared to buffer samples without activators, 1-way ANOVA.*

3.1.2 Cold storage of apheresis-derived platelet concentrates

Next, we explored the impact of storage temperature.

3.1.2.1 Agonist-induced activation

3.1.2.1.1 Aggregation

Based on the previous data in 3.1.1.1.1, reporting a fully activation in the presence of 1.0 mg/ml ristocetin and 20 µM TRAP, we investigated the impact of both inductors in a long-term storage time.

Of note, the increased storage time or the storage in cold could not improve the aggregation with ADP and collagen. On day 4, the aggregation of ADP and collagen was decreased and there were no statistical differences in storage temperature. The max. aggregation of cold-stored collagen was twice as high as that of RT, yet under 20 % and without significance [max. aggregation (mean ± SEM) of RT vs. 4 °C: 10.2 ± 1.1 vs. 19.3 ± 2.2; P = 0.0669; n = 4; Figure 9 A]. ADP induced max. aggregation of around 10 % at RT as well as at 4 °C (RT vs. 4 °C: 8.7 ± 1.1 % vs. 1.8 ± 1.3, P = 0.2037; n = 4; Figure 9 B).

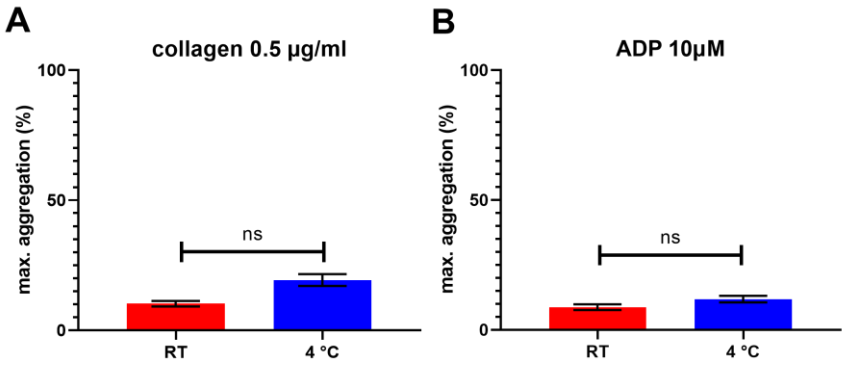

Figure 9. ***Apheresis-derived platelet concentrate (APC) reacts neither to collagen nor to ADP on day 4.*** *Maximal (max.) aggregation (mean ± standard error mean) of APC on day 4 incubated with 0.5 µg/ml collagen (n = 4) (**A**), and 10 µM adenosine diphosphate (ADP) (n = 3) (**B**). ns = not significant.*

CS improved the ristocetin-triggered aggregation from day 4 compared to RT (RT vs. 4 °C: day 4: 41.4 ± 13.3 % vs. 97.6 ± 1.9 %, P = 0.0237; day 7: 7.8 ± 3.5 % vs. 94.7 ± 2.7 %, P < 0.0001; day 10: 5.5 ± 0.9 % vs. 57.7 ± 19.2 %, P = 0.0229; n = 3; Figure 10 A). Observing the results in a chronological manner, we can report that CS maintained the max. aggregation constantly over 90 % until day 7 (day 1: 92.2 ± 6.1 %;

day 4: 97.6 ± 1.9 %; day 7: 94.7 ± 2.7 %; day 10: 57.7 ± 19.2 %; n = 3; Figure 10 B) and provided no significant difference of aggregation for each time period (day 1 vs. 4: P = 0.4447, day 1 vs. 7: P = 0.7316; day 1 vs. 10: P = 0.1615; n = 3; Figure 10 A). In contrast, the aggregation of RSPs dropped earlier with significance, during the storage time, starting from day 4 (day 1: 94.1 ± 2.2 %; day 4: 41.4 ± 13.3 %; day 7: 7.8 ± 3.5 %; day 10: 5.5 ± 0.9 %; day 1 vs. 4: P = 0.0007; day 1 vs. 7: P < 0.0001; day 1 vs. 10: P < 0.0001; n = 3; Figure 10 A). Consequently, the correlation between the RT storage and the storage time was strongly negative (storage time vs. max. aggregation: Pearson r = -0.8688; P < 0.0001; n = 3) those of 4 °C weaker and not significant (r = -0.5155; P = 0.0863; n = 3).

The difference of max. aggregation with TRAP was already visible from the beginning (RT vs. 4 °C on day 1: 89.5 ± 1.9 % vs. 96.2 ± 2.0 %, P = 0.0447; n = 3 Figure 10 B). On day 4, the cold buffer APC showed a strong aggregation over 90 % to TRAP, while RSPs markedly lost the ability of TRAP-induced aggregation over 10% and improved the aggregation significantly (RT vs. 4 °C: day 4: 11.3 ± 1.0 % vs. 93.4 ± 6.6 %, P < 0.0001; n = 3; Figure 10 B). Still on day 7, CSPs showed significant improvement of the aggregation (10.0 ± 0.8 % vs. 17.8 ± 2.8 %, P = 0.0162; n = 3; Figure 10 B). Analyzing each temperature based on the storage time, the buffer APC at 4 °C retained the aggregation until day 4. In fact, the aggregation induced by TRAP on day 1 and 4 was over 90 %, but it dropped significantly to less than 20 % on day 7 (day 1: 96.2 ± 2.0 %; day 4: 93.4 ± 6.6 %; day 7: 17.8 ± 2.8 %; day 10: 8.6 ± 0.7 %; day 1 vs. 4: P = 0.6376; day 1 vs. 7: P < 0.0001; day 1 vs. 10: P < 0.0001; n = 3; Figure 10 B). Meanwhile, the RT storage resulted to an earlier impairment of PLT aggregation of less than 15 % from day 4 which did not recover during the measurement. The RT storage provided a significant drop of maximal aggregation upon day 4 (day 1: 89.5 ± 1.9 %; day 4: 11.3 ± 1.0 %; day 7: 10.0 ± 0.8 %; day 10: 8.4 ± 0.8 %; day 1 vs. 4: P < 0.0001; day 1 vs. 7: P < 0.0001; day 1 vs. 10: P < 0.0001; Figure 10 B). In both cases, the storage time was negatively correlated to the aggregation ability (RT: r = -0.788, P < 0.0001; 4 °C: r = -0.9241, P < 0.0001; storage time vs. max. aggregation, respectively; n = 3).

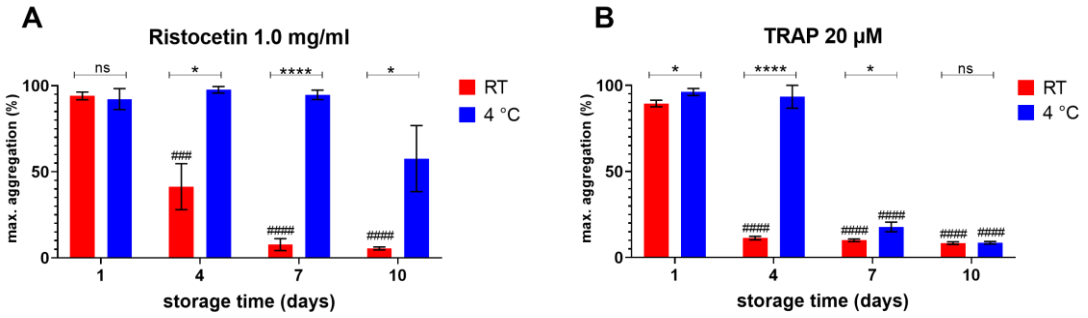

Figure 10. ***Cold storage improves the aggregation on day 4 and 7.*** *(**A** and **B**) Maximal (max.) aggregation of apheresis-derived platelet concentrate (APC) incubated with ristocetin (**A**) and thrombin receptor-activating peptide (TRAP) (**B**) (mean ± standard error mean, n = 3). APCs with buffer were stored at room temperature (RT) or at 4 °C. ###P < 0.001; ####P < 0.0001 compared to day 1 (baseline) of the same treatment. *P < 0.05; ****P < 0.0001. If not indicated, the differences were not significant (ns).*

3.1.2.1.2 Granule releases and glycoprotein activation

Based on the previous results in 3.1.1.1.2, we analyzed the activation with 20 µM TRAP in detail for the stored APCs.

Analyzing the different storage time point of RSPs and CSPs, TRAP induced strong α-granule release on day 1 [FI (mean ± SEM) of RT: 4.4 ± 0.8; 4 ºC: 3.5 ± 0.3; n = 3; Figure 11 A]. The CSPs were able to be activated by TRAP until day 7 (4 °C: day 4: 1.9 ± 0.3; day 7: 1.5 ± 0.3; day 10: 1.0 ± 0.03; n = 3; Figure 11 A), while RSPs were not activatable on day 7 (day 4: 1.2 ± 0.2; day 7: 1.0 ± 0.03; day 10: 1.1 ± 0.1; n = 3; Figure 11 A). Yet, the responsiveness of the PLTs to TRAP and the consequent α-granule release reduced significantly with the storage time for RT as well as 4 °C storage (RT: day 1 vs. 4: $P = 0.0017$; day 1 vs. 7: $P = 0.0062$; day 1 vs. 10: $P = 0.0133$; 4 °C: day 1 vs. 4: $P = 0.015$; day 1 vs. 7: $P = 0.0064$; day 1 vs. 10: $P = 0.0006$, Figure 11 A). The correlation of RT to storage time was moderately negative (r = -0.667, $P = 0.0025$, n = 3). The FI of 4 °C buffer correlated strongly negatively to storage time (4 °C: r = -0.8927, $P < 0.0001$; n = 3).

Investigating the TRAP-induced CD63 expression, the CS buffer improved the dense-degranulation on day 4 [FI (mean ± SEM) of RT vs. 4 ºC: day 4: 1.0 ± 0.04 vs. 2.1 ± 0.2 %, $P = 0.0004$; n = 3; Figure 11 B]. Furthermore, the CS retained the induced dense-granule release until day 10, even showing a weak level on day 7 and 10 (4 ºC on day 1: 2.3 ± 0.4; day 4: 2.1 ± 0.2; day 7: 1.3 ± 0.2; day 10: 1.2 ± 0.2; n = 3; Figure 11 B). While the FI of RT significantly dropped and the PLTs stored at RT did not release the

dense-granule upon TRAP-incubation from day 4 (day 1: 2.6 ± 0.6; day 4: 1.0 ± 0.04; day 7: 1.0 ± 0.03; day 10: 0.9 ± 0.1; day 1 vs. day 4: *P* = 0.0133; day 1 vs. day 7: *P* = 0.0436; day 1 vs. day 10: *P* = 0.0368; n = 3; Figure 11 B). Finally, both the granule release of RT and 4 °C was negatively correlated to the storage time (RT: r = -0.6032, *P* = 0.008; 4 °C buffer: r = -0.7124, *P* = 0.0093; n = 3).

Exploring the GPIIb/IIIa activation by TRAP, we observed that the FI of PAC-1 was markedly higher than that of CD62 and CD63. The CSPs showed inferior activation by TRAP on the first day, but superior activation from day 4. In particular, CSPs could provide a FI over 5 on day 4 [FI (mean ± SEM): 6.4 ± 2.8; n = 3; Figure 11 C] and still on day 7 and 10, TRAP activated CSPs twice as much as without activator (day 7: 2.5 ± 0.6; day 10: 2.3 ± 1.1; n = 3; Figure 11 C). While the RSPs were activatable only until day 7 and the FI of RT significantly decreased during the storage time (day 4: 1.4 ± 0.1; day 7: 1.8 ± 0.6; day 10: 0.7 ± 0.2; day 1 vs. day 4: *P* = 0.0222; day 1 vs. day 7: *P* = 0.0308; day 1 vs. day 10: *P* = 0.0162; n = 3; Figure 11 C). Consequently, the RT showed a negative correlation with storage time (r = -0.7433, *P* = 0.056; n = 3).

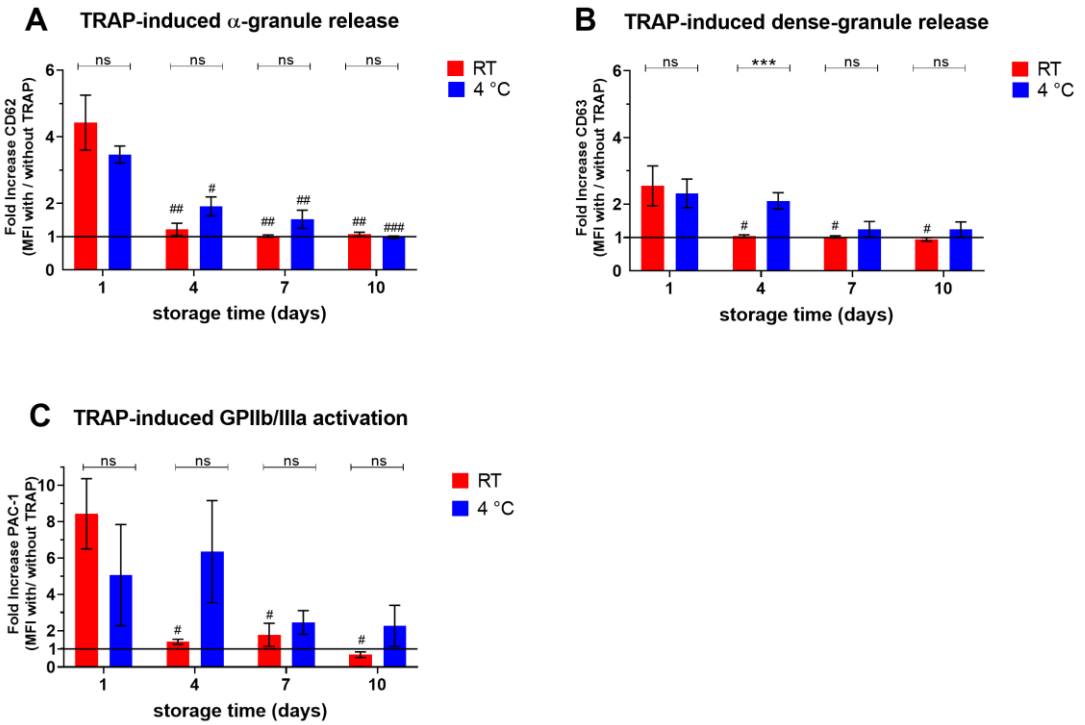

Figure 11. ***Cold storage improves the dense-granule release on day 4.*** *Inducibility of α-granule release (**A**), dense-granule release (**B**), and glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**) by 20 µM thrombin receptor-activating peptide (TRAP) in apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3). APCs with buffer were stored at room temperature (RT) or at 4 °C. MFI = mean fluorescence intensity. #P < 0.05; ##P < 0.01;*

*##P < 0.001 compared to day 1 (baseline) of the same treatment. ***P < 0.001. If not indicated, the differences were not significant (ns).*

3.1.2.2 Platelet storage lesion

First, the PSL was examined measuring the exposure of CD62 on resting PLTs (without activator). The storage temperature led to a statistical difference of pre-release and on day 1 and 7 and CS increased the CD62 expression level (RT vs. 4 °C: day 1: P = 0.0426; day 7: P = 0.0305; Figure 12 A). Analyzing the temperature separately, the expression level of CD62 at RT had a maximum on day 4 while CS tended to increase the granule release with time. This is also indicated by the weakly negative correlation of RT with storage time (storage time vs. rel. MFI: r = -0.2199; P = 0.4131; n = 3) and indicated by the very weak pos. correlation of cold-stored samples (4 ºC buffer: r = 0.09511, P = 0.7687; n = 3), even if they were not statistically significant.

Next, we examined the dense-degranulation without activator. CS provided less CD63 expression than RT storage from day 4 on. In particular, the CSPs showed a significant decrease of dense-granules pre-release compared to untreated RSPs on day 10 (rel. MFI of RT vs. 4 ºC: 6.4 ± 0.4 vs. 3.3 ± 0.6, P = 0.038; n = 3; Figure 12 B). Interestingly, the maximum of CSPs was on day 1 (day 1: 5.3 ± 0.6; day 4: 2.4 ± 0.3; day 7: 3.9 ± 0.7; day 10: 3.4 ± 0.43; n = 3; Figure 12 B). Moreover, the reduction of CD63 on day 4 was significant (day 1 vs. day 4: P = 0.0131; Figure 12 B). In contrast, the CD63 expression showed a pos. correlation between RT and storage time (r = 0.5858, P = 0.0171; n = 3). Moreover, the increase on day 10 was significant compared to the beginning of the storage period (rel. MFI at RT day 1 vs. 10: 3.4 ± 0.4 vs. 6.4 ± 0.4, P = 0.0036; n = 3; Figure 12 B).

Furthermore, PAC-1 expression without inductor was measured. On day 1, cold buffer activated GPIIb/IIIa very strongly over 20 % [PAC-1 pos. cells (mean ± SEM) of 4 °C buffer on day 1: 21.9 ± 13.4 %; n = 3; Figure 12 C]. At the end of the measurement, in contrast, RT had the most PAC-1 expression (RT on day 10: 27.3 ± 12.2 %; n = 3; Figure 12 C). Analyzing the treatments separately, the untreated CSPs had high surface expression on day 1 and 7 (4 °C buffer: day 1: 21.9 ± 13.4 %; day 4: 8.7 ± 5.7 %; day 7: 17.3 ± 7.9 %; day 10: 4.0 ± 1.1 %; n = 3; Figure 12 C) and showed a negative correlation to storage time without significance (PAC-1 pos. cells of 4 °C buffer vs.

storage time: r = -0.3686, *P* = 0.2384; n = 3). RT showed a time dependent pre-activation of GPIIb/IIIa with continuous increase of PAC-1 expression during the storage but without reaching a statistical significance (RT: day 1: 7.4 ± 3.0 %; day 4: 6.6 ± 0.6 %; day 7: 12.77 ± 10.0 %; day 10: 27.3 ± 12.2 %; n = 3; Figure 12 C). The RT storage indicated stronger pos. correlation with storage time, but without reaching statistical significance (PAC-1 pos. cells at RT vs. storage time: r = -0.5226, *P* = 0.0813; n = 3).

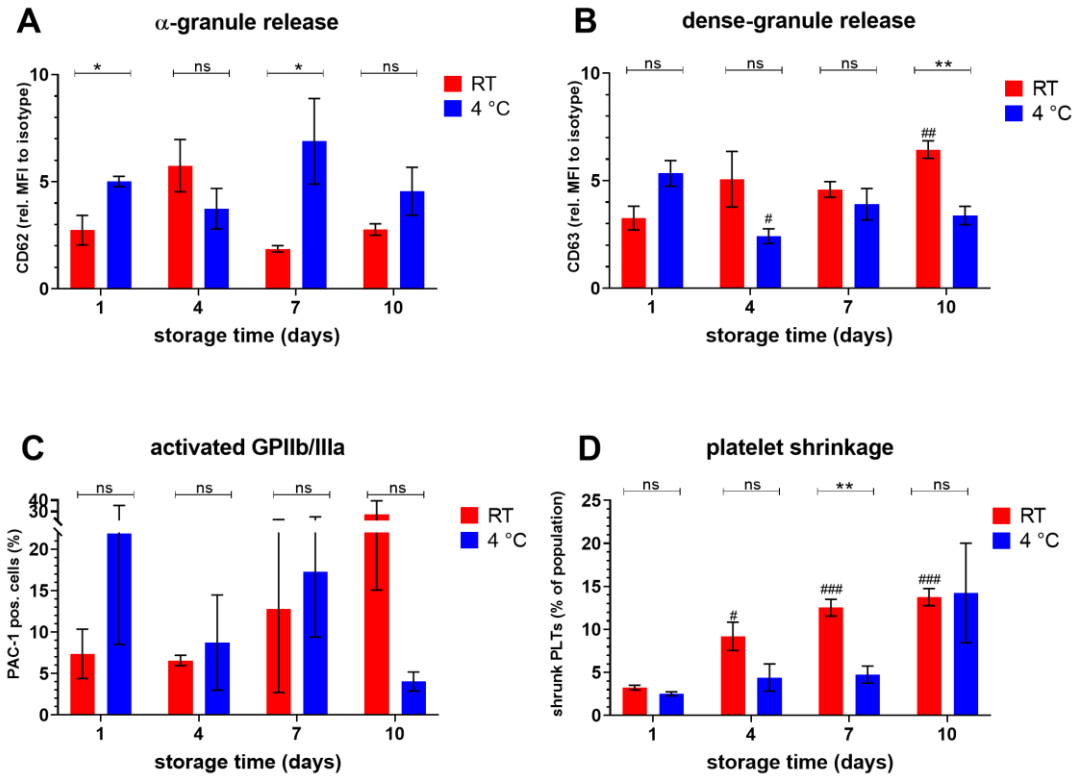

Figure 12. ***The cold storage reduces dense-granule release on day 10 and shrinkage on day 7.*** *α-granule release (**A**), dense- granule release (**B**), glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**), and platelet shrinkage (**D**) of apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3). APCs with buffer were stored at room temperature (RT) or at 4 °C. MFI = relative mean fluorescence intensity. Pos. = positive. #P < 0.05; ###P < 0.001 compared to day 1 (baseline) of the same treatment. *P < 0.05; **P < 0.01. If not indicated, the differences were not significant (ns).*

Finally, the PLT shrinkage was compared. On day 1, the percentage of shrunk PLTs was under 4 % in both storage type. On day 4, CSPs shrank only half as much as the RSPs [shrunk cells (mean ± SEM): RT: 9.2 ± 1.7 %; 4 ºC: 4.4 ± 1.6 %; n = 3; Figure 12 D]. The CS provided significantly less damage on day 7 (RT vs. 4 ºC: 12.5 ± 1.0 % vs. 4.7 ± 1.0 %; *P* = 0.005; n = 3; Figure 12 D). At the last measure point, both caused an increased shrunk PLT population over 10 % (RT: 13.8 ± 1.0 %; 4 ºC: 14.2 ± 5.8 %; n

= 3; Figure 12 D). As shown in the Figure 24 B, the RT storage significantly enhanced the lesion from day 4 on (RT on day 1: 3.2 ± 0.3 %; day 4: 9.2 ± 1.7 %; day 7: 12.5 ± 1.0 %; day 10: 13.8 ± 1.0 %; day 1 vs. 4: P = 0.0233; day 1 vs. 7: P = 0.0008; day 1 vs. 10: P = 0.0005; n = 3; Figure 12 D). The shrunk PLTs population increased in both samples, regardless of the storage temperature, in a time-dependent manner. The storage time correlated with the increase of PLT shrinkage (RT: r = 0.8953, P < 0.0001; n = 3; 4 °C: r = 0.6327, P = 0.0272; n = 3)

3.2 Impact of apoptosis inhibitors on cold-stored platelets

We used 150 µM G04, 0.75 µM forskolin and 40µM z-LEHD-fmk as apoptosis inhibitors. Since the optimal concentrations of G04 and forskolin were unknown, we titrated them at first which as shown in the supplementary data in 3.3.2.

3.2.1 Inhibition of RhoA-GTPase

G04 is a RhoA-GTPase-inhibitor which interacts in the upper stream of intrinsic apoptotic pathway (Figure 13).

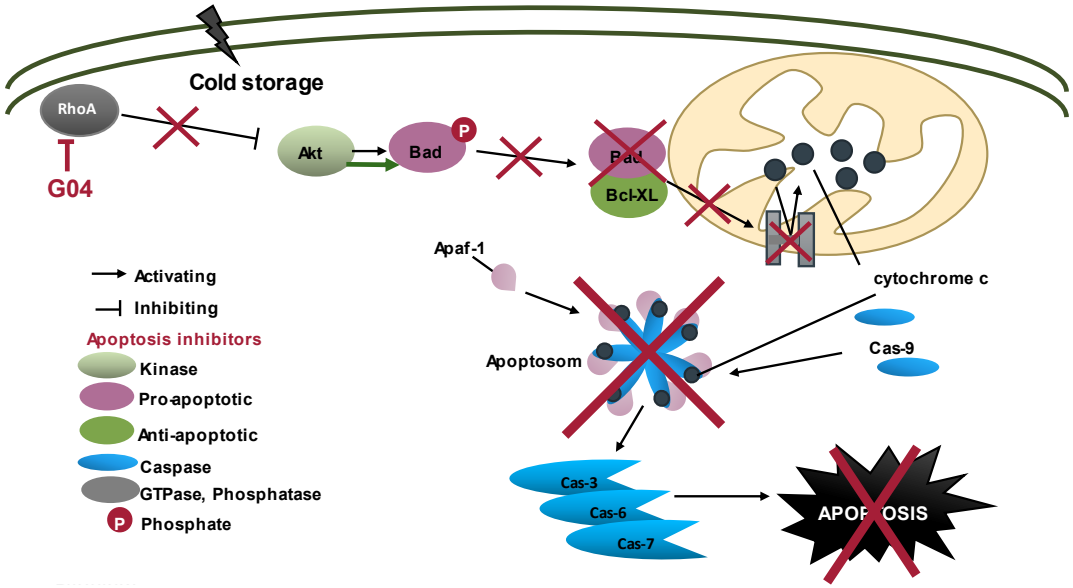

Figure 13. ***Signal pathway of G04.*** *G04 inhibits the GTPase RhoA and this enables to activate the kinase Akt. The kinase inactivates the pro-apoptotic protein Bad: the phosphorylation of Bad by Akt inhibits the translocation of Bad to mitochondria, leading to its cytosolic sequestration.*

3.2.1.1 Agonist-induced activation

3.2.1.1.1 Aggregation

The RhoA-inhibitor retained the ristocetin-induced aggregation, presenting no statistical differences. Likely to the buffer control, the max. aggregation was over 85 % until day 7 [max. aggregation (mean ± SEM) G04: day 1: 90.2 ± 3.3 %, P = 0.9904; day 4: 87.6 ± 4.6 %, P = 0.4699; day 7: 92.0 ± 1.0 %, P = 0.8786; compared to buffer, respectively; n = 3; Figure 14 A]. On day 10, RhoA-inhibitor showed improving tendence of aggregation without significance (buffer vs. G04: 57.7 ± 19.2 % vs. 64.0 ± 18.6 %, P = 0.9016; n = 3; Figure 14 A). Alike to the control, RhoA-inhibitor did not show significant correlation with time (buffer: r = -0.5155, P = 0.0863; G04: r = -0.5194, P = 0.0836; storage time vs. max. aggregation, respectively; n = 3).

TRAP-induced aggregation was neither impacted by RhoA-inhibitor. In particular, G04 retained the aggregation over 90 % on day 1 (G04: 92.4 ± 1.4 %, P = 0.2399 compared to buffer; n = 3; Figure 14 B). The observed partial reduction on day 4 provided no statistical significance (G04: 54.7 ± 24.3 %, P = 0.3514 compared to buffer; n = 3; Figure 14 B). More interestingly, RhoA-inhibitor significantly improved the PLT aggregation on day 7 (buffer vs. G04: 17.8 ± 2.8 % vs. 67.3 ± 11.6 %, P = 0.0017; n = 3; Figure 14 B). Consequently, RhoA-inhibitor was able to maintain a partial aggregation ability over 60 % until day 7 (day 1 vs. 4: P = 0.3551; day 1 vs. 7: P = 0.0983; day 1 vs. 10: P < 0.0001; n = 3; Figure 14 B), while the buffer APC retained the aggregation only until day 4 (n = 3; Figure 14 B). Likely to buffer, the storage time showed a negative correlation to the aggregation ability of RhoA-inhibitor (buffer: r = -0.9241, P < 0.0001; G04: r = -0.8159, P = 0.0012; storage time vs. max. aggregation, respectively; n = 3).

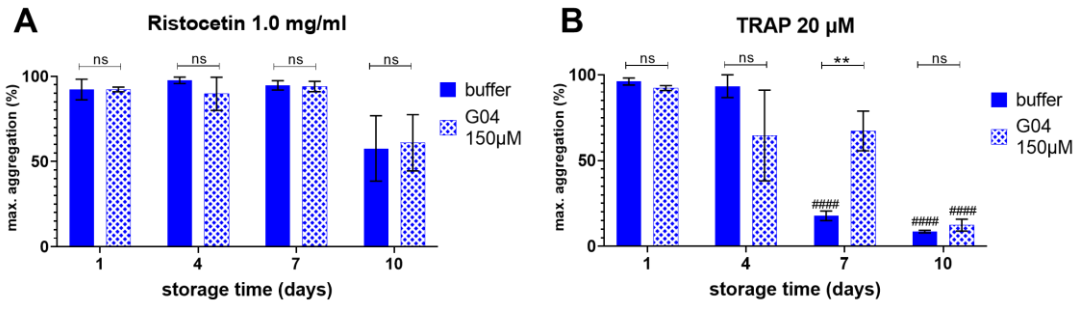

Figure 14. ***The RhoA-inhibitor improves the TRAP-induced aggregation on day 7.*** *(**A** and **B**) Maximal (max.) aggregation of cold-stored apheresis-derived platelet concentrate (APC) incubated with ristocetin (**A**) and thrombin receptor-activating peptide (TRAP) (**B**) (mean ± standard error mean, n = 3). ####P < 0.0001 compared to day 1 (baseline) of the same treatment. **P < 0.01. If not indicated, the differences were not significant (ns).*

3.2.1.1.2 Granule releases and glycoprotein activation

RhoA-inhibitor retained the TRAP-induced α-granule release until day 10 providing no significant differences to control. Furthermore, it improved the activation on day 10 since the buffer control was not more activatable on day 10 (buffer vs. G04: 1.0 ± 0.03 vs. 1.1 ± 0.1, P = 0.0225; n = 3; Figure 15 A). RhoA-inhibitor induced high TRAP activation of FI > 3 on the first [FI (mean ± SEM): 3.3 ± 0.4; n = 3; Figure 15 A] and showed a FI over 1.5 on day 4 and 7 (day 4: 1.9 ± 0.1; day 7: 1.6 ± 0.2; n = 3; Figure 15 A). We observed a general decrease of FI with storage time. Likely to buffer, the activation by TRAP of RhoA-inhibitor was significantly reduced with time (day 1 vs. 4: P = 0.0059; day 1 vs. 7: P = 0.004; day 1 vs. 10: P = 0.0013; n = 3; Figure 15 A) and G04 correlated strongly negatively to storage time (r = -0.8731, P < 0.0002, storage time vs. FI; n = 3).

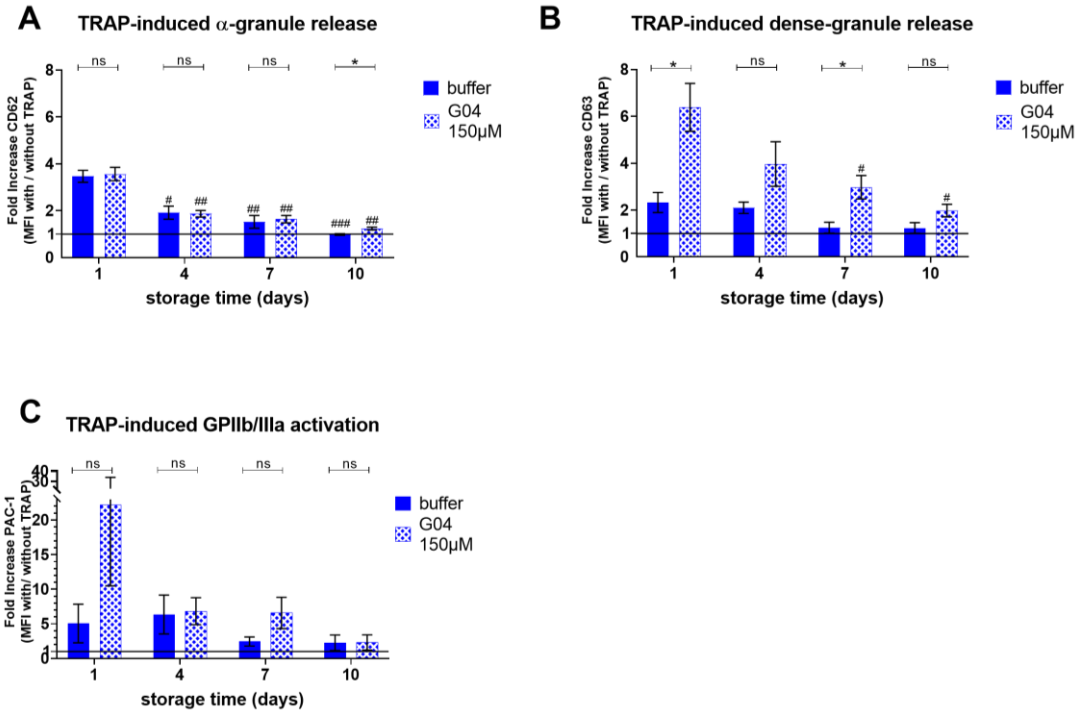

Figure 15. ***RhoA-inhibitor improves the dense-granule release on day 1 and 7.*** *Inducibility of α-granule release (**A**), dense-granule release (**B**), and glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**) by 20 µM thrombin receptor-activating peptide (TRAP) in cold-stored apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3). MFI = mean fluorescence intensity. #P < 0.05; ##P < 0.01; ###P < 0.001 compared to day 1 (baseline) of the same treatment. *P < 0.05. If not indicated, the differences were not significant (ns).*

Furthermore, RhoA-inhibitor improved the TRAP-induced dense-granule release on day 1 and 7. G04 increased the TRAP-induced dense-granule release during the entire the measurement Analyzing each time point, on day 1 and 7, RhoA-inhibitor significantly increased the FI (buffer vs. G04: day 1: 2.3 ± 0.4 vs. 6.4 ± 1.0, P = 0.0251; day 7: 1.3 ± 0.2 vs. 3.0 ± 0.5; P = 0.0353; n = 3; Figure 15 B). On day 4 and 10, RhoA-inhibitor activated more dense-granule release without significance (buffer vs. G04: day 4: 2.1 ± 0.2 vs. 4.0 ± 0.9, P = 0.1292; day 10: 1.2 ± 0.2 vs. 2.0 ± 0.3, P = 0.1024; n = 3; Figure 15 B). The FI decreased with time (day 1 vs. 4: P = 0.1518; day 1 vs. 7: P = 0.0403; day 1 vs. day 10: P = 0.0141; n = 3; Figure 15 B). RhoA-inhibitor correlated negatively to storage time with statistical significance (G04: r = -0.8472, P = 0.0005; storage time vs. FI; n = 3).

Finally, RhoA-inhibitor also retained the TRAP-induced GPIIb/IIIa activation presenting no statistical difference to control. Moreover, RhoA-inhibitor showed enhanced TRAP-induced activation on day 1 and 7 without significance (buffer vs. G04: day 1: 5.1 ± 2.8 vs. 22.2 ± 11.7, P = 0.2269; day 7: 2.5 ± 0.6 vs. 6.6 ± 2.3, P = 0.1533; compared to buffer, respectively; n = 3; Figure 15 C). On day 4 and 10, RhoA-inhibitor retained the activation, presenting no statistical difference to control (day 4: P = 0.8951; day 10: P = 0.9798; n = 3; Figure 15 C). The FI of RhoA-inhibitor showed a significant negative correlation with time (r = -0.5864; P = 0.0451; storage time vs. FI; n = 3).

3.2.1.2 Platelet storage lesion

Investigating the PSL by the exposure of CD62 on resting PLTs, RhoA-inhibitor showed no statistical difference of α-pre-release to buffer control [rel. MFI (mean ± SEM) buffer vs. G04: day 1: 2.7 ± 0.7 vs. 5.4 ± 0.4; day 4: 5.8 ± 1.2 vs. 7.2 ± 2.6; day 7: 1.9 ± 0.2 vs. 6.2 ± 1.8; day 10: 2.8 ± 0.3 vs. 7.0 ± 3.3; n = 3; Figure 16 A).

RhoA-inhibitor maintained the expression level of CD63 compared to 4°C buffer throughout the measurement, presenting no statistical differences, keeping its expression level almost constant during the entire storage (day 1: 3.7 ± 0.4; day 4: 3.8 ± 0.7; day 7: 3.6 ± 0.7; day 10: 4.3 ± 1.1; n = 3; Figure 16 B).

RhoA-inhibitor referred difference of PAC-1 expression level without TRAP-incubation, even if these differences did not reach a statistical significance (Figure 16 C): RhoA-inhibitor showed reduced expression on day 1 and 7 [PAC-1 pos. cells (mean ± SEM) buffer vs. G04: day 1: 21.9 ± 13.4 % vs. 4.2 ± 1.5 %; day 7: 17.3 ± 7.9 % vs. 10.4 ± 6.9 %; n = 3; Figure 16 C), maintained the level on day 4 (buffer vs. G04: 8.7 ± 5.7 % vs. 9.1 ± 4.1 %; n = 3; Figure 16 C), but enhanced expression on day 10 compared to buffer control (buffer vs. G04: 4.0 ± 1.2 % vs. 8.6 ± 4.9 %; n = 3; Figure 16 C). Furthermore, RhoA-inhibitor showed a moderate increase depending on storage time with a peak on day 7, showing a weak pos. correlation without reaching the statistical significance (G04: r = 0.2267, P = 0.4786; storage time vs. PAC-1 pos. cells; n = 3).

There were no significant differences in PLT shrinkage to buffer. Yet, RhoA-inhibitor provided decreased shrinkage from day 4 to day 10 without statistical significance. Especially on day 10, RhoA-inhibitor reduced the shrunk population under 7 %, half as much as the population of buffer control (buffer vs. G04: 14.2 ± 5.8 vs. 6.8 ± 1.2; n = 3; Figure 16 D). Yet, this population was significantly increased compared to day 1 (G04: day 1 vs. day 10: P = 0.0434; n = 3 Figure 16 D). Likely to buffer, RhoA-inhibitor correlated positively with storage time. (buffer: r = 0.6327, P = 0.0272; G04: r = 0.6026, P = 0.0381; storage time vs. shrunk PLTs, respectively; n = 3).

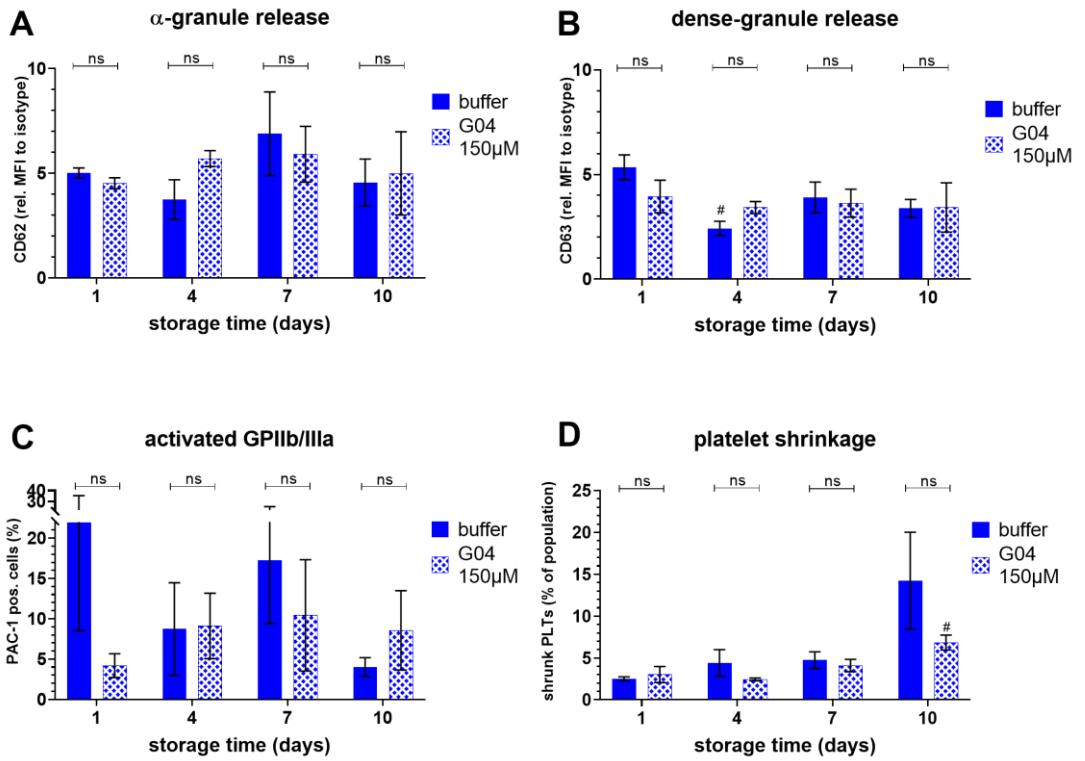

Figure 16. ***RhoA-inhibitor retains platelet storage lesion.*** *α-granule release (**A**), dense- granule release (**B**), glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**), and platelet shrinkage (**D**) of cold-stored apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3). Rel. MFI = relative mean fluorescence intensity. Pos. = positive. #P < 0.05 compared to day 1 (baseline) of the same treatment. If not indicated, the differences were not significant (ns).*

3.2.2 Activation of Phosphokinase A

Forskolin is a phosphokinase A (PKA)-activator which phosphorylates Bad like G04 and leads to apoptosis inhibition (Figure 17).

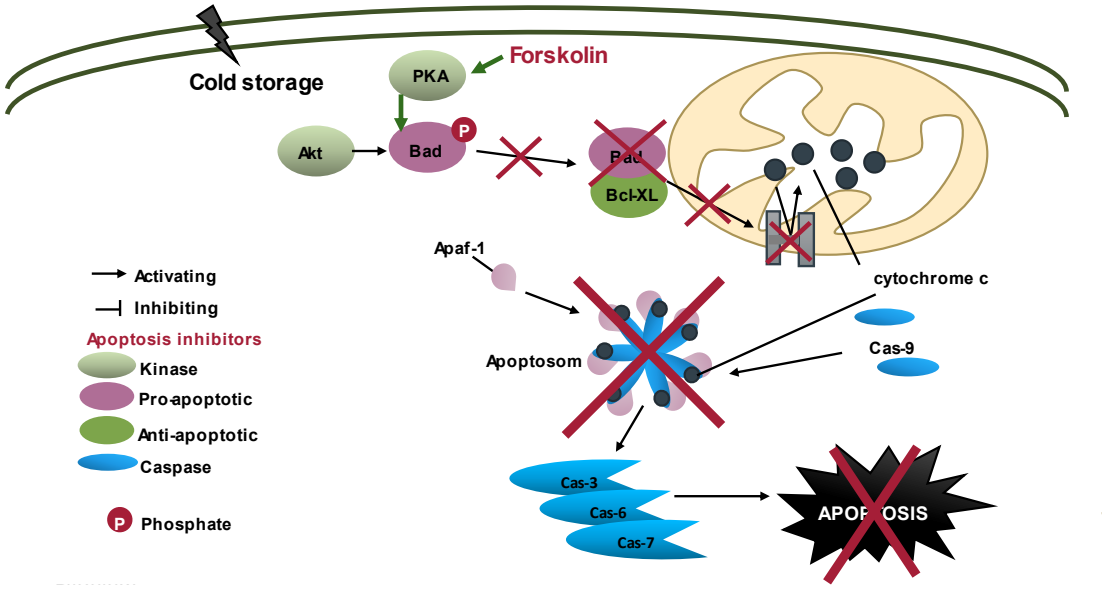

Figure 17. ***Signal pathway of forskolin.*** *Forskolin activates Phosphokinase A (PKA). The kinase inactivates the pro-apoptotic protein Bad: the phosphorylation of Bad by Akt inhibits the translocation of Bad to mitochondria, leading to its cytosolic sequestration.*

3.2.2.1 Agonist-induced activation

3.2.2.1.1 Aggregation

PKA-activator did not impact the ristocetin-induced aggregation. Likely to the buffer, the max. aggregation was over 85 % until day 7 [max. aggregation (mean ± SEM) on day 1: 92.3 ± 1.9 %, P = 0.9784; day 4: 88.3 ± 4.3 %, P = 0.2293; day 7: 91.0 ± 2.4 %, P = 0.3583; compared to buffer, respectively, n = 3; Figure 18 A] and it dropped on day 10 (47.8 ± 8.7%; P = 0.6258; compared to buffer; day 1 vs 10: P < 0.0001; n = 3; Figure 18 A). PKA-Activator showed a significant negative correlation to the storage time (r = -0.6825, P = 0.0002; storage time vs. max. aggregation; n = 3).

Next, TRAP-induced aggregation was examined. PKA-activator retained the aggregation on day 1 (84.4 ± 5.8 %, P = 0.0580; compared to buffer; n = 3; Figure 18 B). While on day 4, the reduction of the maximal aggregation on day 4 was significant (20.8 ± 2.8 %, P < 0.0001 compared to buffer; n = 3; Figure 18 B). On day 7 and 10, PKA-activator provided no differences to buffer (day 7: 16.7 ± 1.6 %, P = 0.7490; day 10: 7.0 ± 2.5 %, P > 0.5722; compared to buffer; n = 3; Figure 18 B). Analyzing each treatment based on the storage time, PKA-activator significantly reduced the aggregation from day 4 (day 1: 84.4 ± 5.8 %; day 4: 20.8 ± 2.8 %; day 1 vs. 4: P <

0.0001; day 1 vs. 7: $P < 0.0001$; day 1 vs. 10: $P < 0.0001$; n = 3; Figure 18 B) while the aggregation of control significantly decreased from day 7. The correlation to the storage time was less negative than for the buffer (buffer: r = -0.9241, $P < 0.0001$; forskolin: r = -0.7588, $P = 0.0002$; storage time vs. max. aggregation, respectively; n = 3).

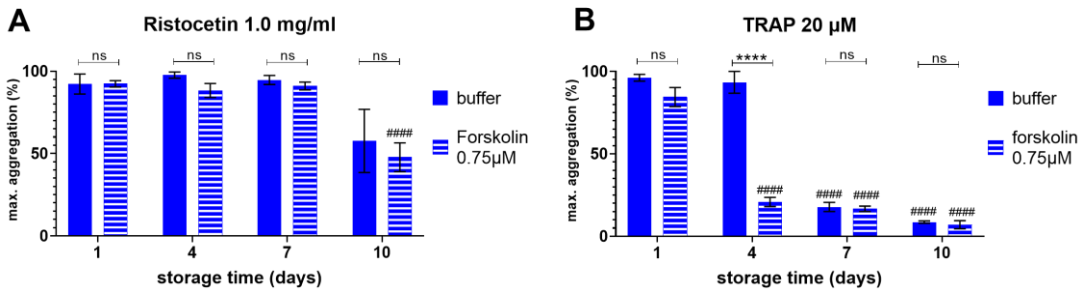

Figure 18. ***Phosphokinase A activator retains the ristocetin-induced aggregation.*** *(**A** and **B**) Maximal (max.) aggregation of cold-stored apheresis-derived platelet concentrate (APC) incubated with ristocetin (**A**) and thrombin receptor-activating peptide (TRAP) (**B**) (mean ± standard error mean, n = 3). ####P < 0.0001 compared to day 1 (baseline) of the same treatment. ****P < 0.0001. If not indicated, the differences were not significant (ns).*

3.2.2.1.2 Granule releases and glycoprotein activation

The PKA-activator retained the TRAP-triggered CD62-expression until day 4. On the first day, PKA-activator induced better TRAP activation with higher FI of around 4 than the control [FI (mean ± SEM) of buffer vs. forskolin: 3.5 ± 0.3 vs. 3.9 ± 1.3; n = 3; Figure 19 A]. PKA-activator provided moderate TRAP activation on day 4 (buffer vs. forskolin: 1.9 ± 0.3 vs. 1.4 ± 0.2; n = 3; Figure 19 A) From day 7, the PLTs of PKA-activator were not more activatable and thus did not retain the activation on day 7 compared to control (buffer vs. forskolin: day 7: 1.5 ± 0.3 vs. 1.0 ± 0.1; day 10: 1.0 ± 0.03 vs. 1.0 ± 0.1; n = 3; Figure 19 A). Even if the time-dependent decrease was not significant compared to day 1, the weak negative correlation (r = -0.4485, $P = 0.0028$; storage time vs. FI; n = 3) indicates the observed a general decrease of FI with storage time.

In contrast, PKA-activator reduced the TRAP-induced dense-granule release, presenting a statistical difference on day 4 (buffer vs. forskolin: day 1: 2.3 ± 0.4 vs. 1.3 ± 0.2, $P = 0.0515$; day 4: 2.1 ± 0.2 vs. 1.4 ± 0.1, $P = 0.0163$; n = 3). From day 7, the PLTs were not more activatable (day 7: 1.0 ± 0.03; day 10: 1.0 ± 0.06; n = 3; Figure 19 B).

The TRAP-induced GPIIb/IIIa activation was completely retained by PKA-activator. Moreover, forskolin provided stronger and superior activation response without statistical significance (buffer vs. forskolin: day 1: 5.1 ± 2.8 vs. 15.3 ± 7.9; day 4: 6.4 ± 2.8 vs. 9.9 ± 4.5; day 7: 2.5 ± 0.6 vs. 7.0 ± 5.0; day 10: 2.3 ± 1.1 vs. 2.5 ± 1.0; n = 3; Figure 19 C).

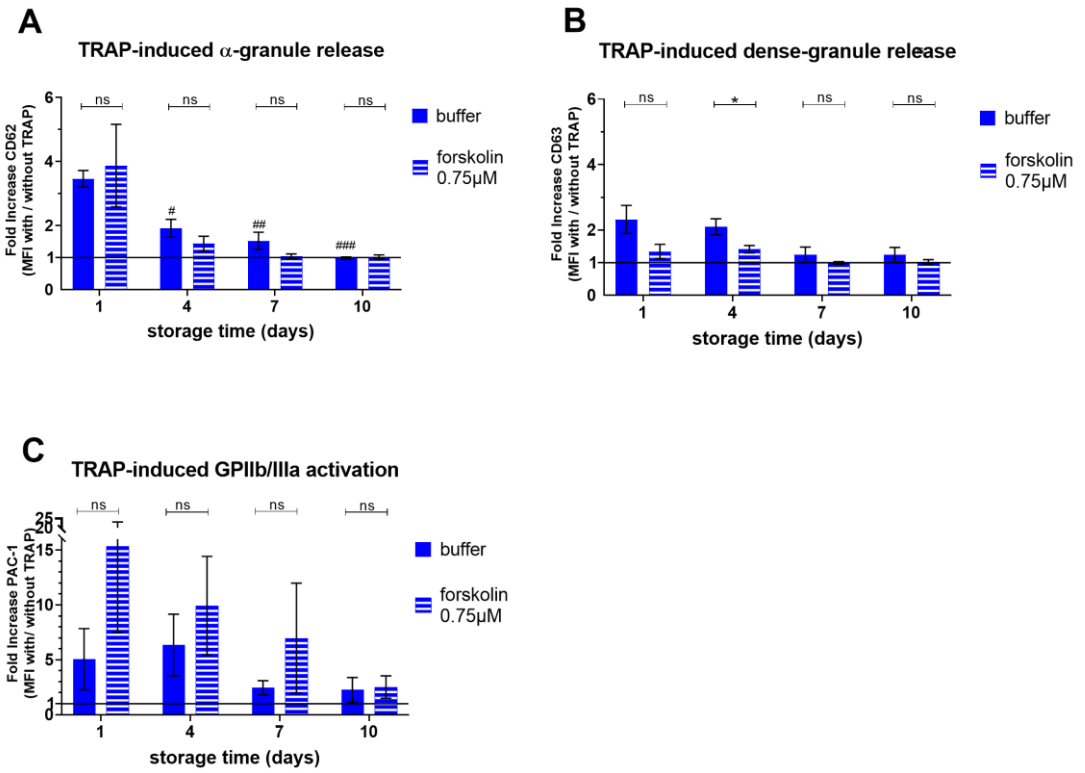

Figure 19. ***Phosphokinase A activator partly retains the agonist-induced platelet activation.*** *Inducibility of α-granule release (**A**), dense-granule release (**B**), and glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**) by 20 µM thrombin receptor-activating peptide (TRAP) in cold-stored apheresis-derived platelet concentrates (APC) (mean ± standard error mean; n = 3). MFI = mean fluorescence intensity. #P < 0.05; ##P < 0.01; ##P < 0.001 compared to day 1 (baseline) of the same treatment. *P < 0.05. If not indicated, the differences were not significant (ns).*

3.2.2.2 Platelet storage lesion

Investigating the PSL by the exposure of CD62 on resting PLTs, PKA-activator showed no statistical difference of pre-release to buffer control (Figure 20 A). Yet, the expression level of forskolin was higher than the control [rel. MFI (mean ± SEM) of buffer vs. forskolin: day 1: 2.7 ± 0.7 vs. 3.4 ± 0.4; day 4: 5.8 ± 1.2 vs. 7.6 ± 1.3; day 7: 1.9 ± 0.2 vs. 6.3 ± 0.9; day 10: 2.8 ± 0.3 vs. 7.5 ± 1.9; n = 3; Figure 20 A]. Analyzing in a time dependent manner, PKA-activator significantly increased the α-granule pre-release (day 1 vs. 4: P = 0.0081; day 1 vs. day 7: P = 0.0058; day 1 vs. day 10: P =

0.0142; n = 3; Figure 20 A) and showed a weak pos. correlation to storage time with statistical significance (r = 0.4203, *P* = 0.0409; storage time vs. rel. MFI; n = 3).

The expression level of CD63 compared to buffer was maintained by PKA-activator throughout the measurement, presenting no statistical differences (Figure 20 B). Yet, PKA-activator showed elevated expression of CD63 the on day 10 (day 1 vs. day 10: *P* = 0.026; n = 3; Figure 20 B). Underlining this, PKA-activator provided a pos. correlation with storage time (r = 0.4771, *P* = 0.0184; storage time vs. rel. MFI; n = 3).

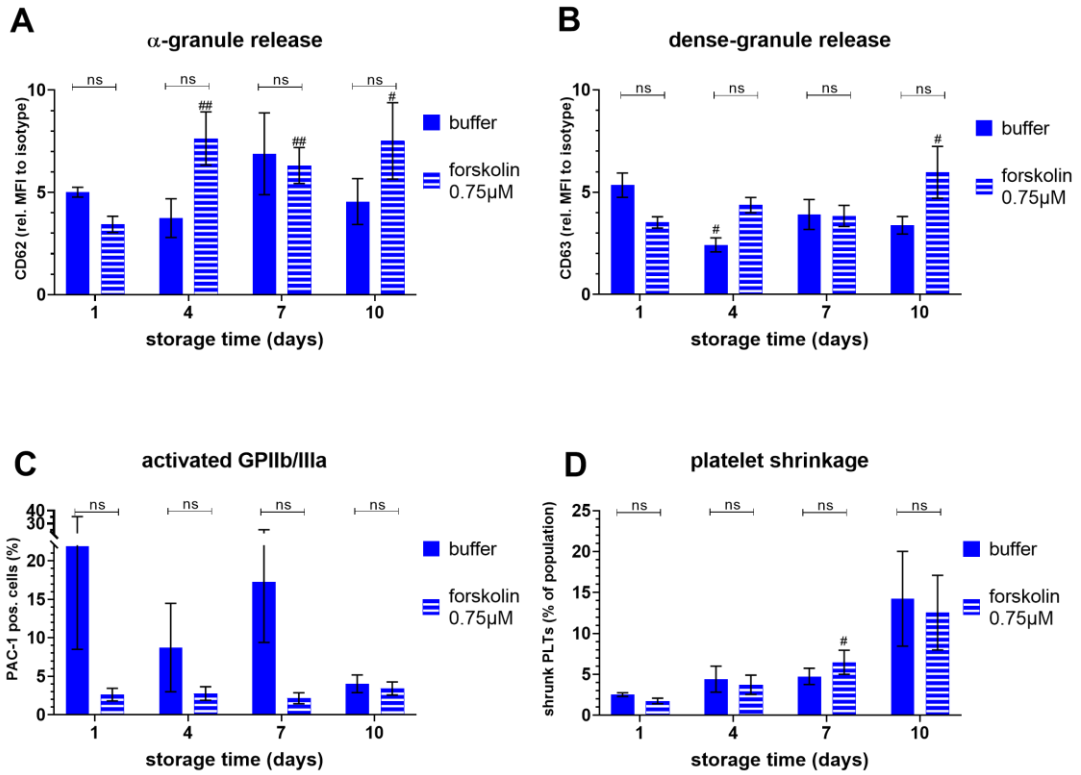

Figure 20. ***Phosphokinase A activator retains the platelet storage lesion.*** *α-granule release (**A**), dense- granule release (**B**), glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**), and platelet shrinkage (**D**) of cold-stored apheresis-derived platelet concentrates (APC) (mean ± standard error mean; n = 3). Rel. MFI = relative mean fluorescence intensity. Pos. = positive. #P < 0.05; ##P < 0.01 compared to day 1 (baseline) of the same treatment. If not indicated, the differences were not significant (ns).*

PKA-activator referred decrease of PAC-1 expression level without TRAP-incubation, even if these differences did not reach a statistical significance (Figure 20 C). PKA-activator treatment seemed to suppress the PAC-1 expression at each time point [PAC-1 pos. cells (mean ± SEM) of buffer vs. forskolin: day 1: 7.4 ± 3.0 % vs. 2.6 ± 0.9 %, *P* = 0.2244; day 4: 6.6 ± 0.6 % vs. 2.8 ± 0.9 %, *P* = 0.3619; day 7: 12.8 ± 10.5 % vs. 2.2 ± 0.7 %, *P* = 0.1287; day 10: 4.0 ± 1.2 % vs. 3.4 ± 0.9 %, *P* = 0.6922; n

= 3; Figure 20 C). PKA-activator showed a weak pos. correlation without reaching the statistical significance (r = 0.1598, *P* = 0.6199; storage time vs. PAC-1 pos. cells; n = 3).

PKA-activator maintained the PLT shrinkage. There were no significant differences to the buffer at any measure point [shrunk cells (mean ± SEM) of buffer vs. forskolin: day 1: 2.5 ± 0.2 % vs. 1.7 ± 0.3 %; day 4: 4.4 ± 1.6 % vs. 3.7 ± 1.2 %; day 7: 4.7 ± 1.0 % vs. 6.5 ± 1.5 %; day 10: 14.2 ± 5.8 % vs. 12.5 ± 4.6 %; n = 3; Figure 20 D). Compared to day 1, the shrinkage of day 7 was significant (day 1 vs. day 7: *P* = 0.0348; n = 3; Figure 20 D). Likely to buffer, phosphokinase A activator treatment correlated positively with storage time. (buffer: r = 0.6327, *P* = 0.0272; forskolin: r = 0.7321, *P* = 0.0068; storage time vs. shrunk cells; n = 3).

3.2.3 Inhibition of Caspase-9

Z-LEHD-fmk is a specific caspase-9-inhibitor and inhibits the apoptosis in the down steam of the signal pathway. Due to the delay of the delivery of z-LEHD-fmk, we examined only TRAP-induced aggregation, degranulation and PLT shrinkage.

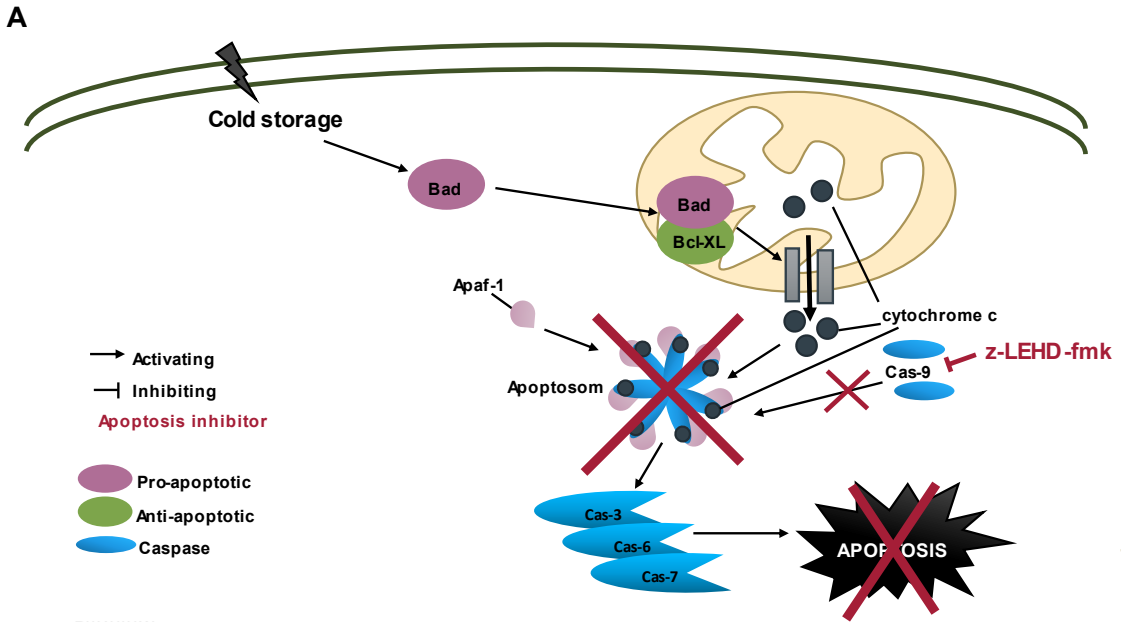

Figure 21. ***Signal pathway of z-LEHD-fmk.*** *Z-LEHD-fmk fmk is a specific caspase-9-inhibitor. The building of apoptosom is not more possible. The inhibition of caspase-9 prohibits the cleavage and activation of further effector caspases, thus the apoptosis in the downstream.*

3.2.3.1.1 Aggregation

The caspase-9-inhibitor retained the TRAP-induced aggregation throughout the measurement. Without significant differences to buffer, the caspase-9-inhibitor showed decreased aggregation at the beginning [max. aggregation (mean ± SEM): buffer vs. z-LEHD-fmk: day 1: 96.2 ± 2.0 % vs. 81.5 ± 15.5 %, *P* = 0.3242; day 4: 93.4 ± 6.6 % vs. 48.9 ± 25.7 %, *P* = 0.1684; n = 3; Figure 22 A], and later increased aggregation (day 7: 17.8 ± 2.8 % vs. 21.6 ± 9.7, *P* = 0.682; day 10: 8.6 ± 0.7 % vs. 9.93 ± 0.3 %, *P* = 0.2835; n = 3; Figure 22 A).

Likely to control, the continuous reduction of aggregation by caspase-9-inhibitor was significant from day 7 (z-LEHD-fmk: day 1 vs. 4: *P* = 0.3015; day 1 vs. 7: *P* = 0.0303; day 1 vs. 10: *P* = 0.0373; n = 3: Figure 22 B). The correlation of aggregation to storage time was significantly negative (r = -0.6825; *P* = 0.0063; n = 3).

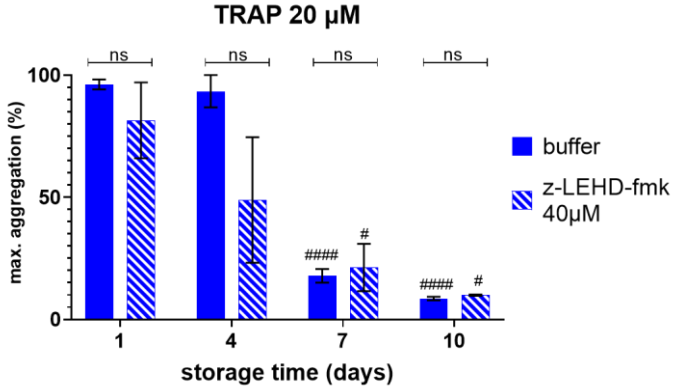

Figure 22. ***Caspase-9-inhibitor retains thrombin receptor-activating peptide (TRAP)-induced aggregation.*** *(**A** and **B**) Maximal (max.) aggregation of cold-stored apheresis-derived platelet concentrate (APC) incubated with TRAP (**A**) (mean ± standard error mean, n = 3). #P < 0.05; ####P < 0.0001; #P < 0.05 compared to day 1 (baseline) of the same treatment. If not indicated, the differences were not significant (ns).*

3.2.3.1.2 Granule releases and glycoprotein activation

The caspase-9-inhibitor retained the TRAP-triggered granule release until day 10. The inhibitor showed enhanced activation without significance on day 1 and 7 [FI (mean ± SEM) buffer vs. z-LEHD-fmk: day 1: 3.5 ± 0.3 vs. 4.7 ± 2.3; day 7: 1.5 ± 0.3 vs. 1.7 ± 0.6; n = 3; Figure 14 A). On day 10, the PLTs with caspase-9-inhibitors were still activatable while those of the control showed no activation (buffer vs. z-LEHD-fmk: 1.0 ± 0.03 vs. 2.0 ± 0.9; n = 3; Figure 23 A).

The caspase-9-inhibitor retained the TRAP-induced dense-granule release until day 7. The PLTs were activated on day 1 and 4 (buffer vs. z-LEHD-fmk: day 1: 2.6 ± 0.6 vs. 1.9 ± 0.2; day 4: 2.1 ± 0.3 vs. 1.5 ± 0.6; n = 3; Figure 23 B) and very weakly on day 7 (1.3 ± 0.2 vs. 1.1 ± 0.02; n = 3; Figure 23 B). Yet, the PLTs were not activatable on day 10 and did not retain the TRAP-triggered dense-granule release (day 10: 1.24 ± 0.23 vs. 1.0 ± 0.01; n = 3; Figure 23 B). Comparing in the time-dependent manner, the decreases of day 7 and 10 of z-LEHD-fmk were significant (day 1 vs. 7: P = 0.0025; day 1 vs. day 10: P = 0.017; n = 3; Figure 23 B).

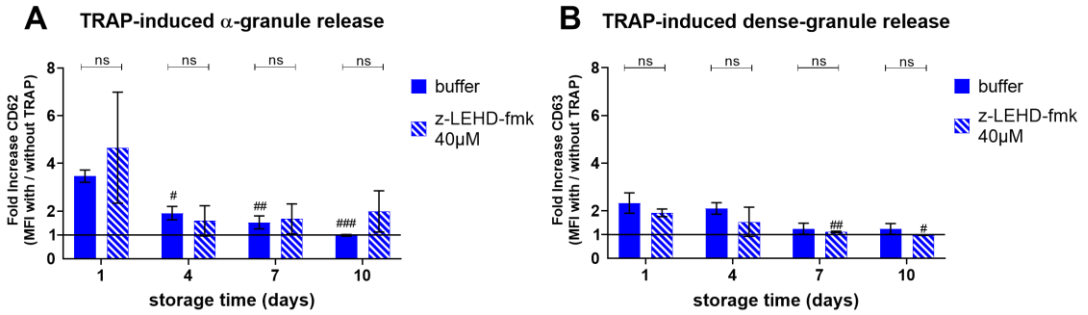

Figure 23. ***Caspase-9-inhibitor partly retains the agonist-induced degranulation.*** *CS provides the thrombin receptor-activating peptide (TRAP)-induced activation on day 4 and 7. Inducibility of α-granule release (**A**) and dense-granule release (**B**) by 20 µM thrombin receptor-activating peptide (TRAP) in cold-stored apheresis-derived platelet concentrates (APC) (mean ± standard error mean; n = 3). MFI = mean fluorescence intensity. #P < 0.05; ##P < 0.01; ###P < 0.001 compared to day 1 (baseline) of the same treatment. If not indicated, the differences were not significant (ns).*

3.2.3.2 Platelet storage lesion

The caspase-9-inhibitor z-LEHD-fmk expressed significantly less CD62 on day 1 [rel. MFI (mean ± SEM) buffer vs. z-LEHD-fmk: day 1: 5.0 ± 0.3 vs. 2.3 ± 0.5, P = 0.0075; n = 3]. In addition, it retained the PSL, presenting lower level of CD62 throughout the measurement; day 4: 3.7 ± 1.0 vs. 2.2 ± 0.5; day 7: 6.9 ± 2.0 vs. 4.1 ± 1.1; day 10: 4.6 ± 1.1 vs. 2.6 ± 1.1; n = 3; Figure 24 A].

The caspase-9-inhibitor maintained the expression level of CD63 compared to buffer throughout the measurement, presenting no statistical differences (buffer vs. z-LEHD-fmk: day 1: 5.3 ± 0.6 vs. 5.5 ± 2.3; day 4: 3.8 ± 0.7 vs. 4.0 ± 1.2; day 7: 3.9 vs. 0.7 vs. 4.9 ± 0.5; day 10: 3.4 ± 0.4 vs. 4.1 ± 0.5; n = 3; Figure 24 B). The CD63 expression was constant and did not correlate positively with time (r = -0.1255, P = 0.669; n = 3).

The caspase-9-inhibitor significantly reduced the shrinkage on the first day [shrunk cells (mean ± SEM) buffer vs. z-LEHD-fmk: day 1: 2.5 ± 0.2 % vs. 1.1 ± 0.2 %, *P* = 0.0232; n = 3]. From day 4, there were no significant differences to the buffer. The caspase-9-inhibitor retained the lesion providing decreased shrinkage on day 4 and 10 (buffer vs. z-LEHD-fmk: day 4: 4.4 ± 1.6 % vs. 3.6 ± 0.8 %; day 7: 4.7 ± 1.0 % vs. 9.4 ± 2.0 %; day 10: 14.2 ± 5.8 % vs. 13.7 ± 2.8 %; n = 3; Figure 24 A]. With storage rime, the shrunk population increased with significance (day 1 vs. 4: *P* = 0.1083; day 1 vs. 7: *P* = 0.0491; day 1 vs. 10: *P* = 0.0398; n = 3; Figure 24 A). The caspase-9-inhibitor correlated more positively with storage time than the control (buffer: r = 0.6327, *P* = 0.0272; z-LEHD-fmk: r = 0.8131, *P* = 0.0004; storage time vs. shrunk cells, respectively; n = 3).

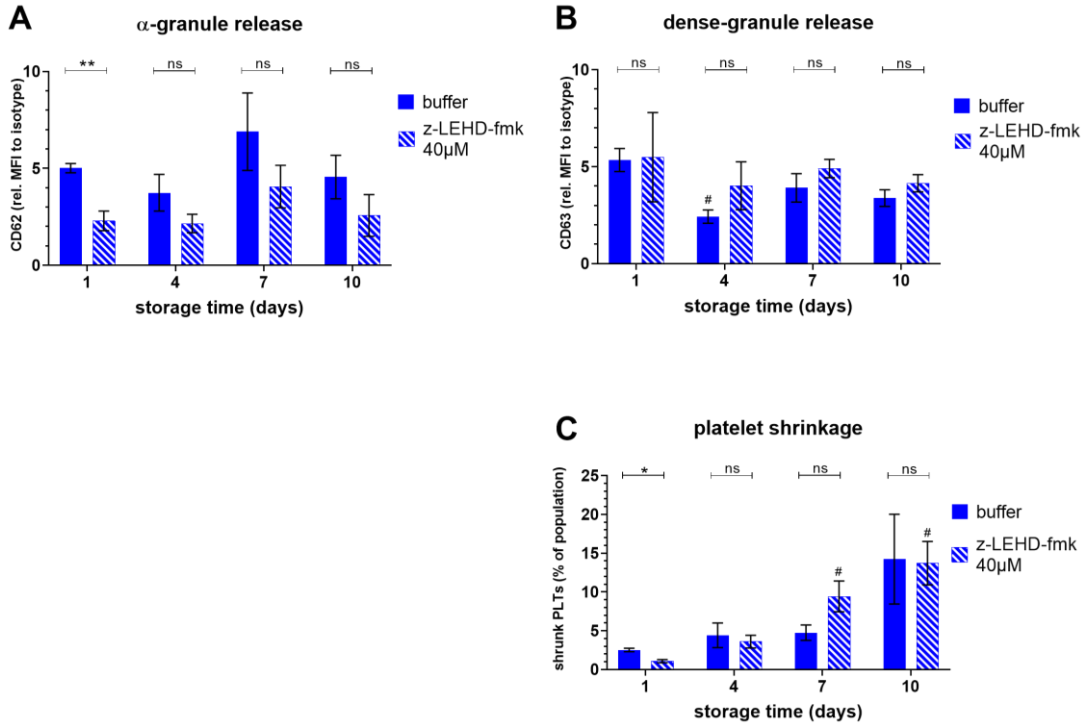

Figure 24. ***Caspase-9-inhibitor reduces the CD62 expression and shrinkage on day 1.*** *α-granule release (**A**), dense-granule release (**B**), and platelet shrinkage (**C**) of cold-stored apheresis-derived platelet concentrates (APC) (mean ± standard error mean; n = 3). Rel. MFI = relative mean fluorescence intensity. Pos. = positive. #P < 0.05 compared to day 1 (baseline) of the same treatment. If not indicated, the differences were not significant (ns).*

3.3 Supplementary data

3.3.1 The impact of vehicle on platelet

Since all inhibitors were diluted in DMSO, we investigated the aggregation in the presence of the corresponding concentration of DMSO in order to exclude a possible impact.

The vehicle did not cause statistical difference in cold-stored APCs activated by ristocetin at each time point (APC without vs. with DMSO: day 1: $P = 0.9048$; day 4: $P = 0.2699$; day 7: $P = 0.6216$; day 10: $P = 0.9817$; n = 3; Figure 25 A). TRAP induced aggregation providing no difference on day 1, 4 and 10 but improved the aggregation on day 7 (day 1: $P = 0.4138$; day 4: $P = 0.3949$; day 7: $P = 0.0336$; day 10: $P > 0.4655$; n = 3; Figure 25 B).

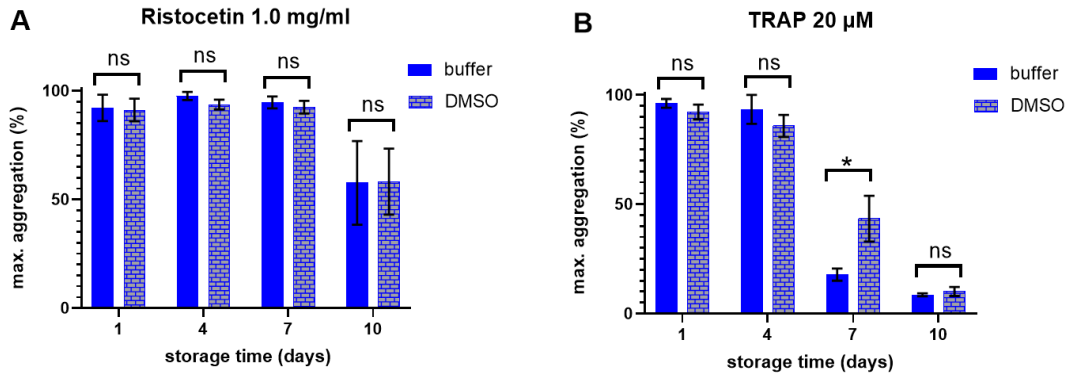

Figure 25. ***Dimethyl sulfoxide (DMSO) does not impact the ristocetin-induced aggregation.*** *(**A** and **B**) Maximal (max.) aggregation of cold-stored apheresis-derived platelet concentrate (APC) incubated with ristocetin (**A**) and thrombin receptor-activating peptide (TRAP) (**B**) (mean ± standard error mean, n = 3). *P < 0.05. ns = not significant.*

Next, we analyzed the impact of DMSO in TRAP-induced PLT activation. No significant difference between cells treated with DMSO and buffer in FI of all markers was observed at each storage time point (Figure 26 A-C).

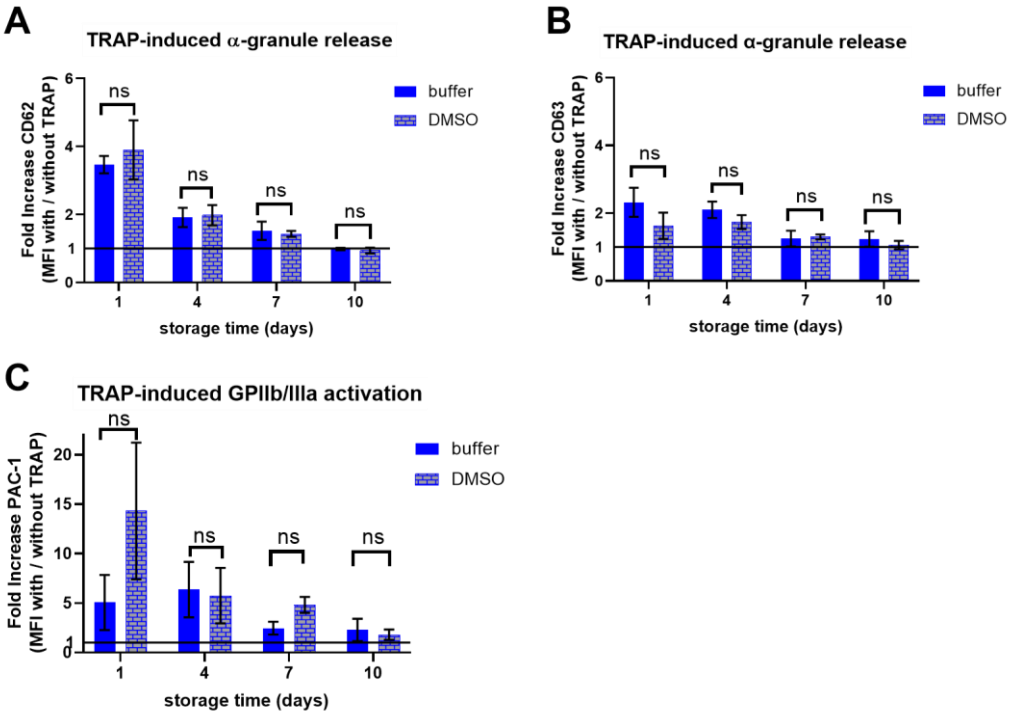

Figure 26. ***Dimethyl sulfoxide (DMSO) does not impact agonist-induced platelet activation.*** *(**A-C**) Inducibility of α-granule release (**A**), dense-granule release (**B**), and glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**) by 20 µM thrombin receptor-activating peptide (TRAP) in cold-stored apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3). MFI = mean fluorescence intensity. ns = not significant.*

DMSO did not cause a significant difference in PSL assays (Figure 27, A-D) apart from day 4, where DMSO increased significantly the CD63 expression [rel. MFI (mean ± SEM) of CD63 in buffer vs. DMSO: 2.4 ± 0.3 vs. 4.2 ± 0.9, P = 0.0148; n = 3; Figure 27 B].

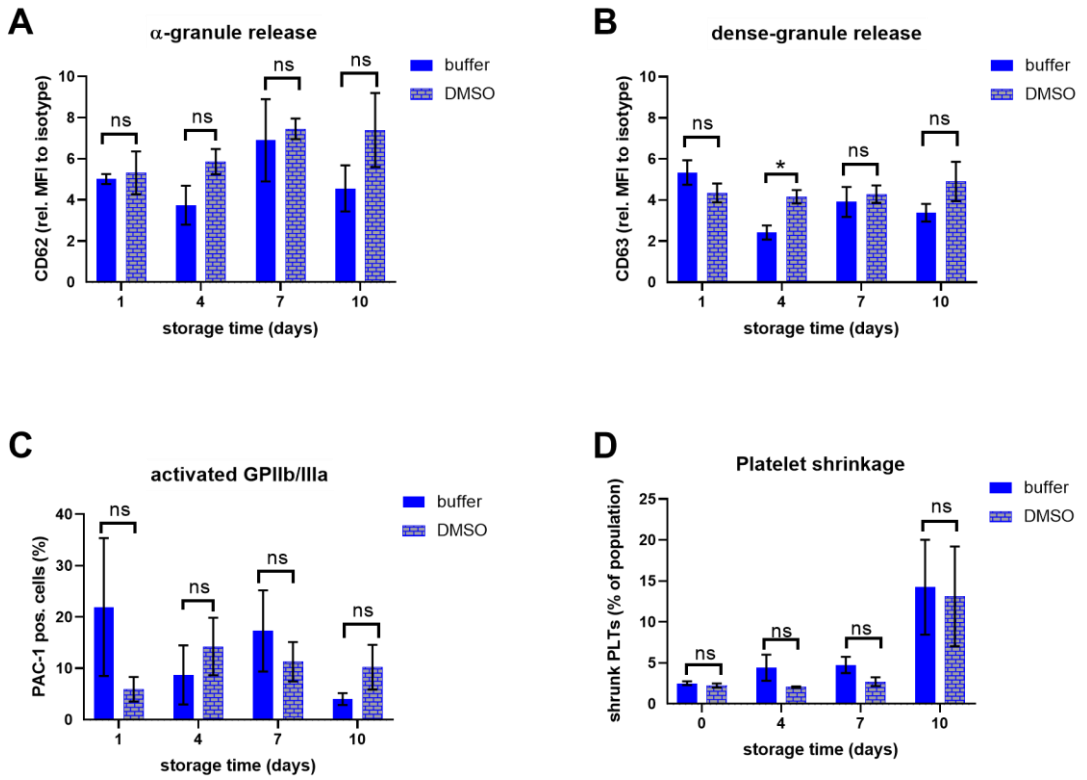

Figure 27. ***Dimethyl sulfoxide (DMSO) does not impact the CD62-, PAC-1 expression and platelet shrinkage.*** *α-granule release (**A**), dense- granule release (**B**), glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**), and platelet shrinkage (**D**) of cold-stored apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3 Rel. MFI = relative mean fluorescence intensity. Pos. = positive. *P < 0.05. If not indicated, the differences were not significant (ns).*

3.3.2 Titration of apoptosis inhibitors

To find out the optimal concentrations for the CS assay, we titrated G04 and forskolin while the optimal concentration of z-LEHD-fmk was already known.

3.3.2.1 G04 Titration

Likely to the result of 3.2.1.1, ristocetin induced a maximal aggregation over 80 % on every concentration until day 7 [max. aggregation (mean ± SEM) on day 7: DMSO: 92.5 ± 2.9 %; 600 µM: 92.0 ± 1.0 %; 300 µM: 87.3 ± 7.2 %; 150 µM: 94.0 ± 3.1 %; n = 3; Figure 28 A]. The aggregation showed a similar behavior between all treatments at each time point with a reduction on day 10 (Figure 28 A).

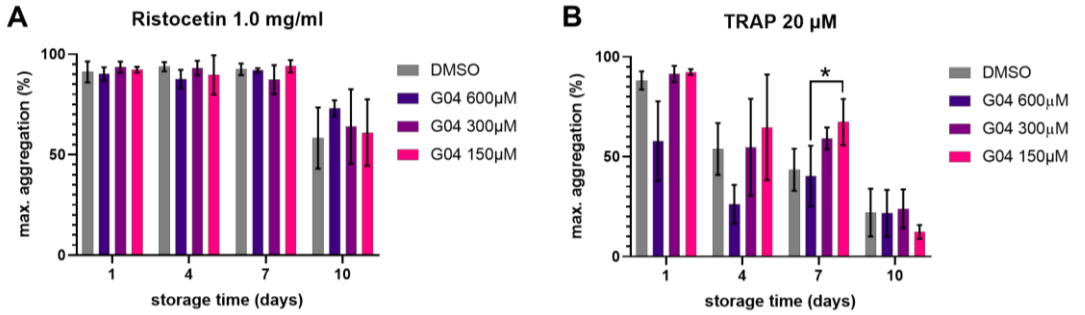

Figure 28. ***150 µM G04 shows the best agonist-induced aggregation on day 7.*** *(**A** and **B**) Maximal (max.) **aggregation** of cold-stored apheresis-derived platelet concentrate (APC) incubated with ristocetin (**A**) and thrombin receptor-activating peptide (TRAP) (**B**) (mean ± standard error mean, n = 3). DMSO = dimethyl sulfoxide. *P < 0.05. If not indicated, the differences were not significant (ns).*

In contrast, 150µM increased the TRAP-induced aggregation on day 4 and 7 without significance (150 µM on day 4: 64 ± 26.5 %; day 7: 67.3 ± 11.6 %; n = 3; Figure 28 B). The aggregation was reduced by 600 µM G04 and these PLTs only reached a max. aggregation of 60 % (day 1: 57.8 ± 19.9; day 4: 26.2 ± 9.7; day 7: ± 40.3 ± 15.1; day 10: 21.7 ± 11.7; n = 3; Figure 28 B). On day 7, 150µM showed improvement of aggregation compared to 600 µM (*P* = 0.0137; n = 3, Figure 28 B).

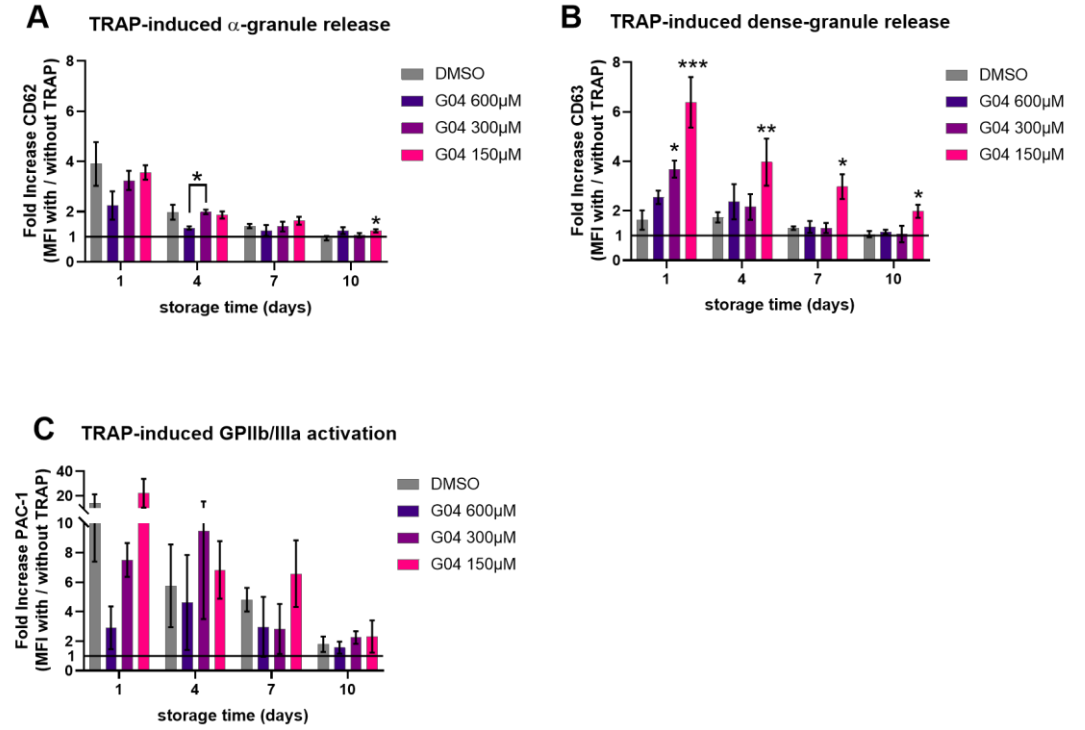

Figure 29. ***150 µM G04 shows the best agonist-induced activation on day 7.*** *Inducibility of α-granule release (**A**), dense-granule release (**B**), and glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**) by 20 µM thrombin receptor-activating peptide (TRAP) in cold-stored apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3). *P < 0.05; **P < 0.01; ***P < 0.001 compared to dimethyl sulfoxide (DMSO) if not otherwise indicated. If not indicated, the differences were not significant (ns).*

For the activation assay with TRAP, the best results were provided by 150 µM. In fact, using this concentration, G04 retained the surface expression and activated the PLTs until day 10 (Figure 29, A-C). In particular, the dense-degranulation of 150 µM G04 was significantly induced throughout the measurement (day 1: $P = 0.0449$; day 4: $P = 0.0004$; day 7. $P = 0.0062$; day 10: $P = 0.0112$; compared to DMSO, respectively; n = 3, Figure 29 B).

Furthermore, 150 µM showed no significant reduction in expression level without TRAP, while 600 µM G04 increased the α- and dense-granule pre-release, and GPIIb/IIIa pre-activation without reaching a statistical significance in any case (Figure 30, A-C).

All concentrations of G04 showed less shrunk population on day 10 than in DMSO without significance (Figure 30 D). However, 600 µM G04 increased the PLT shrinkage until day 7 (Figure 30 D).

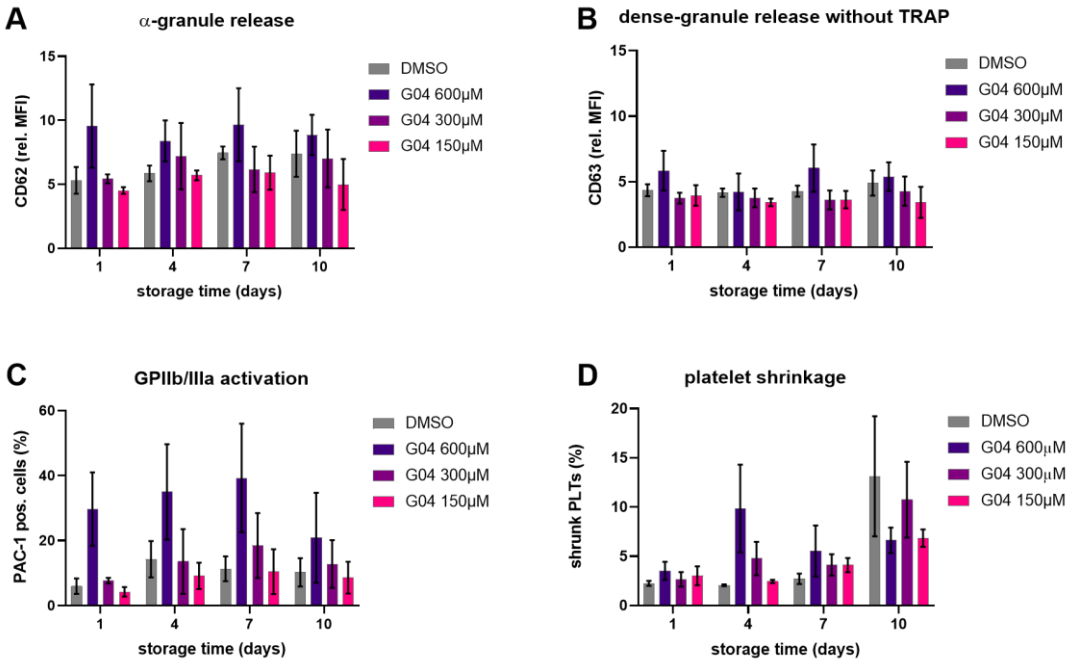

Figure 30. ***600 µM G04 shows highest platelet lesion until day 7.*** *α-granule release (**A**), dense- granule release (**B**), glycoprotein IIb/IIIa (GPIIb/IIIa) activation (**C**), and platelet shrinkage (**D**) of cold-stored apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3 Rel. MFI = relative mean fluorescence intensity. Pos. = positive. If not indicated, the differences were not significant (ns).*

3.3.2.2 Forskolin Titration

The PLT functionality in the presence of Forskolin was examined to find out the optimal concentration.

0.75 µM forskolin kept the TRAP-induced aggregation over 75 % [max. aggregation (mean ± SEM): 76.7 ± 8.0 %; n = 7; Figure 31]. 2.0 µM significantly reduced the max. aggregation (DMSO vs. 2.0 µM: 88.6 ± 1.8 % vs. 30.0 ± 9.6 %, *P* = 0.0025; n = 7; Figure 30).

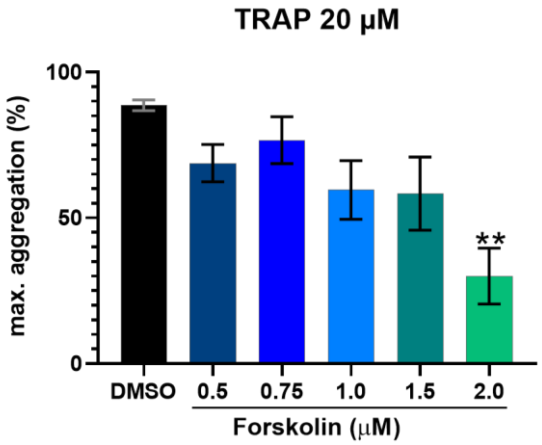

*Figure 31. **0.75 µM forskolin shows the highest aggregation.** Maximal (max.) aggregation of cold-stored apheresis-derived platelet concentrate (APC) incubated with thrombin receptor-activating peptide (TRAP) (mean ± standard error mean, n = 3). **P < 0.01 compared to dimethyl sulfoxide (DMSO) control. 2-way ANOVA. If not indicated, the differences were not significant.*

0.75 µM provided the best response to TRAP-induced granule release [FI (mean ± SEM) of 0.75 µM: CD62: 5.0 ± 2.0; n = 3; Figure 32 A; CD63: 2.6 ± 0.4; n = 3; Figure 32 B].

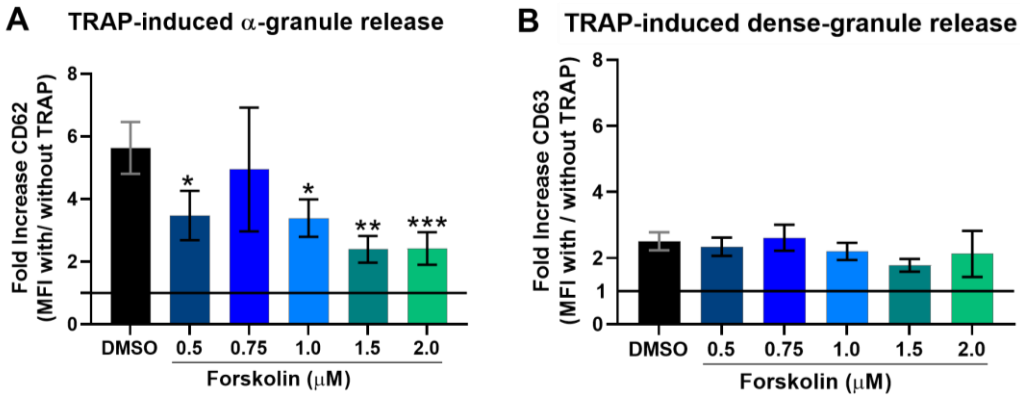

*Figure 32. **0.75 µM retains the agonist-induced platelet activation.** Inducibility of α-granule release (**A**) and dense-granule release (**B**) by 20 µM thrombin receptor-activating peptide (TRAP) in cold-stored apheresis-derived platelet*

*concentrate (APC) (mean ± standard error mean; n = 3). * P < 0.05; **P < 0.01; ***P < 0.001 compared to dimethyl sulfoxide (DMSO) control. If not indicated, the differences were not significant.*

For α-granule release, any other concentration showed significant decrease of granule response (respectively compared to DMSO: 0.5 µM: *P* = 0.0435; 0.75 µM: *P* = 0.9859; 1.0 µM: *P* = 0.0258; 1.5 µM: *P* = 0.0057; 2.0 µM: *P* = 0.0002; n = 7; Figure 32 A).

Without TRAP-incubation, any concentration of forskolin did not reduce the CD62 and CD63 exposure on resting cells at any concentration (Figure 33, A and B).

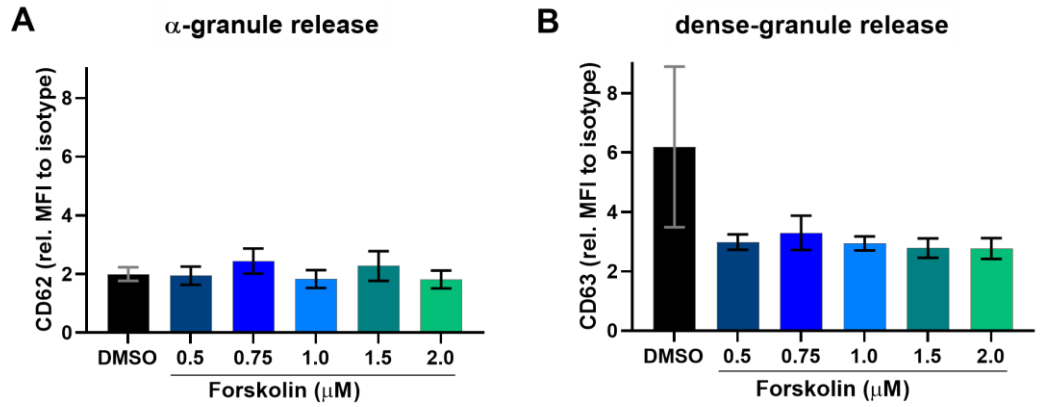

Figure 33. ***Forskolin retains the platelet storage.*** *α-granule release (**A**) and dense-granule release (**B**) of cold-stored apheresis-derived platelet concentrate (APC) (mean ± standard error mean; n = 3. DMSO = dimethyl sulfoxide. Rel. MFI = relative mean fluorescence intensity. If not indicated, the differences were not significant (ns).*

3.4 Summary of the results.

All data are presented in mean ± SEM.

1st question: Does the apheresis-derived platelet concentrate production process impact the platelet functionality?

We explored the function of APC, immediately after their production, and we found out that PLTs markedly increased the aggregation in response to 1.0 mg/ml ristocetin (max. aggregation with ristocetin: 95.2 ± 3.9 %; n = 4) and to 20 µM TRAP (TRAP: 92.4 ± 9.0 %; n = 4). Additionally, upon TRAP incubation, APC significantly released more α- (rel. MFI without vs. with TRAP activation: 2.5 ± 0.6 vs. 15.0 ± 3.8, *P* = 0.007; n = 3) and dense-granule (3.9 ± 0.7 vs. 9.2 ± 0.6, *P* = 0.0194; n = 3), as well as activated more GPIIb/IIIa (2.0 ± 0.9 vs. 29.3 ± 10.8, *P* = 0.008; n = 3). Furthermore, 0.5 µg/ml collagen induced moderate PLT aggregation (max. aggregation of PRP vs. APC: 93.9 ± 1.0 % vs. 49.0 ± 18.5 %, *P* = 0.07; n = 4). While a significant reduction of aggregation

in the presence of 10 μM ADP was observed (PRP vs. APC: 95.3 ± 0.6 % vs. 17.8 ± 6.8 %, $P < 0.001$; n = 3). Moreover, ADP did neither induce the release α- (rel. MFI of APC with vs. without ADP: $P = 0.997$) and dense-granules ($P = 0.4934$) nor activate GPIIb/IIIa ($P = 0.6345$; n = 3).

The APC process did not impact the PLT aggregation with ristocetin, TRAP, and collagen and the activation with TRAP, but it impacted the ADP-induced aggregation and activation.

2nd question: How does the storage temperature impact the platelet functionality?

The CS protected the PLTs from the PSL longer than RT, even if we observed that the PSL occurred in a time-dependent manner for both storage temperatures. In fact, during the storage at 4 ºC, the PSL was detected from day 7 on, while at RT, it was observed starting from day 4.

In particular, the CS significantly improved the aggregation on day 4 and 7 by ristocetin (max. aggregation of RT vs. 4 °C on day 4: 41.4 ± 13.3 % vs. 97.6 ± 1.9 %, $P = 0.0237$; day 7: 7.8 ± 3.5 % vs. 94.7 ± 2.7 %, $P < 0.0001$; n = 3) as well as by TRAP (day 4: 11.3 ± 1.0 % vs. 93.4 ± 6.6 %, $P < 0.0001$; day 7: 10.0 ± 0.8 % vs. 17.8 ± 2.8 %, $P = 0.0162$; n = 3). Analyzing the time-dependent development, the CSPs showed no significant decrease by ristocetin. By TRAP, the reduction of aggregation at 4 °C started first from day 7 (day 1 vs. 7: $P < 0.0001$; n = 3). On the contrary, the maximal aggregation of RSPs dropped significantly from day 4 by ristocetin (day 1 vs. 4: $P = 0.0314$) and by TRAP (day 1 vs. 4: $P < 0.0001$; n = 3). Furthermore, investigating the TRAP-induced platelet activation, we observed that CS retained the dense-degranulation and GPIIb/IIIa activation throughout the measurement while the RT storage decreased the FI from day 4 for α- (FI of day 1 vs. 4: 4.4 ± 0.8 vs. 1.2 ± 0.2, $P = 0.0017$; n = 3), dense-granule release (day 1 vs. 4: 2.6 ± 0.6 vs. 1.0 ± 0.04, $P = 0.0133$; n = 3) as well as for GPIIb/IIIa (day 1 vs. day 4: 8.4 ± 1.9 vs. 1.4 ± 0.1, $P = 0.0222$; n = 3). Moreover, the CS significantly improved the dense-degranulation on day 4 (RT vs. 4 °C: 1.0 ± 0.04 vs. 2.1± 0.2, $P = 0.0004$; n = 3). CSPs released nearly twice as many α-granules on day 4 and 7 without significance (RT vs. 4 ºC: day 4:1.2 ± 0.2 vs. 1.9 ± 0.3; day 7: 1.0 ± 0.03 vs. 1.5 ± 0.3) yet showed significant decrease from day 4 like RT storage (day 1 vs. 4: $P = 0.015$; n = 3).

CS reduced partly the platelet lesion. The CSPs significantly decreased CD63 expression level on day 10 (rel. MFI of RT vs. 4 ºC: 6.4 ± 0.4 vs. 3.3 ± 0.6, P = 0.038; n = 3) and shrinkage on day 7 (shrunk cells: 12.5 ± 1.0 % vs. 4.7 ± 1.0 %; P = 0.005; n = 3). The CS significantly reduced the CD63 expression from day 1 to 4 (rel. MFI of day 1 vs. 4: 5.3 ± 0.6 vs. 2.4 ± 0.3, P = 0.0131; n = 3) while the RT storage increased it on day 10 (day 1 vs. 10: 3.4 ± 0.4 vs. 6.4 ± 0.4, P = 0.0036; n = 3). Moreover, the RT increased the shrinkage from day 4 (shrunk cells of day 1 vs. 4: 3.2 ± 0.3 % vs. 9.2 ± 1.7 %, P = 0.0233; n = 3).

The CS prolonged the PLT function compared to RT storage starting from day 4.

3rd question: Does apoptosis inhibition impact the cold-stored platelet function?

We investigated the PLT function of cold-stored-APC for 10 days using three different apoptosis inhibitors compared to the 4 °C buffer control.

First, the RhoA-inhibitor G04 was investigated. A concentration of 150 μM performed the best of three inhibitors and retained all measure points in all assays.

Concretely, RhoA-inhibitor retained the ristocetin-triggered aggregation until day 10 (max. aggregation of buffer vs. G04 on day 10: 57.7 ± 19.2 % vs. 61.0 ± 8.7 %, P = 0.9016; n = 3). Moreover, RhoA-inhibitor provided significantly elevated aggregation by TRAP on day 7 (buffer vs. G04: 17.8 ± 2.8 % vs. 67.3 ± 11.6 %, P = 0.0017; n = 3). Upon inductor-triggered activation, RhoA-inhibitor retained the α-granule release and GPIIb/IIIa activation until day 10. RhoA-inhibitor showed enhanced the TRAP-induced dense-granule release throughout the measurement and significantly increased the release on day 1 (FI of buffer vs. G04: 2.3 ± 0.4 vs. 6.4 ± 1.0, P = 0.0251) and 7 (1.3 ± 0.2 vs. 3.0 ± 0.5, P = 0.0353; n = 3).

RhoA-inhibitor maintained the platelet storage lesion and provided no difference to control.

Next, the PKA-activator forskolin was tested which retained most of the function of CSPs.

We observed that a concentration of 0.75 μM retained the ristocetin-induced PLT aggregation during the whole measurement time (max. aggregation of buffer vs.

forskolin on day 10: 57.7 ± 19.2 % vs. 47.8 ± 8.7 %, *P* = 0.9988; n = 3). The GPIIb/IIIa activation was maintained until day 10 in the presence of PKA-activator (FI of buffer vs. forskolin on day 10: 2.3 ± 1.0 vs. 2.2 ± 0.4, *P* = 0.8763; n = 3). Until day 4, PKA-activator retained TRAP-triggered α-granule release (buffer vs. forskolin of day 4: 1.9 ± 0.3 vs. 1.4 ± 0.2; *P* = 0.2854; n = 3) However, on day 4, PKA-activator reduced the TRAP-induced aggregation (max. aggregation: 20.8 ± 2.8 %, *P* = 0.0286; n = 3) and dense-degranulation (FI: 1.4 ± 0.1, *P* = 0.0163, compared to buffer; n = 3).

PKA-activator retained the platelet lesion for all assays.

Investigating the caspase-9-inhibitor, 40 µM z-LEHD-fmk partly retained the platelet function.

Caspase-9-inhibitor partly conserved the TRAP-induced activation. Concretely, the inhibitor retained the aggregation until day 10 (max. aggregation of buffer vs. z-LEHD-fmk on day 10: 8.6 ± 0.7 % vs. 9.93 ± 0.3 %, *P* = 0.2835; n = 3), α-granule release until day 10 (FI on day 10: 1.0 ± 0.03 vs. 2.0 ± 0.9, *P* = 0.2201; n = 3), dense-granule release until day 7 (FI on day 7: 1.3 ± 0.2 vs. 1.1 ± 0.02, *P* = 0.5161; n = 3).

The caspase-9-inhibitor retained the platelet lesion throughout the storage. Moreover, on day 1, the inhibitor significantly decreased the CD62 expression (rel. MFI buffer vs. z-LEHD-fmk: 5.0 ± 0.3 vs. 2.3 ± 0.5; n = 3) and the shrinkage (shrunk cells: 2.5 ± 0.2 % vs. 1.1 ± 0.2 %, *P* = 0.0232; n = 3).

In conclusion, RhoA-inhibitor G04 did not impact the CSP function and retained the function completely. Furthermore, RhoA-inhibitor improved the functionality in some assays. Forskolin and z-LEHD-fmk partly retained the functionality of CSPs.

4 Discussion

4.1 1st question: the characteristics of apheresis-derived platelet concentrate

In this work, we found out that the APC, immediately after the production, showed a significant responsiveness to ristocetin and TRAP, moderately to collagen, but a weak response to ADP. Furthermore, the ADP response did not recover after 4 days of storage, neither at RT nor at 4 °C.

As described in the introduction, ristocetin is an *in vitro* activator inducing the bound between VWF and GPIb (Gangarosa et al., 1958; 1960). TRAP is an *in vitro* analogue of the strongest PLT activator, α-thrombin (Factor IIa) and agonize the PAR directly independent of fibrin formation (Gresele et al., 2007)**.** Collagen is the initiator of the activation *in vivo*, and is together with fibrinogen (Factor I) and fibrin (Factor Ia) an agonist for GPVI, α2β1 (GPIa/IIa), and resting GPIIb/IIIa (Jurk and Kehrel, 2005). As shown in the results, ristocetin and TRAP provided a high aggregation over 80 % and collagen led to a moderate aggregation about 50 %. Moreover, TRAP induced the granule release and GPIIb/IIIa activation of fresh APC. Other recent studies underline the aggregation response of APC to ristocetin, TRAP, and collagen (Reddoch et al., 2014; Jiang et al., 2018; Marini et al., 2019). Marini et al. reported max. aggregation of APC with 1.5 mg/ml ristocetin over 70 %, collagen 8µg/ml over 50 %. Our results, in accordance with other studies, indicate successful receptor activation upon these inductors in APC, likely to PRP.

In our work, the ADP, known as a PLT aggregation activator for 70 years (Gaarder et al., 1961), induced only a minor activation on APC. It is an agonist for the purinergic receptors $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ (Jurk and Kehrel, 2005). The g-protein $G_{\alpha q}$, coupling to $P2Y_1$, increases the cytosolic calcium concentration through phospholipase C_β (PLC_β) stimulation. The g-protein $G_{\alpha i}$, linked to $P2Y_{12}$ receptors, activates phosphatidylinositol 3-Kinase (PI3-K) and inhibits adenylate cyclase (Burnstock, 2004, Hollopeter et al., 2001). The $P2Y_{12}$ activity leads to amplification of the aggregation induced by all known PLT agonists including collagen, thrombin, immune complexes, TXA2, adrenaline and serotonin (Conley and Delaney, 2003; Gachet, 2006). Thus, ADP is an essential co-activator to achieve a full hemostatic function. They are expressed on the PLT surface and are both required for the PLT aggregation (Hechler et al., 1998;

Foster et al., 2001; Gachet, 2008). Although ADP is a weak reversible agonist under the condition of physiological external calcium level (Gachet, 2008), it leads to a strong irreversible aggregation *in vitro* with low external calcium concentration (Mustard et al., 1975). In our results, the buffer PLTs in PRP also showed enhanced maximal aggregation over 90 %.

Two factors impact the ADP depending on activation: the first one is the desensitization of the receptor by the agonist. Once activated, the $P2Y_1$ undergoes a shape change and PLTs become transiently unresponsive for 15 to 30 minutes (Gachet, 2008). During the refractory time, $P2Y_{12}$ receptor would still be able to ensure the hemostatic function with additional agonists (Gachet, 2008). Secondly, the degradation of ADP by ectonucleotidases plays important role in the aggregation. To avoid the desensitization, soluble apyrase, a hydrolase degrading nucleotides could be used to limit P2 receptor desensitization within *in vitro* (Jones et al., 2011). The ectonucleotidases of endothelial cells and white blood cells (WBCs) antagonize the desensitization upon stimulation with ADP *in vivo* (Cauwenberghs et al., 2006). Cauwenberghs, 2006 found out that the presence of plasma prevents the purinergic receptors for desensitization *in vitro*. In this plasma, the ectonucleotidases activity was encountered. This mechanism provides the basis for the poor reaction of plasma-lacking washed platelets (wPLTs) to ADP: the responsiveness to 10 μM ADP, standard concentration of ADP for aggregation assay, is rapidly dropped in less than 20 % 60 min after preparation (Koessler et al., 2016).

In our case, we hypothesize that the 35 % residual plasma, present in the APC, did not provide a sufficient amount of ectonucleotidaze removing ADP to prevent the desensitization of PLTs. Of note, we did not use the soluble apyrase. Our results, obtained using 10 μM ADP, provided a maximal aggregation of less than 20 % on day 0 and absence of enhanced expression of PLT activation markers by flow cytometry. Marini et al. (2019) reported slightly superior aggregation ability than our result, with max. aggregation of 35 %, in APC with 35 % residual plasma, but using higher concentration of ADP, 80 μM. In PCs, even 100 % plasma seems to be insufficient to remove the ADP: buffy-coat-derived PC in 100 % plasma showed a max. aggregation of 40 % with 10 μM ADP which was inferior to the reaction in PRP (Kicken et al.,

2017). Due to the mechanical stress during the production and high concentration of PLTs, PC could contain higher concentration of ADP. In future work, it would be interesting to quantify the ADP concentration as well as calcium in PC to verify this hypothesis.

Yet, the important question is whether the desensitization observed in the APC has an impact *in vivo* and in clinical settings. From our results of full PLT aggregation with other agonists such as ristocetin and TRAP, we assume that the function of $P2Y_{12}$ receptor was obtained. As mentioned above, at the injury place, several mediators activate the PLTs *in vivo*. Thus, we suppose that the existence of other agonists *in vivo* setting might compensate the desensitization associated reduced responsiveness to ADP. Therefore, the decreased responsiveness to ADP could be concerned as a phenomenon resulting in no severe clinical impact. To underline this, we encourage *in vivo* trials observing the PLTs responsiveness to ADP and the hemostatic function of transfused APC.

In conclusion, APC with 35 % residual plasma differentiated from PRP in some characteristics. In our study, APC showed high response to ristocetin and TRAP, moderate response to collagen, and decreased response upon ADP.

4.2 2nd question: the impact of storage time and temperature

We report that the storage time and the temperature have a significant impact on the hemostatic function of APC-PLTs *in vitro*.

Analyzing the storage time, the CS provided significant retained function over 4 days. In particular, the maximal aggregation was over 80 % with ristocetin and TRAP, and PLTs activated the TRAP-induced α- and dense-granule release as well as GPIIb/IIIa on day 4. Additionally, PLTs' shrinkage was under 5 % on day 4. While, on day 7, the function decreased partly with a significant drop in the TRAP-induced aggregation. Finally, on day 10, the responsiveness partly decreased, and the shrinkage increased over 10 %.

Next, investigating the impact of different storage temperatures, we observed that the CS better maintains the PLTs especially on day 4 and 7. On day 4 and 7, the CS significantly increased the aggregation ability in response to ristocetin and TRAP

compared to RT. Additionally, the CSP showed better dense-degranulation on day 4 and on day 7, CS reduced the shrunk PLT population in comparison to RT.

In accordance with the study of Marini et. al., 2019, we report in our work that the CS improves the PLT aggregation and activation, especially on day 4, indicating a pos. impact of CS on the PLT functionality *in vitro*.

On the other hand, the storage time of 4 days already reduced the function at RT. With ristocetin as well as with TRAP, the RT storage led to a significant PLT aggregation drop on day 4. Furthermore, on the same day, the activation of RSPs were not more inducible by TRAP. Finally, the cell morphology changed also during the storage time and PLTs lost their volume continuously from day 4.

As discussed in the introduction, in most of the countries, including Germany, the PC are kept at RT with a legal shelf life of 3-5 days (Sireis et al., 2011). Particularly in Germany, the normal shelf life is 4 days after the blood collection, and this could be postponed for one day if the infection screening is done. Unfortunately, this control contains only the testing about infectious risk and not the hemostatic function of the PLTs (Sireis et al., 2011; Bundesärztekammer, 2020). Our work, in accordance with many other studies e.g., Marini et al. (2019) and Rosenfeld et al. (1995), showed that not only the infection risk increases with RT storage time but also that the PSL. Moreover, our result shows that the hemostatic function of PLTs decreases significantly and the reduction is already visible on day 4 which is the legal storage time in Germany. In short, some PCs with significantly reduced quality might be transfused nowadays.

The existence of this critical problem was confirmed by two systematic reviews and meta-analysis of a Dutch group. They examined the effect of storage time of PC on PLTs (Caram-Deelder et al., 2016) and on the patient outcome after transfusion (Kreuger et al., 2017). Because of their inferior hemostatic function, old PLTs shorten the transfusion interval and increase the risk of transfusion reaction. Caram-Deedler et al. (2016) selected 46 RCTs and observational studies in human. They found out a decreased PLT count 1 hour and 24 hours after the transfusion, as well as reduced recovery and survival. Furthermore, Kreuger et al. (2017) reported the following results: in 12 studies, an elevated relative risk of a transfusion reaction after old PLTs compared to fresh PLTs was observed. Next, they observed 0.25 days [confidential interval (CI):

0.13-0.38] shorter transfusion interval in patients that received old PLTs and a relative risk of 1.13 (CI: 0.97-1.32) in 4 studies. Finally, five studies reported an increase of the bleeding time after transfusion of old PLTs.

Considering these findings, the outcome of patients comparing the storage time of RT-stored PCs should be investigated to analyze the functionality. The need of alternatives grows; the RT storage should be discussed widely.

In conclusion, we could confirm the improvement of PLT function through CS. Thus, we encourage the reevaluation of storage temperature of PCs and further trials with CSPs.

4.3 3rd question: the impact of apoptosis inhibition on cold-stored platelet

In our study, we investigated the impact of the three different apoptosis inhibitors on the CSPs' functionality. G04 is a reversible non-cytotoxic Rho-GTPase RhoA inhibitor (Shang et al., 2012). In 2017, a Greek group suggested at the conference of European Hematology Association that the inhibition of CSPs' RhoA through G04 could reduce *in vitro* as well as *in vivo* the CSL and phagocytosis (Hegde et al., 2017). Forskolin is a PKA-activator whose potential of apoptosis inhibition in RSP was recently shown by Zhao et al. (2017). Z-LEHD-fmk is a cell-permeable, competitive and irreversible specific caspase-9-inhibitor which initiate the final step of the mitochondrial pathway of apoptosis (Mullani et al., 2016).

Not only the maintenance of the PLT functionality but the better PLT survival is essential for the usage of the apoptosis inhibitors in clinical settings. In order to verify if those inhibitors can prevent the cold-induced apoptosis, another MD student of our group, Chiara Maettler, investigated the apoptosis inhibition effect *in vitro* at the same storage time. In particular, analyzing specific apoptosis markers, such as Phosphatidylserine expression and mitochondrial membrane depolarization, G04 resulted the best apoptosis inhibitor using a concentration of 150 µM (data not shown). Interestingly, considering the data presented in this work, the same concentration of G04 showed the best result and retained the function in all assays for every measure point until day 10. Some TRAP-induced activation such as aggregation, dense-granule release and GPIIb/IIIa activation were even improved.

While the other two inhibitors partly retained the PLT function: 0.75 µM forskolin maintained the ristocetin-induced PLT aggregation, TRAP-induced GPIIb/IIIa activation and the PSL until day 10 but it reduced the TRAP-induced aggregation and dense-degranulation on day 4. 40 µM z-LEHD-fmk conserved the TRAP-induced aggregation and α-granule release as well as PSL until day 10.

In the last several years CS has been proposed and investigated as alternative storage condition of APCs. However, the biggest problem of CS, as shown in the introduction, is the short PLT survival in the blood circulation after transfusion. To discuss this point, it is important to It is evident that for the therapeutic use, the PLTs should induce hemostasis immediately after the transfusion. But even for the prophylactic transfusion, the PLT function is needed in a short time period about 24-48h: in a clinical RCT with hematooncologic patients, the prophylactic use of PCs prolonged the first bleeding time of thrombopenic patients from 1.2 (therapeutic) to 1.7 days despite of long *in vivo* survival of conventional RSPs for several days (Stanworth et al., 2012; Vostal et al., 2018). This indicates that the hemostatic function of transfused PLT in the first 48 h is the most important criteria for transfused PLTs to intervene bleeding. Also, mentioned in introduction, the use of PCs has shifted more to the therapeutic procedure.

A recent clinical trial of autologous 7-day-old PLTs transfusion with healthy volunteers provides a survival time of CSP over 30 hours [survival (mean ± standard deviation) of cold PLT: 33.7 ± 14.7 hours] (Vostal et al., 2018). Our results suggest the retained function of PLT after treatment with apoptosis inhibitors. The use of apoptosis inhibitors could prolong this survival time and optimize the survival of CSPs.

In conclusion, G04 retains the CSP function until day 10 showing improving tendency of some function on day 7, such as TRAP-induced aggregation and dense-granule release. Therefore, we encourage the *in vivo* and clinical investigations of G04 for CS of APCs.

4.4 Conclusion

In this work, we found out that APC with 35 % residual plasma showed a weak response to ADP. The high variability of all published studies is due to the

heterogeneity of the APC produced worldwide. In fact, the absence of a standard protocol defining all parameters and factors involved in the APC production compound the analysis. It is shown that introduction of PASs or oxygen permeable bag can reduce the PSL (Dumont et al., 2007; Ohto and Nollet, 2011; Capocelli and Dumont, 2014). A standardization of the material condition would allow a better evaluation of APC functionality.

Next, we found out that CS maintained the PLT functionality longer than RT storage. Interestingly, on day 4, which is a legal storage time in many countries, the RSPs provided already reduced functionality. In contrast, we could show that the CS retains the PLT function on day 4 *in vitro.*

However, the impact of CS on PLT survival *in vivo* is still under debate due to studies reporting opposite results (Murphy and Gardner, 1969; Marini et al., 2019, Strandenes et al., 2020). In fact, for therapeutic transfusion, not the long half-life but the immediate hemostatic response is required. For the prophylactic use, the actual need of hemostatic function seems to be mostly during the first 48 h after transfusion (Stanworth et al., 2012; Vostal et al., 2018).

The treatment with apoptosis inhibitors could prolong the survival time of CSPs. Our results of retained function under apoptosis inhibition with G04 is promising for potential use.

Taking together all the data of this work, we can conclude that CSPs represents an interesting potential tool to improve the hemostatic function after transfusion at least until day 4 after the PC production. From a future point of view, further investigations, to analyze the impact of G04 on CSP survival *in vivo*, are highly recommended as well as a clinical trial to test this inhibitor for clinical settings.

5 Summary

5.1 English summary

Introduction: The cold storage (CS) represents one of several strategies to improve the platelet (PLT) quality during the storage. In 2019, our group reported a better PLT functionality after CS but a faster clearance *in vivo*, suggesting apoptosis as a mechanism induced by CS leading to the reduced PLT survival. Therefore, we proposed apoptosis inhibition in cold-stored apheresis-derived platelet concentrates (APCs), in order to improve the survival of cold-stored platelets (CSPs). We focused on the PLT functionality with three questions: *1. Does the APC production process impact the PLT functionality? 2. Does the storage temperature impact the PLT functionality? 3. Does apoptosis inhibition impact the CSP function?*

Methods: The apoptosis inhibition was performed with RhoA-GTPase-inhibitor G04, PKA-activator forskolin, and caspase-9-inhibitor z-LEHD-fmk. APC of healthy donors were incubated with the inhibitors directly after production (day 0) and stored at 4 ºC under continuous agitation. The control buffer APC was once stored at RT, once at 4 ºC. Using PLT aggregometry and flow cytometry, the PLT function was investigated.

Results: We found that the APC-PLTs showed high aggregation, immediately after their production, upon 20µM thrombin receptor-activating peptide (TRAP) and 1.0 mg/ml ristocetin, but they showed decreased activation by 10 µM adenosine diphosphate (ADP).

Furthermore, analyzing the storage temperature, we observed that CS retained the PLT functionality longer than storage at RT. The CS provided maximal aggregation over 90 % on day 4, while it dropped significantly after RT storage on the same day. Furthermore, TRAP-induced activation was examined and the CSPs showed improved α- and dense-degranulation and glycoprotein IIb/IIIa (GPIIb/IIIa) activation. The CS still retained the platelet function on day 7.

Next, we investigated the impact of apoptosis inhibition on PLT functionality, observing a heterogeneous effect depending on the specific inhibitor. We showed that the best candidate is the RhoA-GTPase-inhibitor (G04) that maintained the function of CSPs until day 10 in every assay. Furthermore, TRAP induced elevated aggregation and

dense-granule release of G04 on day 7. The PKA-activator (forskolin) maintained the ristocetin-induced aggregation, GPIIb/IIIa activation, and the shrinkage until day 10. However, it decreased the aggregation with TRAP already on day 4. The caspase-9 inhibitor (z-LEHD-fmk) maintained the aggregation and the shrinkage until day 10, and TRAP-induced α- and dense-granule release on day 7.

Conclusion: CS improves the PLT quality which is entirely retained by addition of G04. Other inhibitors, forskolin and z-LEHD-fmk mostly retained the function of CSPs.

In conclusion, showing significant superior function 4 days after production to RSPs, CSPs possess an enormous potential to prolong the storage time and retain the hemostatic function until day 7. The addition of the G04 to CSPs as apoptosis inhibitor may be an efficient tool to prolong the storage time without affecting the PLT function and survival.

5.2 Zusammenfassung

Einleitung: Die Kaltlagerung (KL) könnte eine bedeutende Strategie sein, um den Qualitätsverlust der Thrombozyten während der Lagerung zu verhindern. In 2019 berichtete unsere Arbeitsgruppe über die verbesserte Thrombozytenfunktion (TF) nach der KL aber mit gleichzeitiger beschleunigter Elimination *in vivo.* Als Mechanismus schlugen wir die Aktivierung der Apoptose in Thrombozyten vor. In dieser Arbeit behandelten wir somit die kaltgelagerten Thrombozyten (KT) mit Apoptoseinhibitoren und gingen auf drei Fragen zur TF ein: *1. Hat die Produktion der Apheresethrombozytenkonzentrate (ATK) Einfluss auf die TF? 2. Wie wirkt die Lagertemperatur auf die TF? 3. Schränkt die Apoptoseinhibition die Funktion der KT ein?*

Methode: Die ATK von gesunden Spendern wurden am Produktionstag (Tag 0) mit drei unterschiedlichen Apoptoseinhibitoren (RhoA-GTPase-Inhibitor G04, Phosphokinase-A-Aktivator Forskolin und Caspase-9-Inhibitor z-LEHD-fmk) behandelt und anschließend bei 4 °C unter ständiger Agitation gelagert. Die Puffer-Proben wurden als Kontrollen jeweils bei Raumtemperatur (RT) und 4 °C gelagert. Wir untersuchten die TF mittels Aggregometrie und Durchflusszytometrie.

Ergebnis: Die Thrombozyten der frischen ATK zeigten eine hohe Aggregationsfähigkeit mit 20 µM Thrombinrezeptor aktivierendem Peptid (TRAP) und 1,0 mg/ml Ristocetin. Wir beobachteten jedoch eine erniedrigte Adenosindiphosphat (ADP)-getriggerte Aktivierung.

Im Lagerungstemperaturvergleich konnten wir einen besseren Funktionserhalt der Thrombozyten bei KL zeigen. Die KT wiesen am Tag 4 eine Aggregation von > 90 % mit TRAP sowie Ristocetin auf, während die Aggregation der RT-gelagerten Thrombozyten (RTT) bereits signifikant abnahm. Außerdem wurde die Thromobozytenaktivierung mit TRAP-Inkubation untersucht und die KT zeigten eine gesteigerte Antwort auf α-, dense-Granula-Ausschüttung sowie Glykoprotein IIb/IIIa (GPIIb/IIIa)-Aktivierung. Die KL erhielt die TF stets am Tag 7 aufrecht.

Die Apoptoseinhibitoren zeigten heterogene Ergebnisse. Wir konnten zeigen, dass die TF unter RhoA-GTPase-Inhibition (G04) bis Tag 10 erhalten blieb. Darüber hinaus induzierte TRAP bei KT mit RhoA-GTPase-Inhibitor erhöhte Aggregation sowie dense-Granula-Ausschüttung am Tag 7. Der PKA-Aktivator (Forskolin) bewahrte die Ristocetin-Aggregation, GPIIb/IIIa-Aktivierung sowie Zellschrumpfung bis Tag 10. Der PKA-Aktivator reduzierte dennoch die TRAP-getriggerte Aggregation und dense-Degranulation am Tag 4. Der Caspase-9-Inhibitor (z-LEHD-fmk) behielt im Vergleich zur Kontrolle die Aggregationsfähigkeit und die Zellschrumpfung bis Tag 10, die α- und dense-Granula-Ausschüttung am Tag 7 bei.

Fazit: KL verbessert die Qualität der Thrombozyten, die auch nach Zugabe von RhoA-GTPase-Inhibitor vollständig erhalten bleibt. Andere Inhibitoren, PKA-Aktivator und Caspase-9-Inhibitor erhalten die meisten Funktionen der KT.

Als Schlussfolgerung kann gesagt werden, dass die KT mit ihrer deutlich besseren Funktion um den 4. Tag nach der Produktion gegenüber den RTT und ihrem Funktionerhalt bis zum Tag 7 ein großes Potential besitzen. Die Zugabe von RhoA-GTPase-Inhibitor an KT als Apoptoseinhibitor könnte eine Verlängerung der Lagerungsdauer ermöglichen, ohne die Funktion oder das Überleben der Thrombozyten einzuschränken.

6 Bibliography

ABONNENC, M., SONEGO, G., KAISER-GUIGNARD, J., CRETTAZ, D., PRUDENT, M., TISSOT, J. D. & LION, N. 2015. In vitro evaluation of pathogen-inactivated buffy coat-derived platelet concentrates during storage: psoralen-based photochemical treatment step-by-step. *Blood Transfus,* 13**,** 255-64.

ABRAMS, C. S., ELLISON, N., BUDZYNSKI, A. Z. & SHATTIL, S. J. 1990. Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood,* 75**,** 128-38.

APELSETH, T. O., HERVIG, T. A., WENTZEL-LARSEN, T. & BRUSERUD, O. 2006. Cytokine accumulation in photochemically treated and gamma-irradiated platelet concentrates during storage. *Transfusion,* 46**,** 800-10.

BECKER, G. A., TUCCELLI, M., KUNICKI, T., CHALOS, M. K. & ASTER, R. H. 1973. Studies of platelet concentrates stored at 22 C nad 4 C. *Transfusion,* 13**,** 61-8.

BLAJCHMAN, M. A., SLICHTER, S. J., HEDDLE, N. M. & MURPHY, M. F. 2008. New Strategies for the Optimal Use of Platelet Transfusions. *Hematology,* 2008**,** 198-204.

BLENNER, M. A., DONG, X. & SPRINGER, T. A. 2014. Structural basis of regulation of von Willebrand factor binding to glycoprotein Ib. *J Biol Chem,* 289**,** 5565-79.

BOUCHARD, B. A., CATCHER, C. S., THRASH, B. R., ADIDA, C. & TRACY, P. B. 1997. Effector cell protease receptor-1, a platelet activation-dependent membrane protein, regulates prothrombinase-catalyzed thrombin generation. *J Biol Chem,* 272**,** 9244-51.

BRECHER, M. E., BLAJCHMAN, M. A., YOMTOVIAN, R., NESS, P. & AUBUCHON, J. P. 2013a. Addressing the risk of bacterial contamination of platelets within the United States: a history to help illuminate the future. *Transfusion,* 53**,** 221-31.

BRECHER, M. E., JACOBS, M. R., KATZ, L. M., JACOBSON, J., RIPOSO, J., CARR-GREER, A., KLEINMAN, S. & FORCE, A. B. C. T. 2013b. Survey of methods used to detect bacterial contamination of platelet products in the United States in 2011. *Transfusion,* 53**,** 911-8.

BUDDE, U. 2002. Diagnose von Funktionsstörungen der Thrombozyten mit Hilfe der Aggregometrie/Diagnosis of Platelet Function Defects with Platelet Aggregometers. *Laboratoriumsmedizin-journal of Laboratory Medicine - LABORATORIUMSMEDIZIN,* 26**,** 564-571.

BUNDESÄRZTEKAMMER 2020. Querschnitts-Leitlinien zur Therapie mit Blutkomponenten und Plasmaderivaten. *In:* BUNDESÄRZTEKAMMER (ed.).

BURNSTOCK, G. 2004. Introduction: P2 receptors. *Curr Top Med Chem,* 4**,** 793-803.

CAP, A. P. & SPINELLA, P. C. 2017. Just chill-it's worth it! *Transfusion,* 57**,** 2817-2820.

CAPOCELLI, K. E. & DUMONT, L. J. 2014. Novel platelet storage conditions: additive solutions, gas, and cold. *Curr Opin Hematol,* 21**,** 491-6.

CARAM-DEELDER, C., KREUGER, A. L., JACOBSE, J., VAN DER BOM, J. G. & MIDDELBURG, R. A. 2016. Effect of platelet storage time on platelet measurements: a systematic review and meta-analyses. *Vox Sang,* 111**,** 374-382.

CAUWENBERGHS, S., FEIJGE, M. A., HAGEMAN, G., HOYLAERTS, M., AKKERMAN, J. W., CURVERS, J. & HEEMSKERK, J. W. 2006. Plasma

ectonucleotidases prevent desensitization of purinergic receptors in stored platelets: importance for platelet activity during thrombus formation. *Transfusion,* 46**,** 1018-28.
CAUWENBERGHS, S., VAN PAMPUS, E., CURVERS, J., AKKERMAN, J. W. & HEEMSKERK, J. W. 2007. Hemostatic and signaling functions of transfused platelets. *Transfus Med Rev,* 21**,** 287-94.
CHEN, W., DRUZAK, S. A., WANG, Y., JOSEPHSON, C. D., HOFFMEISTER, K. M., WARE, J. & LI, R. 2017. Refrigeration-Induced Binding of von Willebrand Factor Facilitates Fast Clearance of Refrigerated Platelets. *Arterioscler Thromb Vasc Biol,* 37**,** 2271-2279.
CHEN, W., LIANG, X., SYED, A. K., JESSUP, P., CHURCH, W. R., WARE, J., JOSEPHSON, C. D. & LI, R. 2016. Inhibiting GPIbα Shedding Preserves Post-Transfusion Recovery and Hemostatic Function of Platelets After Prolonged Storage. *Arterioscler Thromb Vasc Biol,* 36**,** 1821-8.
CONLEY, P. B. & DELANEY, S. M. 2003. Scientific and therapeutic insights into the role of the platelet P2Y12 receptor in thrombosis. *Curr Opin Hematol,* 10**,** 333-8.
CURRIE, L. M., HARPER, J. R., ALLAN, H. & CONNOR, J. 1997. Inhibition of cytokine accumulation and bacterial growth during storage of platelet concentrates at 4 degrees C with retention of in vitro functional activity. *Transfusion,* 37**,** 18-24.
DASGUPTA, S. K., ARGAIZ, E. R., MERCADO, J. E., MAUL, H. O., GARZA, J., ENRIQUEZ, A. B., ABDEL-MONEM, H., PRAKASAM, A., ANDREEFF, M. & THIAGARAJAN, P. 2010. Platelet senescence and phosphatidylserine exposure. *Transfusion,* 50**,** 2167-75.
DE KORTE, D. & MARCELIS, J. H. 2014. Platelet concentrates: reducing the risk of transfusion-transmitted bacterial infections. *Int J Clin Transfus Med,* 2**,** 29–37.
DEPPERMANN, C., KRATOFIL, R. M., PEISELER, M., DAVID, B. A., ZINDEL, J., CASTANHEIRA, F., VAN DER WAL, F., CARESTIA, A., JENNE, C. N., MARTH, J. D. & KUBES, P. 2020. Macrophage galactose lectin is critical for Kupffer cells to clear aged platelets. *J Exp Med,* 217.
DUERSCHMIED, D. & BODE, C. 2009. [The role of serotonin in haemostasis]. *Hamostaseologie,* 29**,** 356-9.
DUMONT, L. J., GULLIKSSON, H., VAN DER MEER, P. F., MURPHY, S., NIXON, J. G., DE WILDT-EGGEN, J., VANDENBROEKE, T. & AUBUCHON, J. P. 2007. Interruption of agitation of platelet concentrates: a multicenter in vitro study by the BEST Collaborative on the effects of shipping platelets. *Transfusion,* 47**,** 1666-73.
ESTCOURT, L. J., HEDDLE, N., KAUFMAN, R., MCCULLOUGH, J., MURPHY, M. F., SLICHTER, S., WOOD, E. M., STANWORTH, S. J. & BIOMEDICAL EXCELLENCE FOR SAFER TRANSFUSION, C. 2013. The challenges of measuring bleeding outcomes in clinical trials of platelet transfusions. *Transfusion,* 53**,** 1531-43.
FLAUMENHAFT, R., DILKS, J. R., ROZENVAYN, N., MONAHAN-EARLEY, R. A., FENG, D. & DVORAK, A. M. 2005. The actin cytoskeleton differentially regulates platelet alpha-granule and dense-granule secretion. *Blood,* 105**,** 3879-87.
FOSTER, C. J., PROSSER, D. M., AGANS, J. M., ZHAI, Y., SMITH, M. D., LACHOWICZ, J. E., ZHANG, F. L., GUSTAFSON, E., MONSMA, F. J., JR., WIEKOWSKI, M. T., ABBONDANZO, S. J., COOK, D. N., BAYNE, M. L., LIRA, S. A. & CHINTALA, M. S. 2001. Molecular identification and characterization of

the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *The Journal of clinical investigation,* 107**,** 1591-1598.
GACHET, C. 2006. Regulation of platelet functions by P2 receptors. *Annu Rev Pharmacol Toxicol,* 46**,** 277-300.
GACHET, C. 2008. P2 receptors, platelet function and pharmacological implications. *Thromb Haemost,* 99**,** 466-72.
GANGAROSA, E. J., JOHNSON, T. R. & RAMOS, H. S. 1960. Ristocetin-induced thrombocytopenia: site and mechanism of action. *Arch Intern Med,* 105**,** 83-9.
GANGAROSA, E. J., LANDERMAN, N. S., ROSCH, P. J. & HERNDON, E. G., JR. 1958. Hematologic complications arising during ristocetin therapy; relation between dose and toxicity. *N Engl J Med,* 259**,** 156-61.
GETZ, T. M. 2019. Physiology of cold-stored platelets. *Transfus Apher Sci,* 58**,** 12-15.
GODYNA, S., DIAZ-RICART, M. & ARGRAVES, W. S. 1996. Fibulin-1 mediates platelet adhesion via a bridge of fibrinogen. *Blood,* 88**,** 2569-77.
GREMMEL, T., FRELINGER, A. L., 3RD & MICHELSON, A. D. 2016. Platelet Physiology. *Semin Thromb Hemost,* 42**,** 191-204.
GRESELE, P., FUSTER, V., LOPEZ, J. A., PAGE, C. P. & VERMYLEN, J. 2007. *Platelets in Hematologic and Cardiovascular Disorders: A Clinical Handbook,* Cambridge, Cambridge University Press.
HANSON, S. R. & SLICHTER, S. J. 1985. Platelet kinetics in patients with bone marrow hypoplasia: evidence for a fixed platelet requirement. *Blood,* 66**,** 1105-9.
HECHLER, B., ECKLY, A., OHLMANN, P., CAZENAVE, J. P. & GACHET, C. 1998. The P2Y1 receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol,* 103**,** 858-66.
HEGDE, S., AKBAR, H., NESTHEIDE, S., WELLENDORF, A., MOHMOUD, F., JOHNSON, J., ZHENG, Y. & CANCELAS, J. A. 2017. Reverdible pharmacological targeting of RhoA allows improved storage, survival and hemostatic activity of platelets in vitro and in vivo, in mice and in primates.
HEUFT, H.-G., MENDE, W. & BLASCZYK, R. 2008. A general change of the platelet transfusion policy from apheresis platelet concentrates to pooled platelet concentrates is associated with a sharp increase in donor exposure and infection rates. *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie,* 35**,** 106-113.
HOFFMEISTER, B., BUNDE, T., RUDAWSKY, I. M., VOLK, H. D. & KERN, F. 2003a. Detection of antigen-specific T cells by cytokine flow cytometry: the use of whole blood may underestimate frequencies. *Eur J Immunol,* 33**,** 3484-92.
HOFFMEISTER, K. M., FELBINGER, T. W., FALET, H., DENIS, C. V., BERGMEIER, W., MAYADAS, T. N., VON ANDRIAN, U. H., WAGNER, D. D., STOSSEL, T. P. & HARTWIG, J. H. 2003b. The clearance mechanism of chilled blood platelets. *Cell,* 112**,** 87-97.
HOFFMEISTER, K. M., JOSEFSSON, E. C., ISAAC, N. A., CLAUSEN, H., HARTWIG, J. H. & STOSSEL, T. P. 2003c. Glycosylation restores survival of chilled blood platelets. *Science,* 301**,** 1531-4.
HOLLOPETER, G., JANTZEN, H. M., VINCENT, D., LI, G., ENGLAND, L., RAMAKRISHNAN, V., YANG, R. B., NURDEN, P., NURDEN, A., JULIUS, D. & CONLEY, P. B. 2001. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature,* 409**,** 202-7.

HONG, H., XIAO, W., LAZARUS, H. M., GOOD, C. E., MAITTA, R. W. & JACOBS, M. R. 2016. Detection of septic transfusion reactions to platelet transfusions by active and passive surveillance. *Blood,* 127**,** 496-502.

HUMBRECHT, C., KIENTZ, D. & GACHET, C. Platelet transfusion: Current challenges. 2018. *Transfus Clin Biol.,* 25(3):151-164.

IGNATOVA, A. A., KARPOVA, O. V., TRAKHTMAN, P. E., RUMIANTSEV, S. A. & PANTELEEV, M. A. 2016. Functional characteristics and clinical effectiveness of platelet concentrates treated with riboflavin and ultraviolet light in plasma and in platelet additive solution. *Vox Sang,* 110**,** 244-52.

JIANG, Q. F., XIAO, Q., JIANG, F. S. & LIU, F. 2018. [Effects of Stored Apheresis Platelet Apoptosis on Aggregation Function]. Zhongguo Shi Yan Xue Ye Xue Za Zhi, 26, 1492-1496.

JOHNSON, L., LOH, Y. S., KWOK, M. & MARKS, D. C. 2013. In vitro assessment of buffy-coat derived platelet components suspended in SSP+ treated with the INTERCEPT Blood system. *Transfus Med,* 23**,** 121-9.

JOHNSON, L., TAN, S., WOOD, B., DAVIS, A. & MARKS, D. C. 2016. Refrigeration and cryopreservation of platelets differentially affect platelet metabolism and function: a comparison with conventional platelet storage conditions. *Transfusion,* 56**,** 1807-18.

JOHNSON, L., WINTER, K. M., REID, S., HARTKOPF-THEIS, T., MARSCHNER, S., GOODRICH, R. P. & MARKS, D. C. 2011. The effect of pathogen reduction technology (Mirasol) on platelet quality when treated in additive solution with low plasma carryover. *Vox Sang,* 101**,** 208-14.

JONES, S., EVANS, R. J. & MAHAUT-SMITH, M. P. 2011. Extracellular Ca(2+) modulates ADP-evoked aggregation through altered agonist degradation: implications for conditions used to study P2Y receptor activation. *British journal of haematology,* 153**,** 83-91.

JURK, K. & KEHREL, B. E. 2005. Platelets: physiology and biochemistry. *Semin Thromb Hemost,* 31**,** 381-92.

KICKEN, C. H., ROEST, M., HENSKENS, Y. M. C., DE LAAT, B. & HUSKENS, D. 2017. Application of an optimized flow cytometry-based quantification of Platelet Activation (PACT): Monitoring platelet activation in platelet concentrates. *PloS one,* 12**,** e0172265-e0172265.

KIM, J., ZHANG, C. Z., ZHANG, X. & SPRINGER, T. A. 2010. A mechanically stabilized receptor-ligand flex-bond important in the vasculature. *Nature,* 466**,** 992-5.

KOESSLER, J., HERMANN, S., WEBER, K., KOESSLER, A., KUHN, S., BOECK, M. & KOBSAR, A. 2016. Role of Purinergic Receptor Expression and Function for Reduced Responsiveness to Adenosine Diphosphate in Washed Human Platelets. *PloS one,* 11**,** e0147370-e0147370.

KOSEOGLU, S. & FLAUMENHAFT, R. 2013. Advances in platelet granule biology. *Curr Opin Hematol,* 20**,** 464-71.

KREUGER, A. L., CARAM-DEELDER, C., JACOBSE, J., KERKHOFFS, J. L., VAN DER BOM, J. G. & MIDDELBURG, R. A. 2017. Effect of storage time of platelet products on clinical outcomes after transfusion: a systematic review and meta-analyses. *Vox Sang,* 112**,** 291-300.

LEVY, J. H., NEAL, M. D. & HERMAN, J. H. 2018. Bacterial contamination of platelets for transfusion: strategies for prevention. *Critical Care,* 22.

MAETTLER, C. 2021. 'The apoptosis inhibition of cold stored platelets', medPhD thesis, University of Tuebingen, Tuebingen.
MANN, K. G. 1999. Biochemistry and physiology of blood coagulation. *Thromb Haemost,* 82**,** 165-74.
MANN, K. G. 2011. Thrombin generation in hemorrhage control and vascular occlusion. *Circulation,* 124**,** 225-35.
MARINI, I., AURICH, K., JOUNI, R., NOWAK-HARNAU, S., HARTWICH, O., GREINACHER, A., THIELE, T. & BAKCHOUL, T. 2019. Cold storage of platelets in additive solution: the impact of residual plasma in apheresis platelet concentrates. *Haematologica,* 104**,** 207-214.
MONTGOMERY, R. K., REDDOCH, K. M., EVANI, S. J., CAP, A. P. & RAMASUBRAMANIAN, A. K. 2013. Enhanced shear-induced platelet aggregation due to low-temperature storage. *Transfusion,* 53**,** 1520-30.
MULLANI, N., SINGH, M. K., SHARMA, A., RAMESHBABU, K., MANIK, R. S., PALTA, P., SINGLA, S. K. & CHAUHAN, M. S. 2016. Caspase-9 inhibitor Z-LEHD-FMK enhances the yield of in vitro produced buffalo (Bubalus bubalis) pre-implantation embryos and alters cellular stress response. *Res Vet Sci,* 104**,** 4-9.
MURPHY, S. & GARDNER, F. H. 1969. Effect of storage temperature on maintenance of platelet viability--deleterious effect of refrigerated storage. *N Engl J Med,* 280**,** 1094-8.
MUSTARD, J. F., PERRY, D. W., KINLOUGH-RATHBONE, R. L. & PACKHAM, M. A. 1975. Factors responsible for ADP-induced release reaction of human platelets. *Am J Physiol,* 228**,** 1757-65.
NG, M. S. Y., TUNG, J. P. & FRASER, J. F. 2018. Platelet Storage Lesions: What More Do We Know Now? *Transfus Med Rev*.
OHTO, H. & NOLLET, K. E. 2011. Overview on platelet preservation: better controls over storage lesion. *Transfus Apher Sci,* 44**,** 321-5.
OLIVER, A. E., TABLIN, F., WALKER, N. J. & CROWE, J. H. 1999. The internal calcium concentration of human platelets increases during chilling. *Biochim Biophys Acta,* 1416**,** 349-60.
OSTROWSKI, S. R., BOCHSEN, L., WINDELOV, N. A., SALADO-JIMENA, J. A., REYNAERTS, I., GOODRICH, R. P. & JOHANSSON, P. I. 2011. Hemostatic function of buffy coat platelets in additive solution treated with pathogen reduction technology. *Transfusion,* 51**,** 344-56.
PEARCE, S., ROWE, G. P. & FIELD, S. P. 2011. Screening of platelets for bacterial contamination at the Welsh Blood Service. *Transfus Med,* 21**,** 25-32.
PERKINS, J. G., CAP, A. P., SPINELLA, P. C., BLACKBOURNE, L. H., GRATHWOHL, K. W., REPINE, T. B., KETCHUM, L., WATERMAN, P., LEE, R. E., BEEKLEY, A. C., SEBESTA, J. A., SHORR, A. F., WADE, C. E. & HOLCOMB, J. B. 2009. An evaluation of the impact of apheresis platelets used in the setting of massively transfused trauma patients. *J Trauma,* 66**,** S77-84; discussion S84-5.
PIDCOKE, H. F., ADEN, J. K., MORA, A. G., BORGMAN, M. A., SPINELLA, P. C., DUBICK, M. A., BLACKBOURNE, L. H. & CAP, A. P. 2012. Ten-year analysis of transfusion in Operation Iraqi Freedom and Operation Enduring Freedom: increased plasma and platelet use correlates with improved survival. *J Trauma Acute Care Surg,* 73**,** S445-52.

QUACH, M. E., CHEN, W. & LI, R. 2018a. Mechanisms of platelet clearance and translation to improve platelet storage. *Blood,* 131**,** 1512-1521.
QUACH, M. E., DRAGOVICH, M. A., CHEN, W., SYED, A. K., CAO, W., LIANG, X., DENG, W., DE MEYER, S. F., ZHU, G., PENG, J., NI, H., BENNETT, C. M., HOU, M., WARE, J., DECKMYN, H., ZHANG, X. F. & LI, R. 2018b. Fc-independent immune thrombocytopenia via mechanomolecular signaling in platelets. *Blood,* 131**,** 787-796.
REDDOCH, K. M., PIDCOKE, H. F., MONTGOMERY, R. K., FEDYK, C. G., ADEN, J. K., RAMASUBRAMANIAN, A. K. & CAP, A. P. 2014. Hemostatic function of apheresis platelets stored at 4 degrees C and 22 degrees C. *Shock,* 41 Suppl 1**,** 54-61.
REDDOCH-CARDENAS, K. M., BYNUM, J. A., MELEDEO, M. A., NAIR, P. M., WU, X., DARLINGTON, D. N., RAMASUBRAMANIAN, A. K. & CAP, A. P. 2019. Cold-stored platelets: A product with function optimized for hemorrhage control. *Transfus Apher Sci,* 58**,** 16-22.
ROSENFELD, B. A., HERFEL, B., FARADAY, N., FULLER, A. & BRAINE, H. 1995. Effects of storage time on quantitative and qualitative platelet function after transfusion. *Anesthesiology,* 83**,** 1167-72.
RUGGERI, Z. M. & MENDOLICCHIO, G. L. 2015. Interaction of von Willebrand factor with platelets and the vessel wall. *Hamostaseologie,* 35**,** 211-24.
RUMJANTSEVA, V., GREWAL, P. K., WANDALL, H. H., JOSEFSSON, E. C., SØRENSEN, A. L., LARSON, G., MARTH, J. D., HARTWIG, J. H. & HOFFMEISTER, K. M. 2009. Dual roles for hepatic lectin receptors in the clearance of chilled platelets. *Nature Medicine,* 15**,** 1273-1280.
SAAD, J. & SCHOENBERGER, L. 2020. Physiology, Platelet Activation. *StatPearls.* Treasure Island (FL).
SANDGREN, P., HANSSON, M., GULLIKSSON, H. & SHANWELL, A. 2007. Storage of buffy-coat-derived platelets in additive solutions at 4 degrees C and 22 degrees C: flow cytometry analysis of platelet glycoprotein expression. *Vox Sang,* 93**,** 27-36.
SANDGREN, P., SHANWELL, A. & GULLIKSSON, H. 2006. Storage of buffy coat-derived platelets in additive solutions: in vitro effects of storage at 4 degrees C. *Transfusion,* 46**,** 828-34.
SAVAGE, B. & RUGGERI, Z. M. 1991. Selective recognition of adhesive sites in surface-bound fibrinogen by glycoprotein IIb-IIIa on nonactivated platelets. *J Biol Chem,* 266**,** 11227-33.
SAVAGE, B., SALDÍVAR, E. & RUGGERI, Z. M. 1996. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell,* 84**,** 289-97.
SCHARF, R. E. 2018. Platelet Signaling in Primary Haemostasis and Arterial Thrombus Formation: Part 1. *Hamostaseologie,* 38**,** 203-210.
SCHOENWAELDER, S. M., YUAN, Y., JOSEFSSON, E. C., WHITE, M. J., YAO, Y., MASON, K. D., O'REILLY, L. A., HENLEY, K. J., ONO, A., HSIAO, S., WILLCOX, A., ROBERTS, A. W., HUANG, D. C., SALEM, H. H., KILE, B. T. & JACKSON, S. P. 2009. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood,* 114**,** 663-6.
SHANG, X., MARCHIONI, F., SIPES, N., EVELYN, C. R., JERABEK-WILLEMSEN, M., DUHR, S., SEIBEL, W., WORTMAN, M. & ZHENG, Y. 2012. Rational design

of small molecule inhibitors targeting RhoA subfamily Rho GTPases. *Chem Biol,* 19**,** 699-710.

SHATTIL, S. J., CUNNINGHAM, M. & HOXIE, J. A. 1987. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood,* 70**,** 307-15.

SHEA, S. M., THOMAS, K. A. & SPINELLA, P. C. 2019. The effect of platelet storage temperature on haemostatic, immune, and endothelial function: potential for personalised medicine. *Blood Transfus,* 17**,** 321-330.

SIREIS, W., RUSTER, B., DAISS, C., HOURFAR, M. K., CAPALBO, G., PFEIFFER, H. U., JANETZKO, K., GOEBEL, M., KEMPF, V. A., SEIFRIED, E. & SCHMIDT, M. 2011. Extension of platelet shelf life from 4 to 5 days by implementation of a new screening strategy in Germany. *Vox Sang,* 101**,** 191-9.

SPINELLA, P. C., DUNNE, J., BEILMAN, G. J., O'CONNELL, R. J., BORGMAN, M. A., CAP, A. P. & RENTAS, F. 2012. Constant challenges and evolution of US military transfusion medicine and blood operations in combat. *Transfusion,* 52**,** 1146-53.

STANWORTH, S. J., DYER, C., CHOO, L., BAKRANIA, L., COPPLESTONE, A., LLEWELYN, C., NORFOLK, D., POWTER, G., LITTLEWOOD, T., WOOD, E. M. & MURPHY, M. F. 2010. Do all patients with hematologic malignancies and severe thrombocytopenia need prophylactic platelet transfusions? Background, rationale, and design of a clinical trial (trial of platelet prophylaxis) to assess the effectiveness of prophylactic platelet transfusions. *Transfus Med Rev,* 24**,** 163-71.

STANWORTH, S. J., ESTCOURT, L., POWTER, G., KAHAN, B. C., DYER, C., BAKRANIA, L., LLEWELYN, C., CHOO, L., BIELBY, L., NORFOLK, D., WOOD, E. M. & MURPHY, M. F. 2012. The Effect of a No-Prophylactic Versus Prophylactic Platelet Transfusion Strategy On Bleeding in Patients with Hematological Malignancies and Severe Thrombocytopenia (TOPPS trial). A Randomized Controlled, Non-Inferiority Trial. *Blood,* 120**,** 1-1.

STANWORTH, S. J., ESTCOURT, L. J., POWTER, G., KAHAN, B. C., DYER, C., CHOO, L., BAKRANIA, L., LLEWELYN, C., LITTLEWOOD, T., SOUTAR, R., NORFOLK, D., COPPLESTONE, A., SMITH, N., KERR, P., JONES, G., RAJ, K., WESTERMAN, D. A., SZER, J., JACKSON, N., BARDY, P. G., PLEWS, D., LYONS, S., BIELBY, L., WOOD, E. M. & MURPHY, M. F. 2013. A no-prophylaxis platelet-transfusion strategy for hematologic cancers. *N Engl J Med,* 368**,** 1771-80.

STRANDENES, G., SIVERTSEN, J., KRISTOFFERSEN, E., BJERKVIG, C., FOSSE T.K., CAP A.P., DEL JUNCO D.J., KRISTOFFERSEN, E.K. , HAAVERSTAD, R., KVALHEIM, V., BRAATHEN, H., LUNDE, T. H., HERVIG, T., HUFTHAMMER, K.O., SPINELLA, P.C. & APELSETH, T. 2020. A Pilot Trial of Platelets Stored Cold versus at Room Temperature for Complex Cardiothoracic Surgery. *Anesthesiology,*133(6):1173-1183.

STUBBS, J. R., TRAN, S. A., EMERY, R. L., HAMMEL, S. A., HAUGEN, A. L., ZIELINSKI, M. D., ZIETLOW, S. P. & JENKINS, D. 2017. Cold platelets for trauma-associated bleeding: regulatory approval, accreditation approval, and practice implementation-just the "tip of the iceberg". *Transfusion,* 57**,** 2836-2844.

VALERI, C. R. 1974a. Factors influencing the 24-hour posttransfusion survival and the oxygen transport function of previously frozen red cells preserved with 40 per cent W-V glycerol and frozen at -80 C. *Transfusion,* 14**,** 1-15.

VALERI, C. R. 1974b. Hemostatic effectiveness of liquid-preserved and previously frozen human platelets. *N Engl J Med,* 290**,** 353-8.

VOLLMER, T., ENGEMANN, J., KLEESIEK, K. & DREIER, J. 2011. Bacterial screening by flow cytometry offers potential for extension of platelet storage: results of 14 months of active surveillance. *Transfus Med,* 21**,** 175-82.

VOSTAL, J. G., GELDERMAN, M. P., SKRIPCHENKO, A., XU, F., LI, Y., RYAN, J., CHENG, C., WHITLEY, P., WELLINGTON, M., SAWYER, S., HANLEY, S. & WAGNER, S. J. 2018. Temperature cycling during platelet cold storage improves in vivo recovery and survival in healthy volunteers. *Transfusion,* 58**,** 25-33.

VOSTAL, J. G. & MONDORO, T. H. 1997. Liquid cold storage of platelets: a revitalized possible alternative for limiting bacterial contamination of platelet products. *Transfus Med Rev,* 11**,** 286-95.

WANDALL, H. H., HOFFMEISTER, K. M., SORENSEN, A. L., RUMJANTSEVA, V., CLAUSEN, H., HARTWIG, J. H. & SLICHTER, S. J. 2008. Galactosylation does not prevent the rapid clearance of long-term, 4 degrees C-stored platelets. *Blood,* 111**,** 3249-56.

WANDT, H., SCHAEFER-ECKART, K., WENDELIN, K., PILZ, B., WILHELM, M., THALHEIMER, M., MAHLKNECHT, U., HO, A., SCHAICH, M., KRAMER, M., KAUFMANN, M., LEIMER, L., SCHWERDTFEGER, R., CONRADI, R., DÖLKEN, G., KLENNER, A., HÄNEL, M., HERBST, R., JUNGHANSS, C. & EHNINGER, G. 2012. Therapeutic platelet transfusion versus routine prophylactic transfusion in patients with haematological malignancies: an open-label, multicentre, randomised study. *Lancet,* 380**,** 1309-16.

WATERS, L., CAMERON, M., PADULA, M. P., MARKS, D. C. & JOHNSON, L. 2018. Refrigeration, cryopreservation and pathogen inactivation: an updated perspective on platelet storage conditions. *Vox Sang,* 113**,** 317-328.

WHITE, J. G. & KRIVIT, W. 1967. An ultrastructural basis for the shape changes induced in platelets by chilling. *Blood,* 30**,** 625-35.

WHITE, N. J., WARD, K. R., PATI, S., STRANDENES, G. & CAP, A. P. 2017. Hemorrhagic blood failure: Oxygen debt, coagulopathy, and endothelial damage. *J Trauma Acute Care Surg,* 82**,** S41-S49.

WINOKUR, R. & HARTWIG, J. 1995. Mechanism of shape change in chilled human platelets. *Blood,* 85**,** 1796-1804.

WOOD, B., PADULA, M. P., MARKS, D. C. & JOHNSON, L. 2016. Refrigerated storage of platelets initiates changes in platelet surface marker expression and localization of intracellular proteins. *Transfusion,* 56**,** 2548-2559.

ZHAO, L., LIU, J., HE, C., YAN, R., ZHOU, K., CUI, Q., MENG, X., LI, X., ZHANG, Y., NIE, Y., ZHANG, Y., HU, R., LIU, Y., ZHAO, L., CHEN, M., XIAO, W., TIAN, J., ZHAO, Y., CAO, L., ZHOU, L., LIN, A., RUAN, C. & DAI, K. 2017. Protein kinase A determines platelet life span and survival by regulating apoptosis. *J Clin Invest,* 127**,** 4338-4351.

ZUCKER, M. B. & BORRELLI, J. 1954. Reversible alterations in platelet morphology produced by anticoagulants and by cold. *Blood,* 9**,** 602-8.

7 Statement on own contribution

7.1 Thesis manuscript

Prof. Dr. Tamam Bakchoul had the presented idea. Tamam Bakchoul and Dr. Irene Marini developed the theoretical formalism and directed the project; I, Yoko Tamamushi designed the study and planned the experiments. The survival and apoptotic events of APC, which is not included in this manuscript, was investigated by Chiara Maettler at the same time. I performed the experiments, then analyzed and discussed the results. I wrote the manuscript.

7.2 DRK Hämotherapie

I, Yoko Tamamushi performed the literature search and Dr. Karina Althaus, Dr. Irene Marini and I wrote the manuscript in consultation with Prof. Dr. Tamam Bakchoul.

7.3 Conflict of interest

There is no conflict of interest to declare.

8 Publication

MARINI, I., TAMAMUSHI, Y. & ALTHAUS K. 2019. Kaltlagerung von Thrombozyten: Aktuelle Herausforderungen und zukünftige Perspektiven. *DRK Hämotherapie*, 33, 4-11.

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