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Establishing a reproducible protocol for thrombogenicity testing of left ventricular assist devices based on bovine blood

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Sema Alzubaidi, BSc.

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Abbreviations

1°	Primary Antibody
2°	Secondary Antibody
AB	Antibody
ACT	Activated Clotting Time
ADP	Adenine Diphosphate
AF 488	Alexa Fluor 488
AF 635	Alexa Fluor 635
AP	Activated Platelets
ATP	Adenosine Triphosphate
AxV	Annexin V
B	Bovine Blood
BTC	Bridge to Candidacy
BTT	Bridge to Transplantation
Ca^{2+}	Calcium Ion
CBM	Cardiopulmonary Bypass Machine
CD	Cluster of Differentiation
CO	Carbon Monoxide
DT	Destination Therapy
ESC	European Society of Cardiology
FACS	Fluorescence Activated Cell Scanning
FSC	Forward Scatter
FVD	Fixable Viability Dye
GP	Glycoprotein
H	Human Blood
HCT	Hematocrit
HM II	HeartMate II
HM III	HeartMate III
HMWK	High Molecular Weight Kininogen
Ig	Immunoglobulin
LMWH	Low Molecular Weight Heparin
LVAD	Left Ventricular Assist Device
LVEF	Left Ventricular Ejection Fraction

MCS	Mechanical Circulatory Support
MP	Microparticles
NYHA	New York Heart Association
P	Platelet Rich Plasma
PFA	Paraformaldehyde
PMA	Phorbol 12-Mysitate 13-Acetate
PRP	Platelet Rich Plasma
ROTEM	Rotational Thromboelastometry
RT	Room Temperature
SSC	Sideward Scatter
TF	Tissue Factor
Ti	Titanium
TxA_2	Thromboxane A_2
VAD	Ventricular Assist Device
vWF	von Willebrand Factor

Table of Contents

1 Abstract

Background: Left ventricular assist devices (LVADs) serve as a valued therapy in patients with advanced heart failure. However, adverse events during LVAD-support limit the success of this therapy and are often linked to device-related thrombogenic complications.

Objective: This study aimed to propose a reproducible in-vitro protocol using bovine blood in a loop setup for the quantification of thrombogenicity in the the HeartMate III (HM III).

Methods: A literature research was conducted to identify proposed methods for the detection of thrombus formation with bovine blood in LVADs. Based on this search, the blood's activated clotting time (ACT) was measured hourly with the HM III operated in pulse- and fixed-modes for 6 hours. Furthermore, a flow cytometric analysis was performed with various staining strategies (CAPP2A, CD61, CD41, CD62-P, Annexin, Apotracker) to detect activated platelets. Samples were stimulated with either Phorbol Myristate Acetate (PMA) or Adenosine Diphosphate (ADP) for positive control.

Results: 3 loop experiments were conducted. A general decrease in ACT values was observed over time. Lower ACT values were observed in the samples of the HM III loops in comparison to the static control. The difference of detected activated platelets between stimulated and unstimulated samples using CAPP2A, CD61, CD41 and CD62P was below 5%. Annexin and Apotracker samples, by contrast, showed a difference of 22% and -26%, respectively.

Discussion: The flow cytometric analysis revealed the complexity of detecting platelets. The combination of platelet rich plasma and staining with Annexin showed the most promising results in the detection of activated platelets.

2 Zusammenfassung

Hintergrund: Linksventrikuläre Unterstützungssysteme (LVADs) haben sich bei Patienten mit fortgeschrittener Herzinsuffizienz als effiziente Therapie erwiesen. Unerwünschte Ereignisse, während der LVAD-Unterstützung schränken den Erfolg dieser Therapie jedoch nach wie vor ein und sind häufig mit gerätebedingten thrombogenen Komplikationen verbunden.

Ziel: Ziel dieser Studie war es, ein reproduzierbares in-vitro-Protokoll unter Verwendung von Rinderblut in einem Kreislauf, zur Quantifizierung der Thrombogenität im Heart Mate III (HM III) – zu etablieren.

Methoden: Eine systematische Suche ergab, verschiedene Methoden um die Thrombenbildung von Rinderblut in LVADs nachzuweisen. Auf Basis dieser Suche wurde die aktivierte Gerinnungszeit (ACT) des Blutes stündlich gemessen, wobei das HM III 6 Stunden lang im Puls- und Festmodus betrieben wurde. Darüber hinaus wurde eine durchflusszytometrische Analyse mit verschiedenen Färbestrategien (CAPP2A, CD61, CD41, CD62-P, Annexin, Apotracker) zum Nachweis aktivierter Thrombozyten durchgeführt. Die Proben wurden entweder mit Phorbol-Myristat-Acetat (PMA) oder Adenosindiphosphat (ADP) als Positivkontrolle stimuliert.

Resultate: Es wurden insgesamt 3 Schleifenversuche durchgeführt. Ein allgemeiner Rückgang der ACT-Werte der Kreisläufe im Laufe der Zeit, war zu beobachten. Bei den aus den HM III-Schleifen extrahierten Proben wurden im Vergleich zu den statischen Kontrollen generell niedrigere ACT-Werte festgestellt. Der prozentuale Unterschied der nachgewiesenen aktivierten Thrombozyten zwischen stimulierten und unstimulierten Proben unter Verwendung von CAPP2A, CD61, CD41 und CD62P lag unter 5 %. Annexin- und Apotracker- Proben zeigten einen Unterschied von 22 % bzw. -26 %.

Schlussfolgerung: Die durchflusszytometrische Analyse zeigte die Komplexität des Nachweises von Blutplättchen. Die Kombination aus thrombozytenreichem Plasma und Färbung mit Annexin zeigte die vielversprechendsten Ergebnisse beim Nachweis aktivierter Thrombozyten.

3 Introduction

3.1 Heart Failure

Heart failure is a chronic syndrome with a global prevalence of 26 million[1], and denotes a widespread disease in industrialized countries. Among others, risk factors include a high burden of obesity, hyperlipidemia and metabolic syndrome.[2] Heart failure is mainly characterized by structural or functional impairments of ventricular ejection and filling.[2] Systolic or diastolic ventricular dysfunction are caused by abnormalities in cardiac valves, pericardium or endocardium.[3]

Consequently, the blood flow is insufficient to adequately deliver oxygen to peripheral organs.[4] Due to a decrease in cardiac output, the sympathetic nervous system as well as the renin angiotensin-aldosterone system are activated and, to ensure tissue perfusion, the blood pressure is increased. Furthermore, an increased blood volume may further promote an increase in preload to restore cardiac output.[5] This is known as the Frank-Starling mechanism. This compensation mechanism typically leads to further deterioration of the myocardial contractility.[5]

Cardinal symptoms for characterizing heart failure include dyspnea, ankle swelling, and pulmonary rales.[3,5] The New York Heart Association (NYHA) classifies heart failure into four classes. The four classes categorize the patient's symptoms based on their limitations during physical activity, normal breathing, and the presence of angina pain (Table 1).[6]

NYHA class	Symptoms
I	Structural myocardial changes (e.g., left ventricular hypertrophy) No limitations such as breathlessness, fatigue, or palpitations with physical activity
II	Small decrease in exercise tolerance – Comfortable at rest, but physical activity results in undue breathlessness, fatigue, or palpitations
III	Significant decrease in exercise tolerance – Comfortable at rest, but less than ordinary physical activity results in undue breathlessness, fatigue, or palpitations
IV	Symptoms of heart failure in rest or during small exercise – Unable to carry on any physical activity without discomfort.

Table 1. NYHA (New York Heart Association) classification of heart failure.[7,8]

The European Society of Cardiology (ESC) Guidelines for diagnosis and treatment of heart failure suggest the use of angiotensin converting enzyme inhibitors, beta blockers, loop diuretics and their combinations for the treatment of different stages of heart failure. Despite maximal medication therapy suggested by various guidelines, many patients transition into a state of advanced heart failure with persistent symptoms. For patients that are severely symptomatic, cardiac transplantation denotes the preferred treatment option. However, according to the ESC guidelines, donor organ shortages remain to be the main limitation to cardiac transplantation. Accordingly, LVADs emerged as a valuable alternative to biologic cardiac replacement.

3.2 Ventricular Assist Devices

Ventricular assist devices (VADs) serve as a bridge to candidacy, bridge to transplantation, destination therapy or even bridge to recovery (Table 2).[8]

Term	Indication
Bridge to candidacy (BTC)	Use of Mechanical Circulatory Support (MCS) (usually LVAD) to improve end-organ function and/or to make an ineligible patient eligible for heart transplantation.
Bridge to transplantation (BTT)	Use of MCS (LVAD) to keep a patient alive who is otherwise at high risk of death before transplantation until a donor organ becomes available.
Destination therapy (DT)	Long-term use of MCS (LVAD) as an alternative to transplantation in patients with end-stage HF ineligible for transplantation.

Table 2. European Society of Cardiology; Terms describing the indications for implantation of mechanical circulatory devices (MCS)[8] LVAD; Left ventricular assist device.

Parameters that contribute to the eligibility of the implantation of an LVAD are described in the ESC Guidelines. These include a left ventricular ejection fraction (LVEF) < 25%, 3 or more heart failure hospitalizations without a precipitating cause, dependance on intravenous inotropic therapy or temporary MCS, and progressive end organ dysfunction.[8]

The main goal of a LVAD is to unload a failing left ventricle, ensuring continuous blood flow and maintaining end organ function.[12] Ideally, LVADs should be durable, reliable to provide adequate flow and have no external power source to ensure mobility. Hemocompatibility and

histocompatibility are additionally of pivotal importance to avoid thrombotic processes and prevent an immunological response, respectively.[13]

3.2.1 First Generation Ventricular Assist Devices

The concept of a VAD was first proposed in the year 1953 after Dr. John Gibbon's cardiopulmonary bypass machine (CBM) was able to sustain sufficient blood flow to a patient during open heart surgery.[9] The CBM encompassed an oxygenation component responsible for saturating the blood oxygen content, a temperature regulator, and roller pumps. The idea of a durable left ventricular assist device came to place when the majority of heart failure patients that were weaned from the CBM passed away.[9]

The HeartMate HM-VE (vented electric) and the XVE (extended vented electric) both denote first generation LVADs. Both devices are pulsatile blood pumps made of titanium with a polyurethane diaphragm, ensuring hemocompatibility.[14] In addition, modifications to blood contacting surfaces (e.g. sintered titanium layers) promote adhesion of circulating cells, thus establishing a barrier against infection.[9,14] The first generation LVADs served as a successful first step towards bridge to cardiac transplantation.

3.2.2 Second Generation Ventricular Assist Devices

Among others, the HeartMate II (HM II) belongs to the second generation of LVADs (Figure 1A, left). In comparison to its predecessor, it is a continuous flow rotodynamic blood pump with axial displacement of the blood by a rotor (Figure 1A, right). The rotor is the only moving piece in the HM II ensuring enhanced durability in comparison with first generation devices.[9] Furthermore, these devices avoid the necessity of mechanical valves, thus reducing the potential for platelet activation and thrombus formation.

A randomized trial was conducted in 2005 comparing the HM II to a first generation LVAD (HM XVE). Over four times as many patients with the HM II had hit the two-year survival mark in comparison to the patients with the HM XVE. Generally, most outcomes were in favor of the HM II. However, the number of adverse events due to hematologic complications remained high. This is mainly attributed to the thrombogenic and hemolytic risk potential associated with the direct contact of blood to foreign surfaces and exposure to non-physiologically high shear stresses.

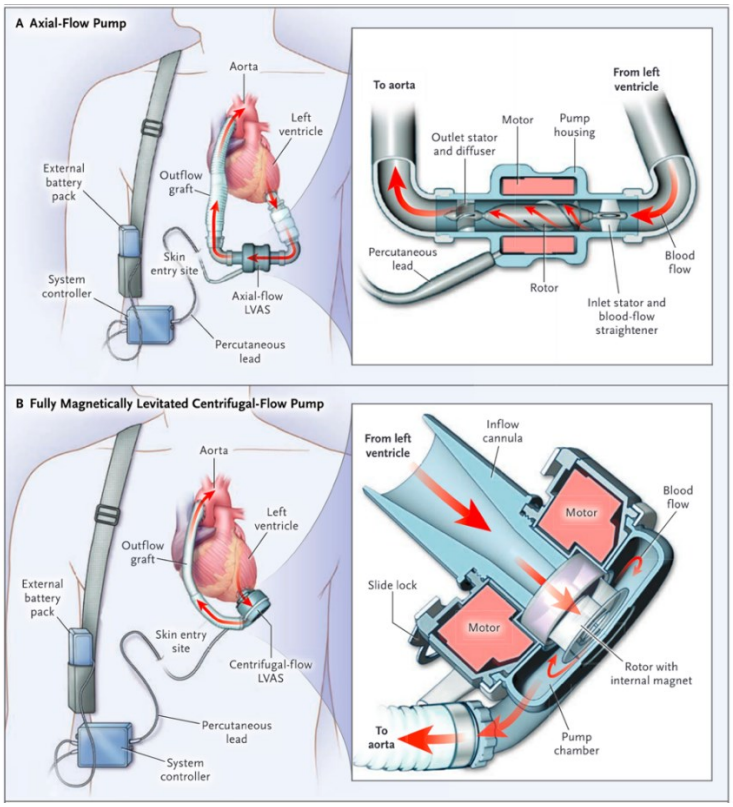


Figure 1. Diagram of a second generation LVAD (A) vs. third generation LVAD (B).[15]

3.2.3 Third Generation Ventricular Assist Devices

Third generation LVADs such as the HeartMate III (HM III) and Heartware HVAD are radial-type pumps that incorporate a magnetically and hydrodynamically levitated rotor, respectively. The HM III (Figure 1B, left) is characterized by lower shear stresses in comparison to its predecessors due to the wide secondary blood flow gaps[16] (Figure 1B, right). The inner and outer surface was designed with a textured surface to permit the establishment of a tissue interface with blood.[16] Another distinguishing characteristic of the HM III is the incorporation of an artificial pulse by altering the rotational speed every two seconds. This constant speed change promotes washout of the pump and addresses the issue of thrombogenesis inside LVADs by reducing events of blood stasis. Figure 2 below shows how the speed is altered every two seconds. The set rotational speed is adjusted by a physician and may range between 4800 – 6000 rpm.[9] The speed increases by 2000 rpm for 0.2 seconds, decreases to the original set speed for 1.65 seconds, decreases it further by 2000 rpm for 0,15s and then repeats the cycle.

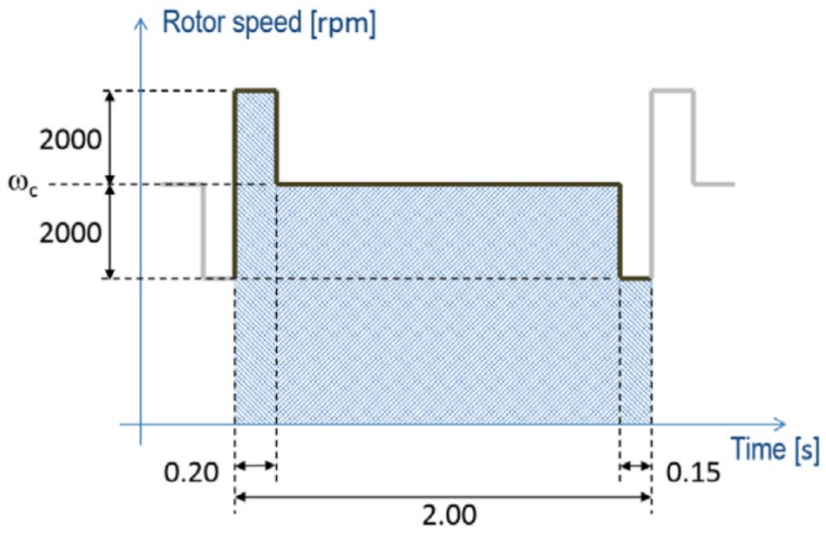


Figure 2. Change in rotor speed of HM III after starting at set point "w_c".[18]

3.3 Adverse Events associated with LVADs

In 2001, first generation LVADs were assessed, within the Randomized Evaluation of Mechanical Assistance for the Treatment of Congestive Heart Failure (RE-MATCH) trial. 129 patients diagnosed with NYHA class IV heart failure were randomly evaluated.[9] One group was randomly assigned optimal medical treatment, the other was assigned optimal medical treatment and a LVAD device. The trial showed a 48% mortality risk reduction for the LVAD-assigned groups.[9] Nonetheless, a number of adverse events have been reported in relation to the LVADs. In addition to the large device size and power inefficiency, the first generation LVADs caused thrombogenesis, hemolysis and infections. Bleeding and neurologic dysfunction such as stroke and transient ischemic attacks were more common in LVAD patients.[9] Also, infections occurred in 28% of LVAD patients. Thus, the development of second generation LVADs was implemented in hopes of therapy improvement and reduction of adverse events.

In the MOMENTUM 3 clinical trial that began in 2014 the HM II was compared to the HM III. The inspected endpoints were suffering of a stroke, pump exchange and pump removal after 6 months. The HM III generally performed better than the HM II with regards to patients surviving without exchange or removal of pump.[9] In addition, the HM III displayed great improvement in the incidences of stroke: stroke rates after 2 years of implantation were 19,4% and 9,9% for HM II and III, respectively. The leading cause of death, however, remains to be heart failure, stroke, and infection.

Among others, the research on the formation of thrombi inside pumps is crucial to reduce the incidences of adverse events. To this end, the extensive understanding towards the pathways of hemostasis and thrombus formation is essential in the development and assessment of LVADs.

3.4 Device-Related Thrombogenicity

3.4.1 Concept of Hemostasis

Hemostasis is a physiological, vital mechanism required to ensure fluidity in blood vessels and prevent excessive bleeding after injuries. It combines several pathways for the formation of the solid thrombus endpoint. Hemostasis is especially initiated by contact with foreign surfaces, high shear stress, and aberrant blood flow (Figure 6).

3.4.1.1 Primary Hemostasis

Platelets

Primary hemostasis is the initial response of the body to vascular injury.[27] It can be divided into three distinct stages: adhesion, activation, and aggregation. Despite the fact that platelets are of the smallest blood component [28], they partake a pivotal role in the primary hemostasis process. Platelet are small anucleate cells with a discoid shape and are the second most abundant circulating blood cells.[21]

Platelets comprehend 3 different types of granules: alpha granules, dense granules, and lysozymes. All 3 granules facilitate the storage of various molecules.[21] Alpha Granules are of the most abundant granules that carry various proteins responsible for modulating the coagulation cascade. Examples of these proteins are FVIII, FIX, FX, FXIII and fibrinogen. Further proteins such as adhesion- and membrane proteins can also be found in alpha granules. For instance, P-Selectin and the von Willebrand Factor (vWF) are essential adhesion proteins for platelet aggregation. Smaller molecules, such as Adenosine Diphosphate (ADP), Adenosine Triphosphate (ATP), Ca^{2+} and polyphosphates are stored in the so called “dense granules”. Lysozymes are also typical granules found in platelets. Lysozymes contain metabolic enzymes. Upon the activation of platelets, content inside all granules is released. In this degranulation process, several granule proteins are released acting as endocrine mediators. In addition to that, membrane proteins are presented at the surface, mediating platelet binding to other platelets or other cells such as leukocytes.[21] The activation process also causes the platelets to undergo a conformational change. The typical discoid shape of resting platelets is converted into a so-

called pseudopodium. By increasing the surface area, this conformational change facilitates the formation of aggregates and shrouds vascular injuries more effectively. In addition, platelets undergo several antigenic changes, in which the expression of various receptors is altered.[29] Table 3 entails a representation of the numerous receptors of the glycoprotein-class found on the surface of platelets, as well as their functions. Other classes, not represented in Table 3 are thromboxane, prostaglandin, thrombin, p-selectin and many more.[28]

Type of Receptor	Family	Ligand	Function
GPIb-IX-V complex	Type I membrane spanning GP	vWF, thrombin, P-selectin	Initiation of platelet recruitment
GPVI	Immunoglobulin (Ig)	Collagen and laminin	Platelet aggregation
GPVI-FcRγ	Transmembrane	Collagen and laminin	Adhesion with collagen
Integrins (αIIbβ3, αvβ3, α2β1, α5β1, and α6β1)	Transmembrane	Fibrinogen or vWF	Platelet aggregations

Table 3. Glycoprotein Receptors found on Platelets.[28] GP; Glycoprotein, vWF; von Willebrand Factor, Ig; Immunoglobulin.

Platelet Adhesion

Upon injury, the subendothelial vascular matrix proteins are exposed to blood flow.[21] Such proteins include: von Willebrand Factor (vWF), collagens, and fibronectin.[30] vWF retains the function of recruiting platelets to the site of injury.[30] The globular form of vWF is freely present in the plasma. Upon vascular injury, immobilization to subendothelial collagen causes the molecule to adopt an unfolded conformation due to high drag forces. [30] As seen in Figure 3 below, unfolded conformation of vWF expresses a higher affinity the GPIb-V-IX complex on platelets than the initial globular conformation.

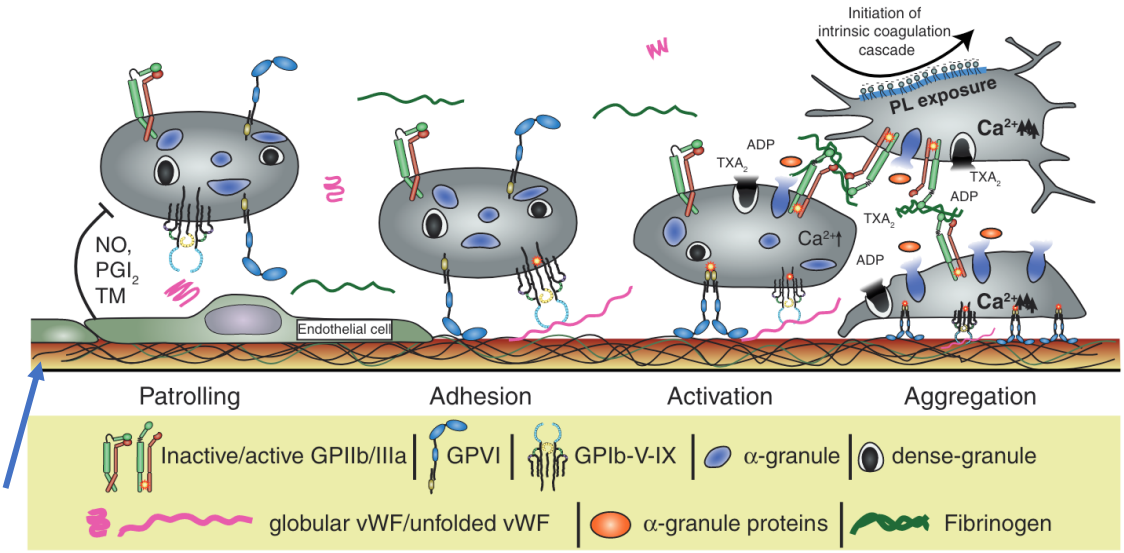


Figure 3. Phases of platelet function in primary hemostasis.[21] Adhesion; Unfolded vWF expresses greater affinity to GPIba than the globular form. Blue arrow shows the subendothelial collagen that binds to GPV1 facilitating patrolling of platelets.

This transient preliminary platelet adhesion serves to induce "platelet patrolling" and reduce the velocity to permit collagen binding via the collagen receptor, GPVI. (Figure 3)

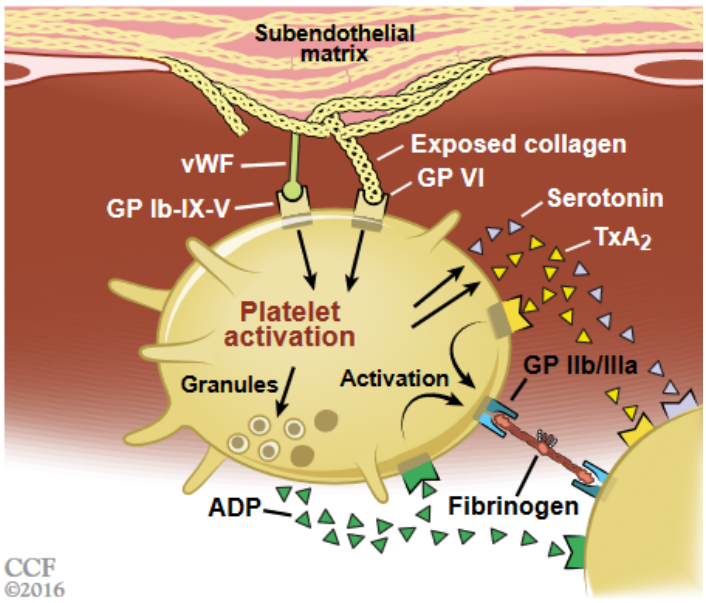


Figure 4. Subendothelial collagen binding to GPV1(adhesion), and von Willebrand Factor (vWF) binding to GPIb-IX-V receptor, inducing platelet activation. Contents inside granules are released and fibrinogen induces crosslinking of platelets (aggregation).vWF;von Willebrand Factor. [31]

Platelet Activation

As mentioned previously, activated platelets undergo a conformational transformation to increase surface area and ensure shrouding of the injured site. Furthermore, the binding interaction between collagen and GPVI induces collagen-mediated activation of platelets. This stimulates an increase of intracellular calcium concentration. Due to the increase in intracellular Ca^{2+}, a series of signal cascades, such as degranulation of the alpha and dense granules and the activation of integrins occur.[21] In addition to the formation of a pseudopodium, the platelet phospholipid membrane is also altered.[21] A membrane "flip-flop" phenomenon (Figure 5) occurs in which the negatively charged phospholipids are exposed on the surface of platelets.[21] A notable example for a negatively charged phospholipid presented on the surface after activation is phosphatidylserine, a procoagulant phospholipid.[32,33] Phosphatidylserine expressed on the surface of platelets facilitates the binding of coagulation factors Va and Xa.[34] This stimulates the formation of the prothrombinase complex, usually formed in the extrinsic and intrinsic pathway of secondary hemostasis, and in turn promotes the formation of thrombin. (See Figure 29 in section 9.1).

Moreover, the GPa2b3 integrin (also known the GPIIbIIIa receptor) is activated and has a relatively high ligand affinity to molecules such as fibrinogen and fibronectin (Figure 3) in comparison to its native state with low ligand affinity for these molecules.[21] GPIIbIIIa also interacts with cytoskeletal proteins.[35] This process is called "inside-out signaling". The Ca^{2+} mediated inside-out activation also promotes the binding of vWF to GPIIbIIIa receptor.[28] Furthermore, fibrinogen is constantly present in circulating blood but is also one of the various components released from a-granules. The binding of fibrinogen to the GPIIbIIIa receptor initiates signals sent back to the platelets, increasing platelet degranulation. This process, on the other hand, is called "outside-in" signaling.[21] Thus, GPIIbIIIa receptor is the most frequent surface expressed integrin on platelets and retains various targets.[28]

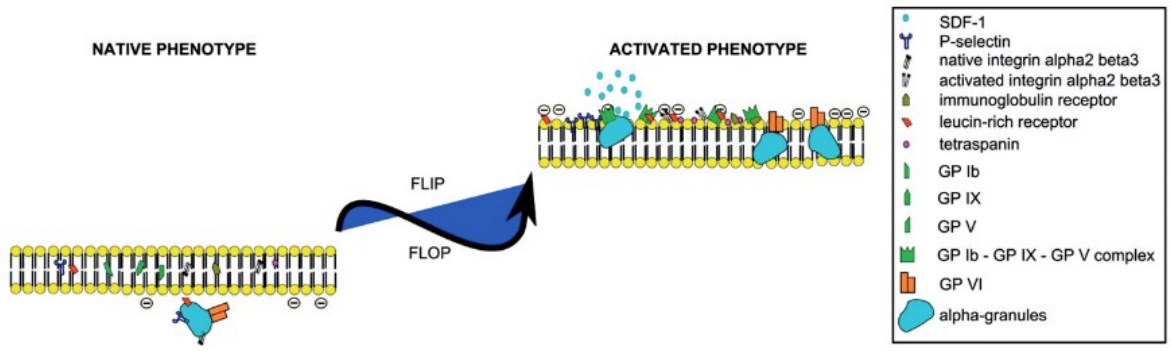


Figure 5. Membrane flip-flop phenomenon as platelets are stimulated and become activated negatively charged proteins such as p-selectin are displayed on the surface of platelets. [33]

In addition, persistent platelet activation is also ensured by secondary feedback from adenosine diphosphate (ADP), released from dense granules, and thromboxane 2 (TXA_2) synthesized by membrane phospholipids.[21] This secondary feedback triggers amplified inside-out signaling. Common antiplatelet therapies such as aspirin and clopidogrel target ADP receptor binding, and TXA_2 synthesis, respectively.

Platelet Aggregation

The replenished monolayer of accumulated activated platelets formed is not stable and requires cross linking of fibrinogen to form a stable white thrombus or platelet plug.[28] Fibrinogen, bound to GPIIbIIIa receptors is capable of cross linking adjacent activated platelets. Antiplatelet GPIIbIIIa inhibitors target the stabilization of white thrombus via inhibition of fibrinogen binding.[21] (Figure 4)

3.4.1.2 Secondary Hemostasis

To prevent hemorrhage, primary hemostasis is followed by secondary hemostasis in which fibrin acts to secure the clot.[36] The coagulation cascade encompasses coagulation factors that are zymogens synthesized by the liver.[21] Zymogens are inactive precursors of enzymes that are activated in step by step protease mediated reactions. [21] The endpoint of secondary hemostasis, which is the formation of fibrin, is usually initiated by two distinct pathways, the extrinsic and intrinsic pathway. A detailed description of both pathways can be found in the Appendix (9.1).

3.4.2 Virchows Triad

The general scheme of pump thrombogenesis is highly similar to the physiological response of endothelial injury.[20] The Virchow's triad describes the three contributing factors for physiological thrombus formation: Venous stasis, vascular injury and hypercoagulability.[21] Analogically, de biasi et al.[22] have defined three major causes for thrombogenesis in blood pumps corresponding to Virchow's Triad (Figure 6): Bioreactive materials, aberrant flow and platelet activation analogously represent the three pillars of Virchow's Triad's tenets, which are described in the following.

Bioreactive Material

The first pillar of pump thrombogenesis is initiated by the foreign material of the LVAD such as e.g. titanium, a biocompatible metal frequently used in blood pumps.[22] Similar to the initial responses upon endothelial injury in which platelets attach to subendothelial collagen, platelet adhesion is initiated in LVADs by the foreign material. Furthermore, titanium-adsorbed proteins such as fibrinogen further mediate platelet activation.[23] Textured surfaces, such as the sintered titanium microspheres utilized in the HM III lineage, have proven beneficial in forming an endothelial lining within the device.[9] The sintered surfaces provides thromboresistance by promoting endothelialization.[9]

Platelet activation

Platelet activation is the second pillar of pump thrombogenesis. Excessive shear stresses cause activation of platelets and eventually the formation of thrombi. Several studies have suggested thresholds for shear stresses at which the activation of platelets is induced if exceeded.[9] Chan et al.[26] suggested that the shear threshold for platelet activation is 50 Pa at 600 s and 12 Pa at 900 s of exposure time. Thus, it is of utmost importance to minimize the magnitude as well the duration of shear stress inflicted on blood. Platelet activation is also facilitated by indirect activation of the coagulation cascade, induced by biomaterials such as titanium (TI).[23] Corresponding to Virchow's proposition, thrombogenesis also occurs in hypercoagulable environments, which are promoted by activated platelets. The presence of circulating activated platelets has been suggested as a marker for increased risk of thrombotic complications.[24]

Aberrant Flow

The third pillar of pump thrombogenesis is aberrant flow. De biasi et al [20] suggested that aberrant flow, analogous to Virchow's third tenet of stasis, contributes towards pump thrombogenesis. When referring to aberrant flow, both excessive shear stresses and blood stasis are meant. Thrombus formation at high shear stresses is not mediated by the coagulation cascade but rather by platelet activation. A high-shear thrombus forms rapidly and is distinct from the slow formation of a red thrombus that occurs in stagnant blood.[25]

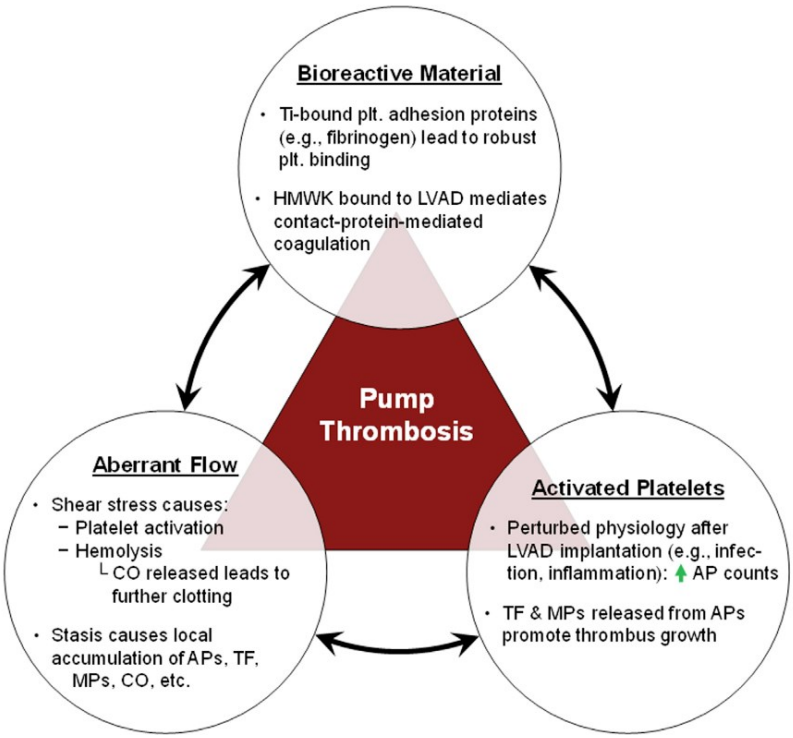


Figure 6. Virchow's triad of endothelial injury, hypercoagulability, and stasis serves as the conceptual framework for understanding the 3 fundamental determinants of pump thrombogenesis. LVAD: Left ventricular Assist Devices; Ti: Titanium; HMWK: High molecular weight kininogen; CO: Carbon monoxide; AP: Activated Platelets; TF: Tissue Factor; MP: Microparticles.[22]

3.5 *Aim of Study*

Currently suggested methods to quantitively asses the thrombogenic potential in LVADs have not been thoroughly established.[37] General challenges in thrombogenicity testing include the definition of 1) an appropriate anticoagulation method, 2) a reasonable stop criterion, and 3) a framework for quantitative evaluation.[37] Furthermore, testing of thrombus formation in LVADs may require large volumes of fresh blood which are challenging to obtain. This study aimed to exploit existing methods proposed in literature in order to define a reproducible protocol and establish a setup for the quantification of thrombus formation in the HM III using bovine blood from a slaughterhouse. The methods used aim to analyze the platelet activation potential of bovine blood.

4 Materials and Methods

4.1 Systematic Literature Search

To assess the available methods for the determination and quantification of thrombus formation in LVADs in-vitro and to inform the conception of our experimental design a systematic literature research was conducted. The systematic search was performed on the PubMed and Scopus electronic database with the keywords thrombus, in vitro and pump. A total of 333 papers were found on PubMed and Scopus. The papers were initially screened by title and abstract. The major inclusion factors were:

- an in-vitro experiment
- a blood-filled test loop
- the usage of a blood pump
- proposed methodology for thrombus detection

309 papers were excluded based on title, abstract and duplication. The remainder of 24 papers were fully screened. Out of those 24 studies, 9 were classified as relevant for our considerations since they described methods that were compatible with quantifying thrombi in bovine blood (Chapter 4.2.2). Table 4 below entails a representation of the summarized detection methods extracted from these 9 studies.

Title of Study	Authors	Year	Thrombus Detection Method
In vitro thrombogenicity testing of pulsatile mechanical circulatory support systems: Design and proof-of-concept [37]	Brockhaus et. al.	2021	Rotational Thromboelastometry [ROTEM]
The CentriMag centrifugal blood pump as a benchmark for in vitro testing of hemocompatibility in implantable ventricular assist devices [38]	Chan et al.	2015	Flow Cytometry, Antibody Labeling of Activated Platelets
Ex Vivo Assessment of Different Oral Anticoagulant Regimens on Pump Thrombosis in a HeartWare Ventricular Assist Device [39]	Hayward et al.	2021	LVAD Power Consumption
Is avoidance of air contact necessary for the in vitro evaluation of thrombogenicity in	Kim et al.	2000	Coagulation Factor VIII & XII, fibrinogen, thromboxane B2

mechanical circulatory assist devices? [40]			
In vitro thrombogenicity testing of artificial organs [41]	Paul et al.	1998	Thrombus size and location were documented photographically
In Vitro Benchmarking Study of Ventricular Assist Devices in Current Clinical Use [42]	Radley et al.	2020	Flow Cytometry, Antibody Labeling of Activated Platelets
Hemocompatibility of Axial Versus Centrifugal Pump Technology in Mechanical Circulatory Support Devices [43]	Schibilsky et al.	2015	ELISA Analysis of Coagulation Marker TAT Complex
In vitro investigation of thrombogenesis in rotary blood pumps [44]	Schima H.	1993	Thrombus size and location were documented photographically
In vitro thrombogenic evaluation of centrifugal pump [45]	Tayama E.	1997	Gravimetric analysis of vacuum dried thrombi

Table 4. Studies found in systematic literature search.

4.2 Experimental Design

The 9 protocols found in the literature search were assessed, and a selection thereof was combined in our experimental design.

4.2.1 Loop Setup

Similar to Brockhaus et al.[37] the setup of the test loops for this experiment originated from the ASTM F1841 standards for in-vitro hemolysis tests in continuous flow blood pumps.[46]

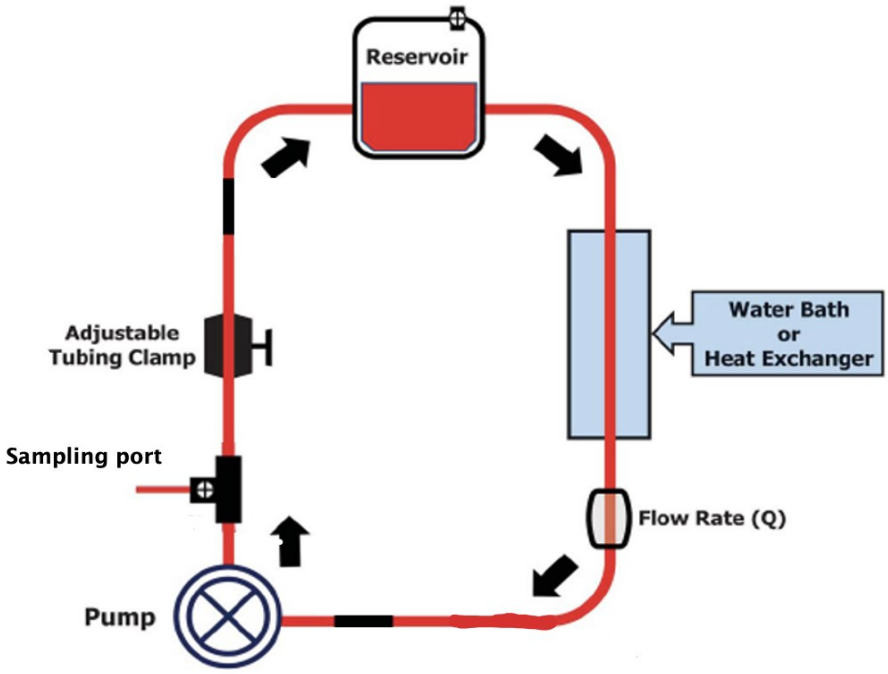


Figure 7. Test Loop Setup in accordance with ASTM F1841 Standard.[46]

A loop setup ensures continuous flow of blood, silicone material ensures biocompatibility, and a water bath set to 37°C aims to keep the blood in reservoirs at a physiologic, constant temperature. Further, the pressure and flow rate can be adjusted by an adjustable tubing clamp. For this purpose, the pump speed was set at 5600rpm and the resistance adjusted to a flow condition of 4,5L/min to replicate a typical clinical pump setting for full support. Further, a sampling port served to enable hourly extraction of blood samples. In our study, the position of the sampling port has been adjusted by placing it after the pump instead of before. This ensures that air is not sucked into the pump.

Three parallel test setups were run per experiment. One HM 3 device was connected to each of the first two loops: One served for testing the HM 3 in pulsatile mode (artificial pulse), the other in regular fixed function mode. Each of the two setups were filled with 500 mL filtered blood, similar to several studies that were found in the search. [37,38,39,42,43] The third setup served as the static control, in which a urine bag filled with the same blood (500 mL) was placed on a rocker. Blood extraction took place every hour from the static control simultaneous to the other two loops.

4.2.2 Blood Collection

In this experiment, bovine blood from a local slaughterhouse was used. Without addition of an anticoagulating solution, freshly drawn blood would clot within a few minutes of collection.[47] The blood (approx. 2L per experiment) was thus instantly anticoagulated with 2000 IU/L INHIXA (Techdow, *Guangdong, CHN*), a low molecular weight heparin LMWH suggested by Brockhaus et al.[37] at the collection site. Similar to Chan and Radley et al., 50 mg/mL gentamycin solution (1 ml/L) from Sigma (*Missouri, USA*) and 10 mL/L antimycotic solution from Sigma (*Missouri, USA*) was added to the whole blood. This served to prevent bacterial formation in overnight stored samples.

Upon arrival in the laboratory, the blood-filled tared canister was weighed, and the density of the blood at approximately 1050 kg/m^3 was used to calculate the approximate volume of blood collected from the slaughterhouse. If needed, additional INHIXA was added to reach an end concentration of 2000 IU/L. This ensured that the anticoagulation of all experiments always remained constant, irrespective of the amount of blood collected. This is beneficial in instances upon collection at the slaughterhouse in which the exact collected volume of blood in the canister cannot be controlled. Basic hematology assessment and filtration of blood to remove microthrombi was performed before filling of the loop setups for hourly measurements of activated clotting time (ACT).

4.3 Blood Analysis

In this study a total of 3 loop experiments were performed. In all loop experiments, baseline blood measurements were performed. After discarding the first 2 mL extracted from the sampling port, further 2 mL of blood were extracted and used for the hematology assessments. The pH, the hematocrit (HCT), and platelet count were determined using the Sysmex XN350 (*Kobe, Japan*). The pH was determined using a pH 1000 L device (*VWR, Pennsylvania, USA*). Blood cell counts as well as the hematocrit was determined using the Sysmex XN350.

4.3.1 Activated Clotting Time

The activated clotting time (ACT) is a test method widely used in clinics and in point of care to monitor anticoagulation effects in blood. The ACT assessment is a method that can be used to determine the thrombogenicity of bovine blood collected from the slaughterhouse. Several studies found in the systematic literature search (e.g. the studies from Paul et al[41] and Schima

et al[44]) used ACT measures to determine the thrombogenicity of the blood used in their studies. Here, ACT measurements were used to monitor the anticoagulation of blood before filling, and thereafter in all the loops. A total of 3 loop experiments were conducted with HM III pumps and static control, in which hourly blood samples were taken and the ACT was measured. To determine the ACT, special ACT test tubes (*Werfen HRFTK-ACT Test Tube, Barcelona, ESP*) were filled with 2 mL (2 cc) of blood. The clot formation of the whole blood is activated by the Kaolin present in the test tubes. Kaolin activates factor XII and thus the intrinsic pathway. The test tube is inserted into the test well of the ACT detector (*Hemochron Response, Acrivva, California, USA)*, and is rotated and incubated at 37°C by the Hemochron system. The time duration required to form a blood clot is measured. The principle of operation relies on the displacement of a magnet present inside the test tube. When a clot is formed, the displacement of the magnet in the test tube is precisely sensed by the two magnet detectors present in the test tube well. The elapsed time between the beginning of the test and the formation of a clot is displayed as the activated coagulation time in seconds.

4.3.2 Flow Cytometry

Chan et al.[38] and Radley et al.[42] proposed an assay to quantify activated platelets in bovine blood samples obtained from the slaughterhouse using flow cytometry. One of the major advantages of flow cytometry is that platelet activation can be quantified using antibodies. These antibodies label specific receptors on platelets in an activated state. Flow cytometry analyzes cells as they flow past lasers.[49] Visible light is used in various directions to sort cells by their size and granularity. The forward scatter (FSC) indicates the relative size of the cell. The sideward scatter (SSC) in which light scatter is measured at 90° gives indications of the granularity of the cell. Typical forward/sideward scatters as seen in Figure 8 show the upward shift of platelets in the dot plot upon activation and gain of size as aggregates form. [50]

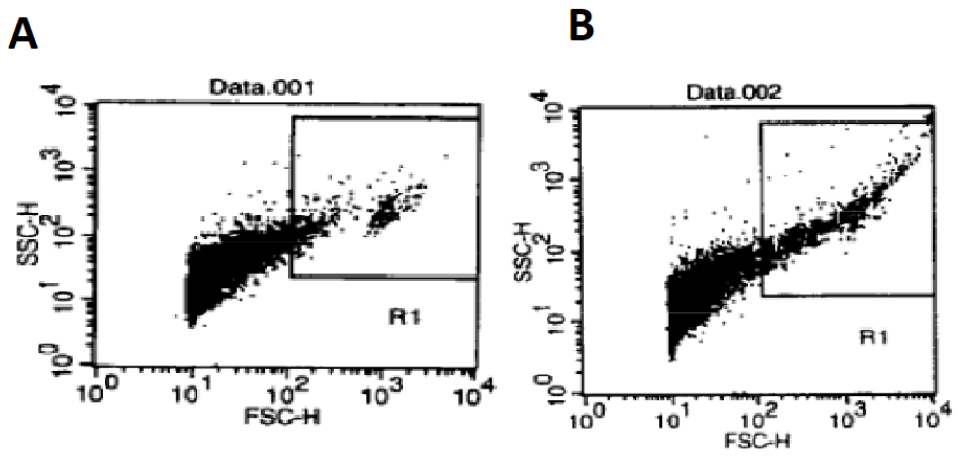


Figure 8. Typical dot plots of forward vs. sideward scatter light for (A) ovine resting platelets (B) ovine activated platelets stimulated with 1 unit of thrombin per mL.[50]

4.3.2.1 Platelet Activation Assay

The optimal staining procedure should be able to detect bovine activated platelets but should also be cost-effective. We iteratively tested various antibody Panels (various antibody combinations) and lysing/fixing protocols. The activated control samples were stimulated by either, phorbol myristate acetate (Sigma, *Missouri, USA*) or adenosine diphosphate. The various protocols were also tested with human blood, to verify the binding of antibodies. In this study, a total of 6 Panels were tested (Table 5).

Both Radley et al.[42] and Chan et al.[38] made use of antibody labeling and flow cytometric analysis to quantitatively determine the percent of platelets in an activated state in hourly taken blood samples. The initial protocol of our study was derived from the Chan/Radley procedure. In our study, the protocol for platelet activation was initially conducted with hourly extracted blood samples obtained from the loop experiments (Panel 1-3). Due to the complexity of the staining procedure, we pursued to test and refine our protocol with just baseline blood samples obtained from the loops (Panels 2 & 3). Afterwards, the focus of this study shifted towards finding the optimal staining protocol, so static experiments were performed without test loops and pumps (Panel 4-6). Accordingly, blood obtained from the slaughterhouse was directly used for the staining protocol in the last few Panels of this study (Panel 4-6). In all Panels with exception to Panel 6, the volume of one blood sample was 20 uL. After evaluating the results of Panel 5, the volume for one blood sample in Panel 6 was reduced to 10 uL. This was done to attempt to reduce the risk of unspecific binding, the number of events presented in the dot plots and the background noise.

Staining of Activated Platelets

In several Panels of this study, the CAPP2A (*Novus Biologicals, Colorado, USA*) bovine platelet specific antibody suggested by Radley and Chan et al [42,38] was used to determine the percent of platelets in an activated state. According to Radley et al.[42], CAPP2A binds to the GPIIbIIIa receptor of resting inactivated bovine platelets. This implies that the percentage of negative events of CAPP2A samples is equivalent to the percent of activated platelets. Other antibodies, such as human and murine p-selectin antibodies, CD62-P and CD61, CD41, Annexin and Apotracker were also tested to assess binding to bovine activated platelets.

	Blood Type	**Primary Antibody (dilution)**	**Secondary Antibody / Fluorophore (dilution)**	**Positive Activated Control**	**Lysing & Fixing Protocol**
Panel 1	B	CAPP2A (1:50)	AF 488 (1:20)	PMA	1°, 2°, Lyse, Fix
Panel 2	B	CAPP2A (1:50)	AF 488 (1:20)	PMA	1°, 2°, Fix, Lyse
Panel 3a	B	CAPP2A (1:50)	AF 488 (1:100)	PMA	1°, Lyse, 2°, Fix
Panel 3b	B	CAPP2A (1:50)	AF 488 (1:100)	PMA	1°, Fix, Lyse, 2°
Panel 4a	B	CAPP2A(1:50, 1:100, 1:250, 1:500)	AF 488 (1:100)	PMA	1°, 2°, Lyse, Fix
Panel 4b	B	CAPP2A (1:50, 1:100, 1:250, 1:500)	AF 488 (1:100)	ADP	1°, Fix, Lyse, 2°, Fix
Panel 5a	B, H, P	CAPP2A (1:250)	AF 635 (1:100) Annexin-F (1:500)	ADP, PMA	1°, Fix, Lyse, 2°
Panel 5b	B, H, P	CAPP2A (1:250)	AF 635 (1:100) Apotracker-F (1:200)	ADP, PMA	1°, Fix, Lyse, 2°
Panel 5c	B, H, P	-	CD61-FITC CD62P-AF 647 (1:75)	ADP, PMA	2°, Fix, Lyse Fix
Panel 5d	B, H, P	-	CD41-FITC CD62P-PEC7 (1:75)	ADP, PMA	2°, Fix, Lyse, Fix
Panel 6	H	-	CD61-FITC CD62P-AF647 (1:75)	ADP	Fix, 2°, Lyse, Fix

Table 5. Panels and antibodies used during the study. B; Bovine whole blood, H; Human whole blood, P; Bovine Platelet rich plasma, AF; Alexa Fluor, PMA; Phorbol 12-Myristate 13-Acetate, ADP; Adenosine Diphosphate, 1°; Primary Antibody, 2°; Secondary Antibody, Annexin-F; Annexin-FITC, Apotracker-F; Apotracker-FITC

Viability staining

In all Panels, 10 uL Fixable Viability Dye (FVD) eFluor 506 (*Invitrogen, Massachusetts, USA)* was added to the primary antibody stock to eliminate the gating of dead cells. FVD permeates the damaged membranes of dead cells and stain interior amines. The antibody stock solutions for all Panels were prepared on the same day as staining. The stock solutions were prepared with fluorescence activated cell scanning (FACS) buffer (*R&D Systems, FC001*). Each of the samples in all Panels, with exception to samples in Subpanel 5a, were covered with parafilm, stored at 4°C overnight and measured the next day.

Fixing and Lysing

FACS Lysing solution (*BD Bioscience, New Jersey, USA*) used by Chan[42] and Radley et al. [38] diluted in 1:10 manner (with FACS buffer) was used for the lysis of red blood cells in samples. For the fixing of platelets, several approaches were tested. Streck cell preservative (*Streck, Nebraska, USA*) as well as various concentrations of paraformaldehyde (PFA) were used.

As provided in Table 5, the various protocols tested included modifications in the lysing, fixing and staining procedure. After the measurement of each Panel, the results were assessed and iterations with modified protocols were conducted in the next Panels.

Panel 1 CAPP2A – Chan/Radley et al.

In Panel 1, the exact procedure published by Radley et al.[42] and Chan et al.[38] et al. was followed. CAPP2A (host: mouse) was diluted with FACS buffer in a 1:50 manner. The secondary antibody used was Alexa Fluor 448 goat anti-mouse (*Life Technologies,California, USA*) and was diluted in a 1:20 manner with FACS buffer. The activated positive control blood sample (20 uL) was incubated with 80 uL of 4uM phorbol myristate acetate (PMA*, Sigma, Missouri, USA)* for 1 hour. The sample was then washed with 2 mL of FACS buffer, centrifuged at 1200 rpm and 8°C for 4 minutes and aspirated thereafter. In the next steps the activated samples were treated the same way normal blood samples were treated: Staining with 80 uL primary antibody stock and 10 uL FVD occurred first. The samples were then incubated for 30 minutes on ice, washed, centrifuged (1200 rpm, 4°C, 8 min.), and aspirated. The samples were then stained with secondary antibody stock (80 uL) and incubated for 20 minutes on ice. The washing procedure was then repeated. 2 mL of lysis buffer was added for 10 minutes

before washing the sample a third time. Finally, 200 uL streck cell preservative was added, samples were covered with parafilm and stored at 4°C overnight.

Flow cytometric measurements were taken with BD FACSCanto II (*BD Bioscience, New Jersey USA*). The data was analyzed the next day manually by gating using the FACS Diva Software (*BD Bioscience, New Jersey, USA*) and Kaluza (*Beckman, California, USA*). This process allows to specifically look at cells with specific characteristics, such as the expression of CAPP2A antibodies. To compensate the overlapping of fluorochromes and autofluorescence of samples, unstained samples for each Panel were also prepared parallel to regular samples. The unstained samples were treated in the exact same procedure as samples in the corresponding Panel, with the exception of adding FACS Buffer instead of antibody stocks upon staining. An approximate gating strategy to locate platelets in forward and sideward scatter was developed (see 4.3.2.2).

Panel 2 CAPP2A – alteration of lysis and positive control

Based on the findings of Panel 1, the lysis procedure and positive control was refined in the scope of Panel 2. In comparison to Panel 1, in which lysing occurred before fixing, cells in Panel 2 were fixed first, then lysed. In Panel 2, cells were fixed with PFA instead of streck cell preservative. For the positive control, 4uM of PMA as well as a higher concentration of PMA (12uM) were tested (only 4uM PMA was used in Panel 1).

The samples were first stained with primary antibodies, washed, and stained with secondary antibody (AF 488 1: 20). Then the cells were fixed with 4% PFA, washed and lysed for 10 minutes with lysis buffer. The samples were washed again and stored in 350 ul FACS buffer with parafilm at 4°C overnight. For the positive controls of Panel 2 a higher concentration of PMA was tested. Both 4uM (same as Panel 1) and 12uM PMA were used. 80 uL of PMA was added to the 20ul blood sample, incubated for 1 hour (4uM) and 5 hours (12uM), and then stained and treated as a regular sample.

Panel 3 CAPP2A – testing for different concentrations

Due to the lack of success in detection of activated platelets in the stimulated (positive control with PMA) and unstimulated samples of Panels 1 and 2, a different approach was tested in Panel 3. In Panel 3, a lower concentration of secondary antibody was tested (1:100 in comparison 1:20 previously used in Panels 1 and 2) in an attempt to reduce unspecific binding of secondary antibodies. Furthermore, different lysing/fixing procedures were tested. In

previous Panels (Panels 1 and 2) lysing and fixing occurred after staining with primary and secondary antibodies. In Panel 3, the lysing and fixing occurred between the staining steps. This was done to increase the efficiency of staining, by eliminating the red blood cells before staining with the secondary antibody. This way, unspecific binding is further reduced.

The concentration of PMA used for this Panel was 4uM, in which samples were incubated for 1 hour at room temperature (as in Panel 1). In Panel 3a, the samples were firstly stained with CAPP2A and FVD (1:50), washed and then lysed 10 minutes with lysis buffer. The samples were then stained with AF-488 secondary antibody (1:100), washed and fixed with streck cell preservative. In Panel 3b, the samples were firstly stained with CAPP2A and FVD (1:50) and fixed with 90 uL 2% PFA for 10 minutes. Then they were lysed with 1,8 mL lysis buffer for 5-8 minutes at room temperature. The samples were centrifuged at 500xg RT, the supernatant was discarded, and the samples were stained with secondary antibody (1:100), washed, fixed again with 350 ul of 1% PFA, covered with parafilm and stored overnight at 4°C. Flow cytometric measurement of samples was done the next day.

Panel 4 CAPP2A – positive control strategy

In Panel 4 Adenosine Diphosphate (ADP, 4uM), an alternative reagent (than used in previous Panels), was tested. PMA was used in Subpanel 4a, ADP was used in Subpanel 4b. This is done to test which reagent is most effective in activating platelets and can better be utilized as a control for the detection of activated platelets. Furthermore, in Panel 4, different concentrations of CAPP2A Antibody were tested (1:50, 1:100, 1:250, 1:500). This is to determine the optimal saturation concentration of primary antibody at which platelets can still be detected. Costs for antibody stocks may also be reduced if proven lower concentrations are deemed to bind acceptably.

In Panel 4a, for the positive control, 80 uL 4uM PMA was added to a whole blood sample and incubated for 1 hour on a roller mixer to achieve gentle agitation. Then, samples were washed and treated similar to the rest of the samples. Samples were stained with primary antibody, washed, stained immediately with secondary antibody AF-488 (1:100), lysed with 2 mL lysis buffer for 10 minutes, and fixed with 200 uL of streck cell preservative to be stored overnight. On the contrary, in Panel 4b, 80 uL 4uM ADP was used for the positive control, in which the sample was also incubated for 1 hour on a roller mixer. The next steps were identical to that of Panel 4a.

Panel 5 Annexin, Apotracker, CD41, CD61 and CD62P

In Panel 5, different staining approaches to detect activated platelets were tested and both ADP and PMA were used as positive controls, in which the incubation time was 1 hour. The staining approaches tested in Panel 5 were Annexin, Apotracker, and a combination of CD41/CD61 and CD62-P, for the detection of activated platelets. In this Panel, both human and bovine blood were used. Human blood was obtained with a vacutainer needle and tube. Each Subpanel consisted of samples with whole bovine blood, whole human blood and platelet rich plasma (PRP) extracted from bovine blood. Platelet rich plasma was prepared by centrifuging a citrate tube (*Greiner Bio One, Kremsmünster, AUT*) filled with 3,5 mL bovine whole blood at 120xg for 15 minutes. The upper 2/3 of the supernatant was extracted using a pipette, and 20 uL was transferred to a FACS tube and treated as a regular sample. The benefit of adding platelet rich plasma samples to the Panel is the ability to view only platelets and eliminate the disturbance of any other blood cells in the flow cytometric scatter plot. This is also beneficial when setting the gating areas for platelets of whole blood samples. In all Subpanels of Panel 5, the concentrations for the positive control reagents, PMA and ADP were 4uM and 500uM, respectively.

Both CAPP2A and Annexin V (*Biolegend, California, USA)* were used in Panel 5a. Annexin is known to bind to phosphatidylserine on the surface of activated platelets.[34] In Panel 5a, the primary antibody stock consisted of CAPP2A diluted in a 1:250 manner with FACS buffer.[34] Annexin-FITC (1:500) was added to the antibody stock and was also diluted with FACS buffer. The secondary antibody for the CAPP2A primary Antibody used was Alexa Fluor 635 (1:100). The positive control samples (bovine whole blood, bovine PRP and human whole blood) were each incubated for 1 hour at room temperature on a roller mixture with 80 uL of 4uM and 500uM, PMA and ADP respectively. After incubation, the positive controls were washed and centrifuged. The positive controls were then treated as the regular samples. Samples were firstly stained with primary antibody and incubated for 30 minutes. After primary staining, the samples were fixed with 2% PFA and incubated for additional 10 minutes. Lysis of red blood cells occurred immediately after fixing, with 1,8 mL lysis buffer for 10 minutes. The samples were washed and centrifuged. Then, 80 uL of the secondary antibody stock with Annexin was used to stain the samples for 20 minutes in the dark at room temperature. The cells were then washed with binding buffer (contains substantial amounts of calcium, recipe can be found in section 9.3). 350 uL binding buffer was added to each FACS tube. Annexin's fluorochrome is instable and is dependent on a calcium-rich environment (present in the binding buffer). Fixing

the cells after staining with Annexin V is not possible, due to the fixation solution eliminating the calcium rich environment present in samples. Thus, the measurement of the samples in this Panel proceeded on the same day of staining.

In Panel 5b Apotracker Green (*Biolegend,California, USA)* which (similar to Annexin V) also binds to phosphatidyl serine expressed on activated platelets. The difference is that Apotracker Green stained samples can be fixed and measured the next day. This is due to the fact that Apotracker Green is calcium independent. This allows samples to be fixed with a fixation solution and measured the next day. The procedure was identical to that of Panel 5a. After staining with secondary antibody stock which includes Apotracker Green, the samples were washed, and 350 uL, 1% PFA was added. The samples were covered with parafilm and stored at 4°C. The measurement of the samples occurred the next day.

In Panel 5c, a different staining approach was tested. Directly conjugated antibodies were tested. Thus, the benefit is that a second staining step with secondary antibodies and associated washing steps included are eliminated. This reduces complexity of the protocol and the risk of activating platelets during the experiment. Anti-human CD61 and CD62P Antibodies (*Biolegend, California, USA*) were used. According to the producer's data sheet, both antibodies have a cross reactivity for human platelets. A cross reactivity for bovine platelets is not explicitly stated. Therefore, this Panel is an attempt to check for cross reactivity with bovine platelets. CD61 is also known as the integral glycoprotein IIIa of the GPIIbIIIa complex (Table 3) present on all platelets independent of their activation state. CD62P represents the P-selectin protein that is expressed on the surface of platelets upon activation.[51] Both the CD61 and CD62P antibodies are directly conjugated to a fluorochrome with each having a different emission wavelength range. This two-color approach is used to firstly identify all platelets independent of their activation state with the CD61 antibody. CD6P is then used to label only the platelets in an activated state. The procedure for Panel 5c is identical to Panel 5a and 5b with the elimination of the second staining step. Samples were firstly stained, fixed, lysed, washed then stored with 350uL 1% PFA, and covered with parafilm at 4°C. Measurement of the samples was done the next day.

CD41 and CD62P (*Biolegend,California, USA*) were used in Panel 5d. The difference in this Panel in comparison the previous one is the difference in cross reactivity of the antibodies used. According to the manufacturer`s datasheet, both antibodies are anti-mouse. It is also not

explicitly stated that a cross reactivity with bovine or human blood is present. Thus, in Panel 5c the aim is to test for cross reactivity of these antibodies to bovine blood in an attempt to detect activated bovine platelets. The procedure is identical to that of Panel 5c.

Panel 6 Anti-human CD61 and CD62P

In Panel 6, the procedure of Panel 5c is repeated with same antibodies and slight modifications. Firstly, the samples of Panel 6 were all tested using 10 uL human whole blood instead of the 20 uL blood, used in previous Panels. The reason for this is to reduce the risk for unspecific binding of antibodies. The human whole blood was obtained in a very careful manner. The positive samples of this Panel were only treated with 40 uL 10uM ADP for 1 minute at 37°C. In Panel 6, during the incubation of the positive samples, the regular samples were treated with the same amount (40uL) of FACS buffer for 1 minute at 37°C. This ensures, that all samples are treated timely in the same manner. After that the samples were immediately fixed with 200 uL 2% PFA for 10 minutes at room temperature. The samples were then washed. Then the staining of the samples occurred with the previously prepared antibody stock (1:75 for both CD61 and CD62P). The samples were incubated for 15 minutes in the dark at room temperature. Lysing the occurred with 0,5 mL lysis buffer for 10 minutes at room temperature. The samples were washed and stored with 200 uL 1% PFA with parafilm at 4°C. Measurement of the samples was done the next day. Stopping time was after 240 seconds. Events to display were set to a maximum of 100000 events, events to record were set at 2500000 events. The flow rate was set to medium.

4.3.2.2 Gating Strategy

Gating of dot plots in all Panels was conducted with the Kaluza software (*Beckmann, California, USA*). Figure 9 below, shows the gating strategy for Panels 1 through 4, in which CAPP2A antibody was used for the detection of percent activated platelets in blood samples.

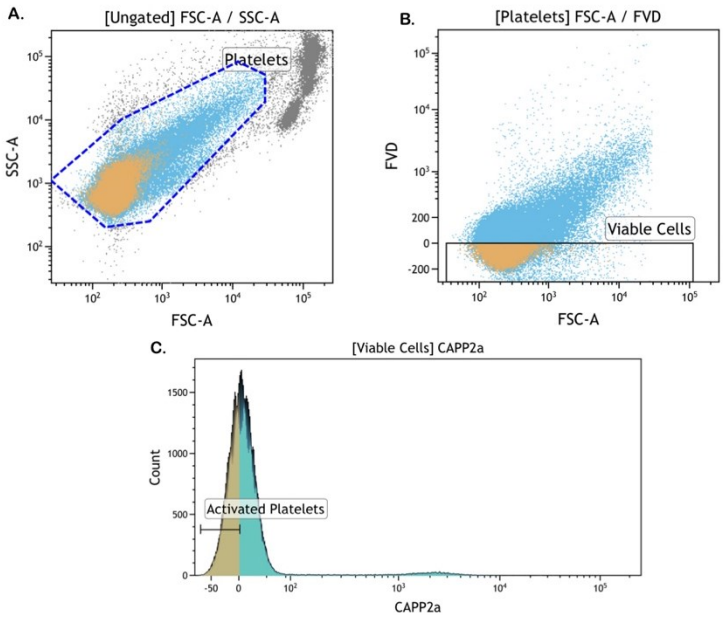


Figure 9. Exemplary gating strategy using the Kaluza Software for Panels 1-4. SSC-A: Sideward Scatter Area; FSC-A: Forward Scatter Area; V500-A: Fixable Viability Dye efluor 506. A: Ungated forward and sideward scatter with platelet gating derived from platelet rich plasma sample. B: Negative events of viability dye labeled as "viable cells". C: Negative events of CAPP2A antibody labeled as "activated platelets".

First a dot plot with sideward area (SSC-A) and forward scatter area (FSC-A) is constructed (Figure 9A). In the SSC/FSC plot all types of cells present in whole blood (such as platelets, leukocytes, and thrombocytes), are displayed, and sorted by their size and granularity. Approximate gating of platelets in the SSC/FSC plots, in accordance with published studies[50], was used for the gating of platelets (Figure 9A). In Panel 5, dot plots of platelet rich plasma (PRP) were measured, in which all other cell types have been eliminated in the plot. Later during this study, the SSC/FSC plots of PRP in Panel 5 were used to determine the final platelet gating region for SSC/FSC plots of all samples in all Panels (Figure 9A, Platelets). The gated region of the SSC/FSC dot plot is then applied to a dot plot displaying FSC-A and fluorescence of the viability stain used. In the case of this study, V500-A was used. With this dot plot, the cells previously gated in SSC/FSC plots are sorted as viable and non-viable. The viability stain used (Fixable Viability Dye eFluor 506) is known to stain dead cells. Thus, the negative events of this plot are gated as "viable cells" (Figure 9B). The gated viable cells are then used to display a histogram of positive and negative CAPP2A events. According to Radley et al. [42], CAPP2A binds to resting inactivated platelets. Thus, the negative events of this dot plot, are gated as activated platelets (Figure 9C). The overall percentage of activated platelets from total platelets initially gated through the SSC/FSC plots is automatically calculated in the software.

In Panels 5a and 5b, in which Annexin V and Apotracker was used, the gating through SSC/FSC and viable cells was utilized to detect the positive events of Annexin and Apotracker. These events are labeled as "Activated Platelets" because these cells are viable and express phosphatidyl serine to which Annexin or Apotracker has bound to. In this study, both Annexin and Apotracker were conjugated with FITC, which was used in the final gating plot.

Similarly, in Panels 5c, 5d, and 6, SSC/FSC as well as a viable plot were used for gating. Next, the viable cells were plotted against the platelet specific antibody which was CD61 and CD41 in Panels 5c and 5d, respectively. The positive events of these plots are labeled as "Platelets CD61 or CD41 +". This gate is then used in the next plot, in which a CD62P histogram is displayed. The positive events of this plot are labeled as "activated platelets". The percent of CD62P from CD61/41 positive events is calculated by the software.

5 Results

5.1 Hematology Tests

Table 6 above shows the mean hematologic measurements obtained from the baseline samples of Panel 1-3.

	Average	Standard Deviation
pH	7,55	± 0,02
HCT (%)	41	± 4
Platelet count (10^3 / uL)	101,08	± 2,24

Table 6. Mean Hematologic measurements obtained in baseline blood measurements from the first 3 Panels. HCT: Hematocrit.

5.2 Activated Clotting Time

Figure 10 below shows the mean ACT of all three loop experiments of the HM3 with artificial pulse-mode (pulsatile), the HM3 in fixed-mode (fixed), and the setup without HM3 (static). Generally, in all 3 experiments the clotting time of the static control was higher than that of the loops with the HM III in fixed and artificial pulse mode. The mean ACT values of the loops including a HM3 are quite similar, yet the loop with the HM3 in pulse-mode showed a slightly higher activated clotting time than the loop with the HM3 in fixed-mode. A slight decrease in ACT over time is observed in all three setups: The average decrease of ACT after 6 hour run

time in comparison to the first sample taken for pulsatile, fixed and static are 2,47%, 9,90 % and 5,06% respectively.

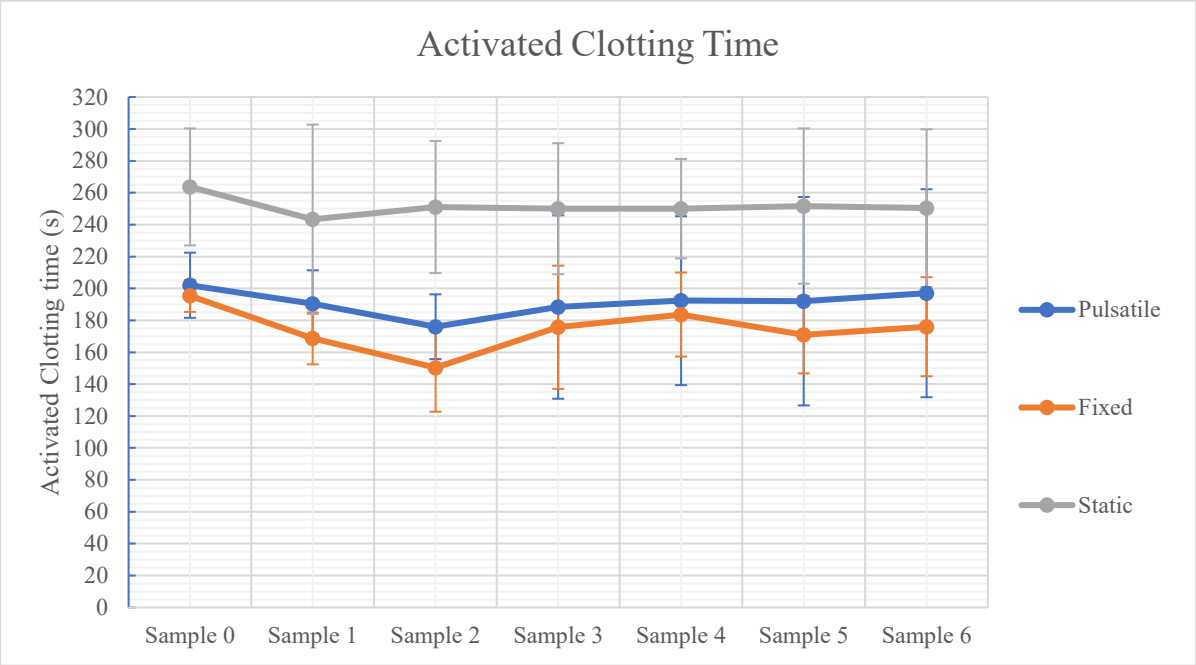


Figure 10. Activated clotting time mean values of three loop experiments, with 1 hour extracted samples.

5.3 Platelet Activation Assay

As mentioned earlier, throughout this study various staining, lysing and fixing protocol were tested. The results of each Panel were evaluated, and the procedure was refined accordingly. The results of Panels 1-6 are displayed below.

Panel 1 and 2 CAPP2A Chan/Radley et al.

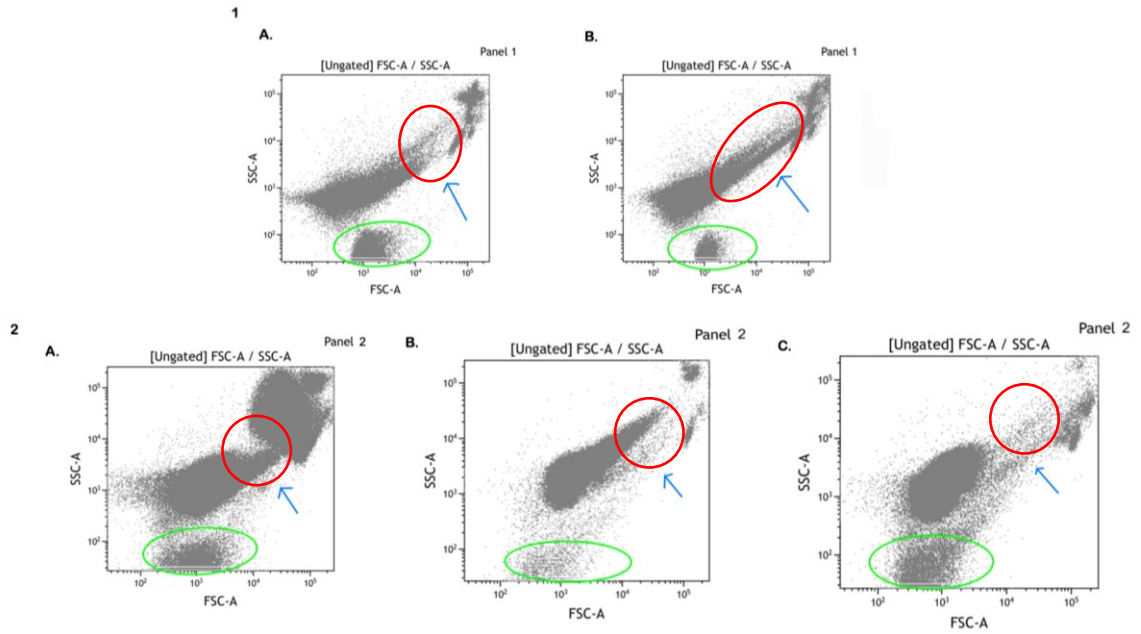
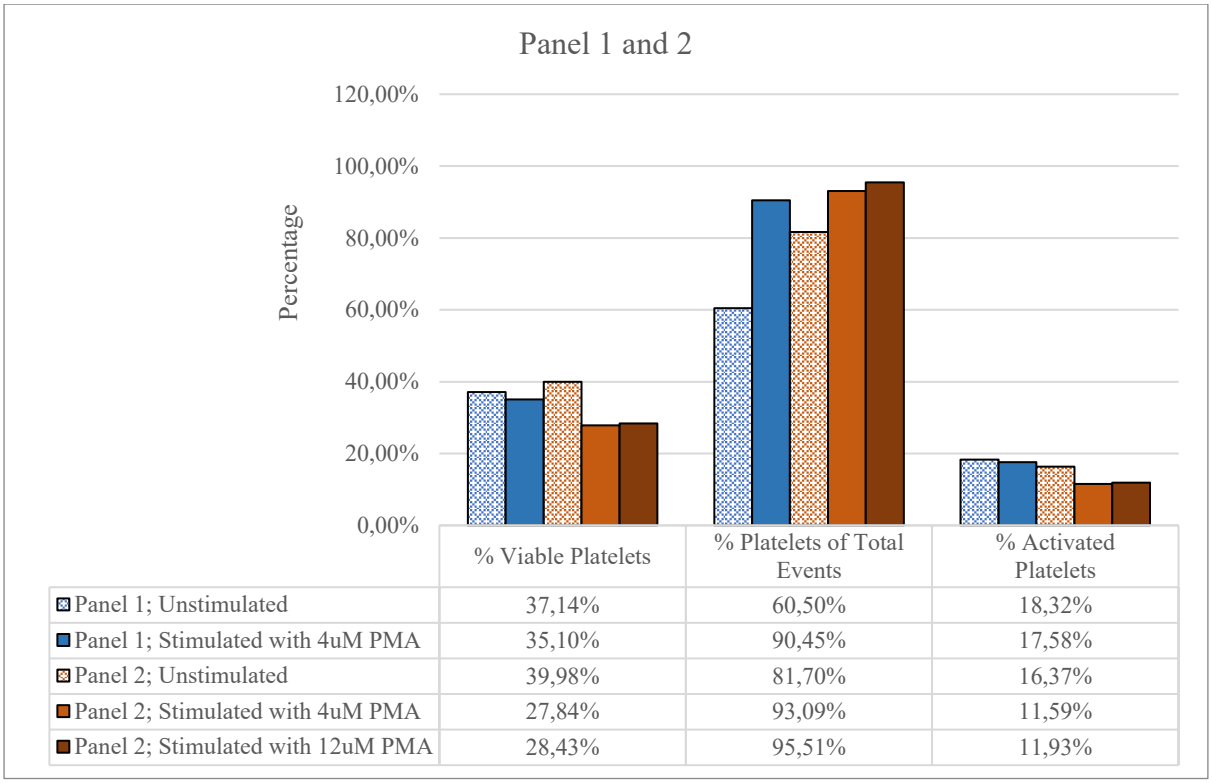


Figure 11. Forward and sideward scatter of Panel 1 and 2 with bovine whole blood; 1A: Unstimulated sample of Panel 1; 1B: Stimulated with 4uM PMA; 2A: Unstimulated sample of Panel 2; 2B: Stimulated with 4uM PMA; 2C: Stimulated with 12uM PMA. Arrows indicate the shift of platelets in dot plot upon activation. Circle: Presence of Artifacts and Background noise

In Figure 11, exemplary forward and sideward scatters for Panels 1 and 2 are displayed. In Figure 11.1.B a slight upward shift can be seen upon stimulation of sample with 4uM PMA (in comparison with Figure 11.1.A and blue arrow). A similar trend can be seen when comparing Figure 11.2.A and Figure 11.2.B. Further upward shift is not present in Figure 11.2.C which was stimulated with a higher concentration of PMA (12uM) in comparison to Figure 11.2.B. Due to the presence of artifacts and various disturbances of stacked red blood cells presented in the dot plots of Panel 1, the lysing procedure was altered in Panel 2. In Panel 2, fixing occurred before lysing. Artifacts, though slightly attenuated, remained to be present in the dot plots of Panel 2 (Figure 11.2, green marked circle). Using the gating strategy described earlier (4.3.2.2), the percentage of platelets, viable platelets, and activated platelets in whole blood samples was determined (Figure 12).



	% Viable Platelets	% Platelets of Total Events	% Activated Platelets
Panel 1; Unstimulated	37,14%	60,50%	18,32%
Panel 1; Stimulated with 4uM PMA	35,10%	90,45%	17,58%
Panel 2; Unstimulated	39,98%	81,70%	16,37%
Panel 2; Stimulated with 4uM PMA	27,84%	93,09%	11,59%
Panel 2; Stimulated with 12uM PMA	28,43%	95,51%	11,93%

Figure 12. Percent platelets, viable platelets and activated platelets of unstimulated and stimulated samples of Panel 1 (blue) and Panel 2 (orange) with bovine whole blood. Percentages were determined using flow cytometry, fixable viability dye and CAPP2A Antibody.

In both Panels, there is no increase in percent of activated platelets upon stimulation with 4/12uM PMA present. The percent difference of activated platelets in Panel 1, between stimulated and unstimulated samples, is -0,74 %. In Panel 2, the difference in percentage of activated platelets between stimulated and unstimulated samples is -4,78 % for 4uM PMA samples, and -4,44 % for 12uM PMA samples. Furthermore, a general decrease in percent of viable platelets is observed upon stimulation in both Panels. The decrease in Panel 1 is at approximately 2,04 %, in Panel 2 12,14% for the 4uM PMA sample and 11,55% for the 12uM PMA sample.

Panel 3 CAPP2A – testing of different concentrations

Due to the lack of success in Panel 1 and 2 in the detection of activated platelets and the presence of artifacts with no clear populations in SSC/FSC dot plots, the procedure was altered in Panel 3. As mentioned earlier (4.3.2.1), in Panel 3 the concentration of secondary antibody used in staining was altered. Furthermore, different lysing and fixing protocols were tested.

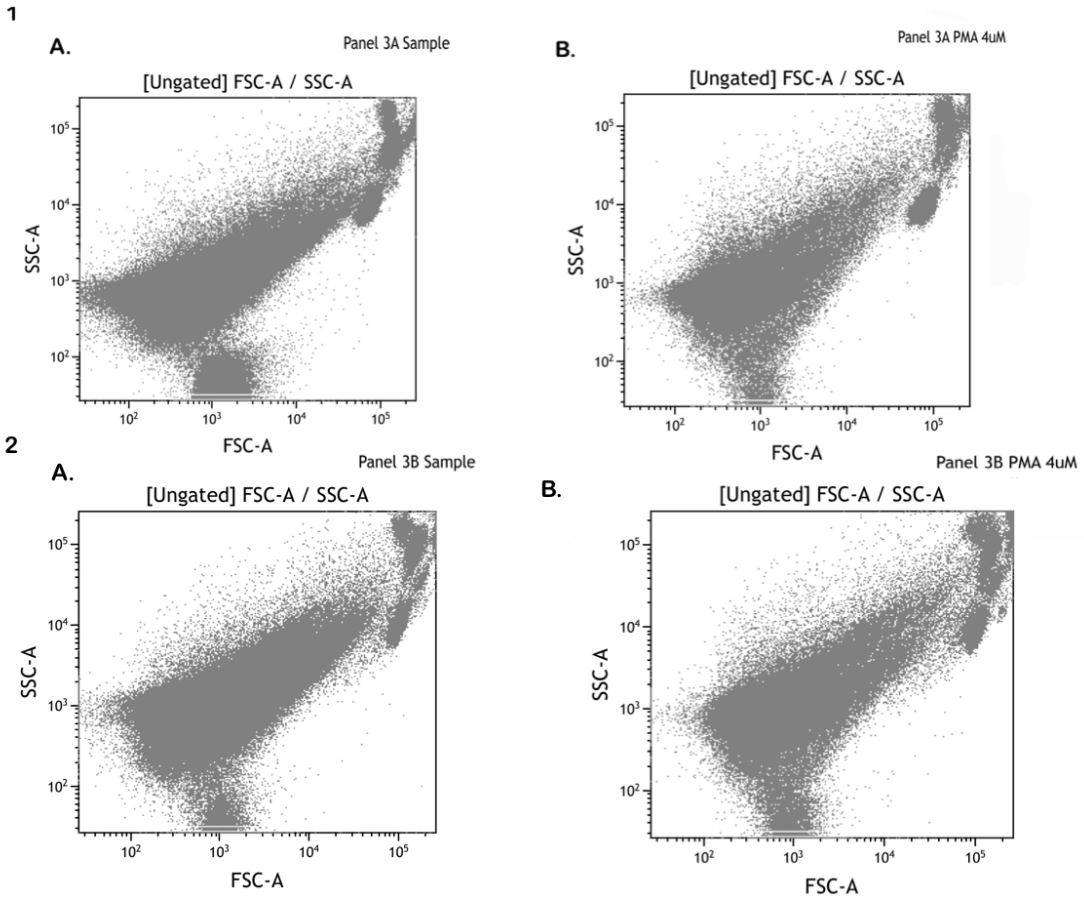
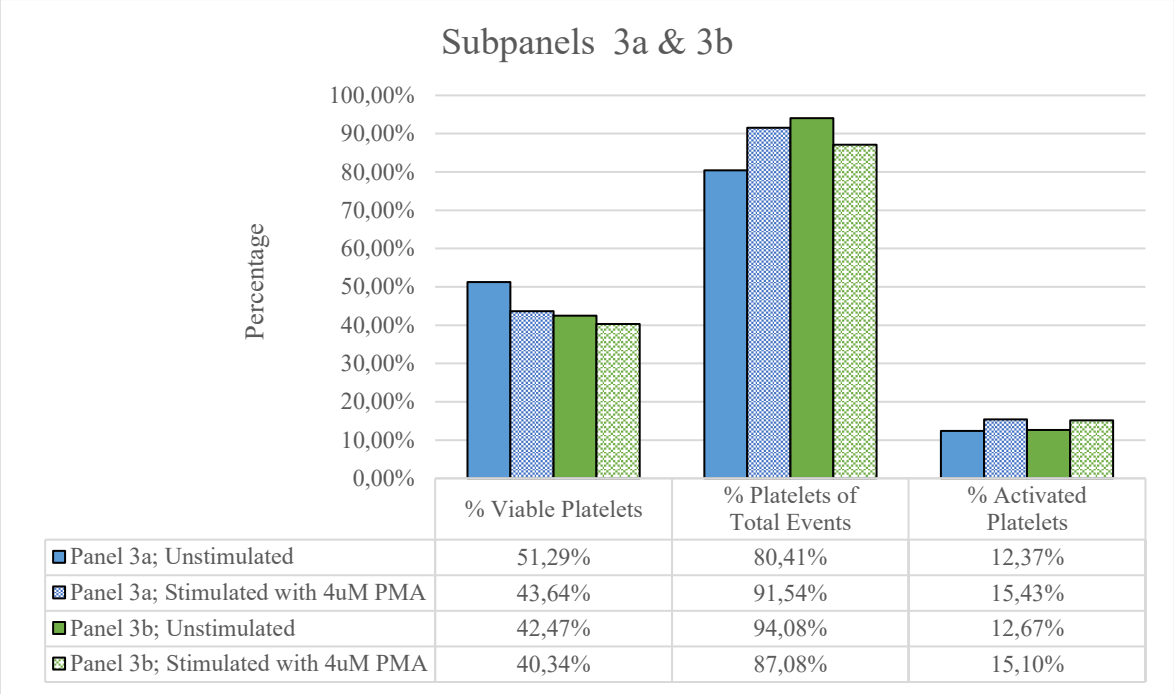


Figure 13. Forward and Sideward Scatter of Panel 3 with bovine whole blood. SSC-A: Sideward Scatter Area; FSC-A: Forward Scatter Area; PMA: Phorbol Myristate Acetate. 1A: Unstimulated Sample of Panel 3a; 1B: Stimulated sample of Panel 3a with 4uM PMA; 2A: Unstimulated sample of Panel 3b; 2B: Stimulated sample of Panel 3b with 4uM PMA

Figure 13 shows the SSC/FSC scatter plots of Subpanels 3A and 3B. A clear upward shift of events upon stimulation with PMA, is not present in either Panel. Artifacts similar to the ones present in Panels 1 and 2, remain to be present in both Subpanels of Panel 3.



	% Viable Platelets	% Platelets of Total Events	% Activated Platelets
Panel 3a; Unstimulated	51,29%	80,41%	12,37%
Panel 3a; Stimulated with 4uM PMA	43,64%	91,54%	15,43%
Panel 3b; Unstimulated	42,47%	94,08%	12,67%
Panel 3b; Stimulated with 4uM PMA	40,34%	87,08%	15,10%

Figure 14. Bovine Whole Blood: Percent platelets, viable platelets and activated platelets of unstimulated and stimulated bovine whole blood samples of Panel 3a (blue) and Panel 3b (green) after the lysing procedure and the concentration of secondary antibody was altered. Percentages were determined using flow cytometry, fixable viability dye and CAPP2A Antibody.

The same gating strategy used for Panels 1 and 2, described in 4.3.2.2, was used in Panel 3. Figure 14 above, shows the percent platelets, activated platelets and viable platelets in stimulated and unstimulated samples of Panels 3a and 3b. The percent difference of activated platelets in stimulated and unstimulated samples was 3,06% for Panel 3a and 2,43% for Panel 3b. Similar to Panels 1 and 2, a general decrease of percent viable platelets can be observed in both Subpanels 3a and 3b, upon stimulation with 4uM PMA. The percent decrease of viable cells in stimulated and unstimulated samples is 7,65% and 2,13% in Subpanels 3a and b, respectively.

Panel 4 CAPP2A – Positive control strategy

As mentioned earlier (4.3.2.1), due to the lack of success in the stimulation and detection of activated platelets, samples in Panel 4 were stimulated in a different manner, than attempted in previous Panels. The activated control sample in Subpanel 4a was stimulated with 4uM PMA, for 1 hour, this time with gentle agitation on a roller mixer. The activated sample of Subpanel 4b was stimulated with 4uM adenosine diphosphate (ADP). Furthermore, an attempt to test for different saturation concentrations of the CAPP2A antibody was conducted in Panel 4. The different concentrations tested were 1:50, 1:100, 1:250, 1:500.

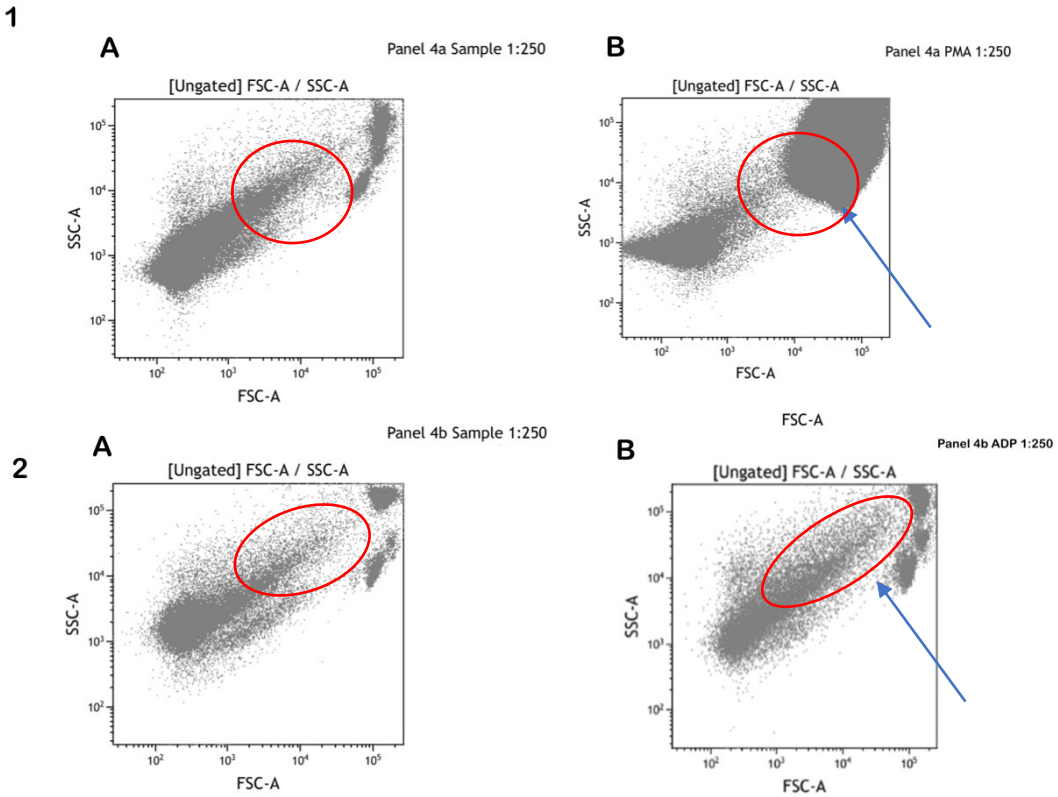
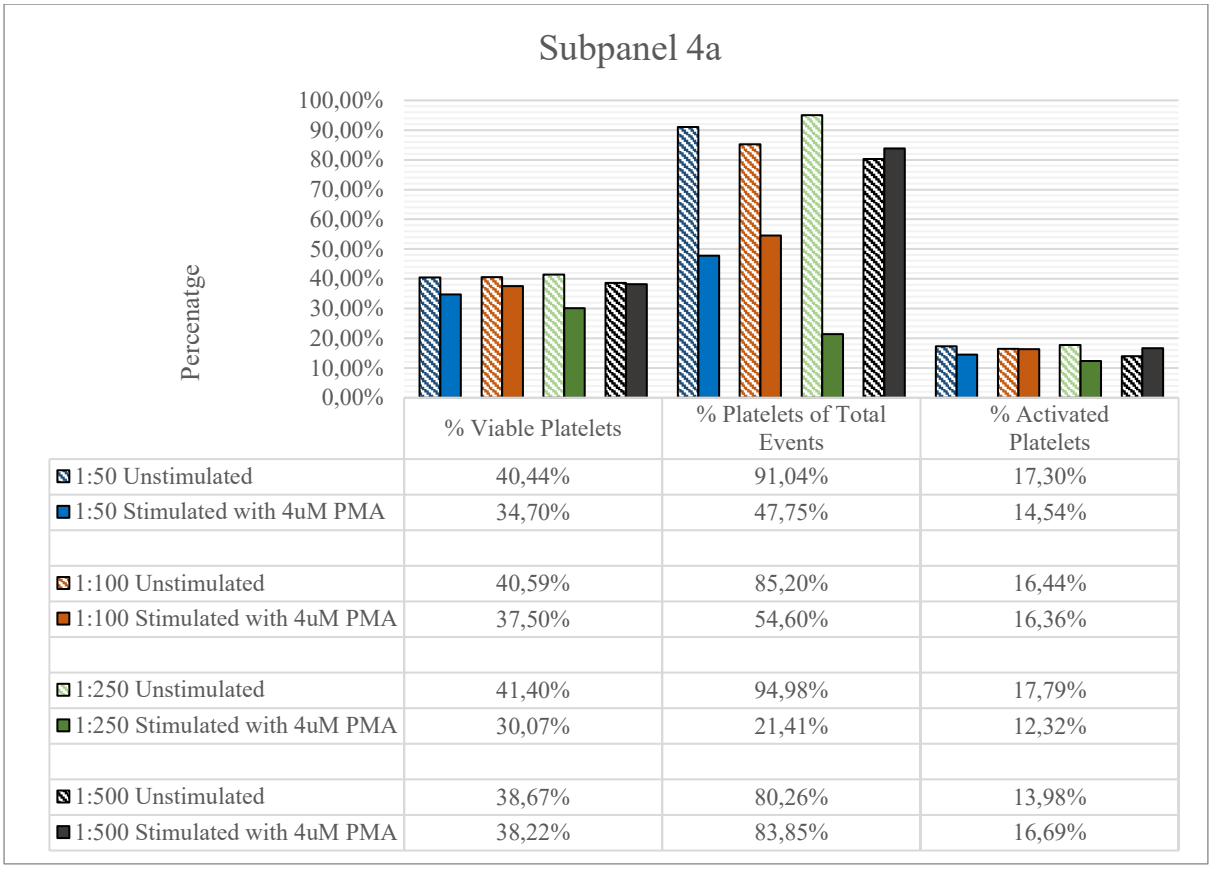


Figure 15. Forward and Sideward Scatter of Subpanels 4a (1) and 4b (2) with bovine whole blood. SSC-A: Sideward Scatter Area; FSC-A: Forward Scatter Area; PMA: Phorbol Myristate Acetate; ADP: Adenosine Diphosphate. 1A: Unstimulated Sample of Panel 4a; 1B: Stimulated sample of Panel 4a with 4uM PMA; 2A: Unstimulated sample of Panel 4b; 2B; Stimulated sample of Panel 4b with 4uM ADP.

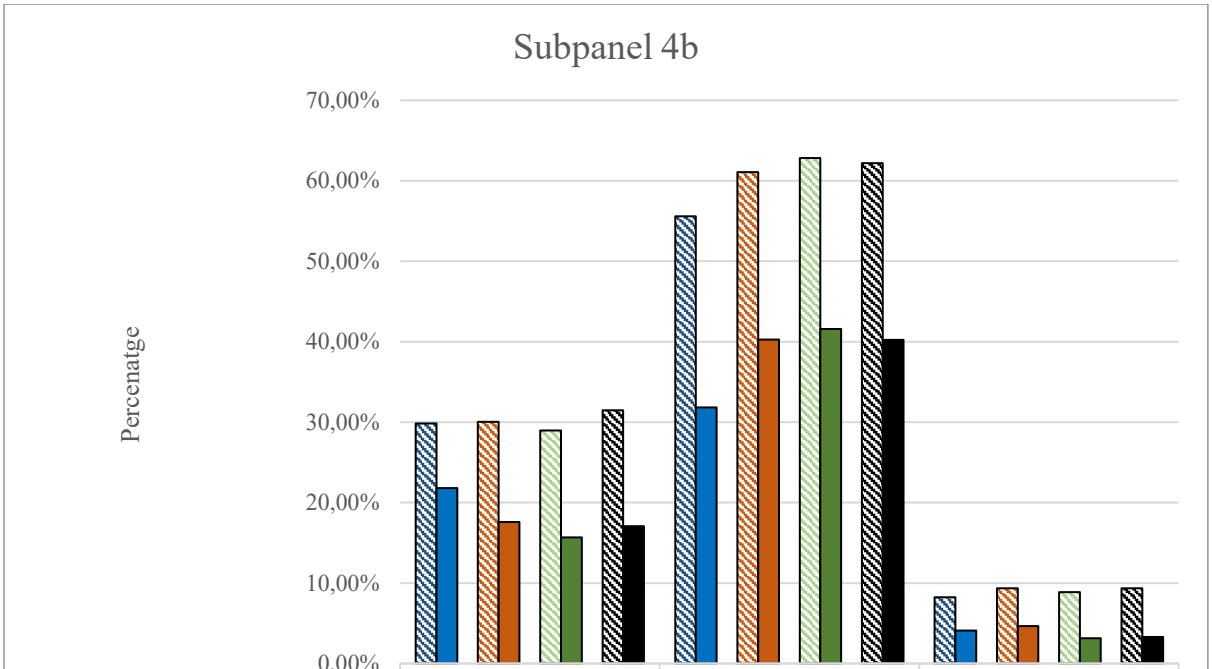
Figure 15 shows the forward and sideward scatter of Subpanels 4a and 4b with a 1:250 dilution of CAPP2A. The remainder dot plots of Panel 4 can be found in the appendix (9.4.1). In both Panels 4a and b, a clear reduction of background noise and artifacts can be seen in comparison to the background present in the previous Panels (1-3). In both Panels 4a and 4b, an upward shift of events can be seen upon stimulation with PMA/ADP (blue arrow Figure 15). Furthermore, Figure 15.1.A and B show more dense populations than Figure 15.2.A and B. All plots with different concentrations were compared with each other in terms of density and detection of platelets, viable platelets, and percent platelets (Figure 16).



	% Viable Platelets	% Platelets of Total Events	% Activated Platelets
1:50 Unstimulated	40,44%	91,04%	17,30%
1:50 Stimulated with 4uM PMA	34,70%	47,75%	14,54%
1:100 Unstimulated	40,59%	85,20%	16,44%
1:100 Stimulated with 4uM PMA	37,50%	54,60%	16,36%
1:250 Unstimulated	41,40%	94,98%	17,79%
1:250 Stimulated with 4uM PMA	30,07%	21,41%	12,32%
1:500 Unstimulated	38,67%	80,26%	13,98%
1:500 Stimulated with 4uM PMA	38,22%	83,85%	16,69%

Figure 16. Bovine Whole Blood: Viable platelets, percent platelets and activated platelets of unstimulated and stimulated with PMA samples of Panel 4a. Blue: 1:50 dilution of CAPP2A Antibody; Orange: 1:100 dilution of CAPP2A; Green: 1:250 dilution of CAPP2A; Black: 1:500 dilution of CAPP2A. Percentages were determined using flow cytometry, fixable viability dye and CAPP2A.

Figure 16 shows the percent platelets, activated platelets and viable platelets present in Panel 4a, with the various concentrations of CAPP2A tested. In general, the percentage of activated and viable platelets do not differ when the concentration of CAPP2A is altered. The standard deviations for the values of percent activated platelets in the different concentrations of CAPP2A of Panel 4a are 1,69% and 2,01% for the unstimulated and stimulated samples, respectively. The standard deviations for percent viable platelets are 1,15% and 3,69% for unstimulated and stimulated samples in Panel 4a, respectively. Moreover, the percent differences of activated platelets in stimulated and unstimulated samples of Panel 4a are: 2,76% for 1:50 dilution, -0,08% for the 1:100 dilution, 5,47 % for the 1:250 dilution, and -2,71 % for the 1:500 dilution of CAPP2A antibody. The mean decrease of viability observed among the samples of Panel 4a in stimulated and unstimulated samples is 5,15%.



	% Viable Platelets	% Platelets of Total Events	% Activated Platelets
1:50 Unstimulated	29,84%	55,59%	8,23%
1:50 Stimulated with 4uM ADP	21,81%	31,82%	4,08%
1:100 Unstimulated	30,05%	61,06%	9,36%
1:100 Stimulated with 4uM ADP	17,59%	40,27%	4,63%
1:250 Unstimulated	28,95%	62,82%	8,85%
1:250 Stimulated with 4uM ADP	15,67%	41,59%	3,15%
1:500 Unstimulated	31,48%	62,18%	9,33%
1:500 Stimulated with 4uM ADP	17,08%	40,24%	3,30%

Figure 17. Bovine Whole blood; Viable platelets, percent platelets, and activated platelets of unstimulated and stimulated with ADP samples of Panel 4b after lysing procedure was altered. Blue: 1:50 dilution of CAPP2A Antibody; Orange: 1:100 dilution of CAPP2A; Green: 1:250 dilution of CAPP2A; Black: 1:500 dilution of CAPP2A.Percentages were determined using flow cytometry, fixable viability dye and CAPP2A Antibody.

Similarly, Figure 17 shows the percent platelets and activated and viable platelets in stimulated and unstimulated samples of Panel 4b. Analogously to the previous Panel, the values for the percent of activated and viable platelets do not differ when the concentration of CAPP2A is altered. The standard deviations for the percent of activated platelets with different concentrations of CAPP2A antibody in Panel 4b, are 0,53% and 0,69% for unstimulated and stimulated samples, respectively. The standard deviation for percent of viable platelets in unstimulated and stimulated samples of Panel 4b are 1,05% and 2,64%. Moreover, a general decrease in percent of activated platelets is observed upon stimulation with ADP in all

concentration of CAPP2A antibody. Additionally, a decrease in viable platelets is also observed upon stimulation with 4uM ADP in all concentrations of antibody.

Panel 5 Annexin, Apotracker

As mentioned earlier (4.3.2.1), due to the ineffectiveness of CAPP2A in the detection of activated platelets in all previous Panels, different staining approaches were tested in Panel 5. In Panel 5a and b, Annexin V and Apotracker were used to detect activated platelets.

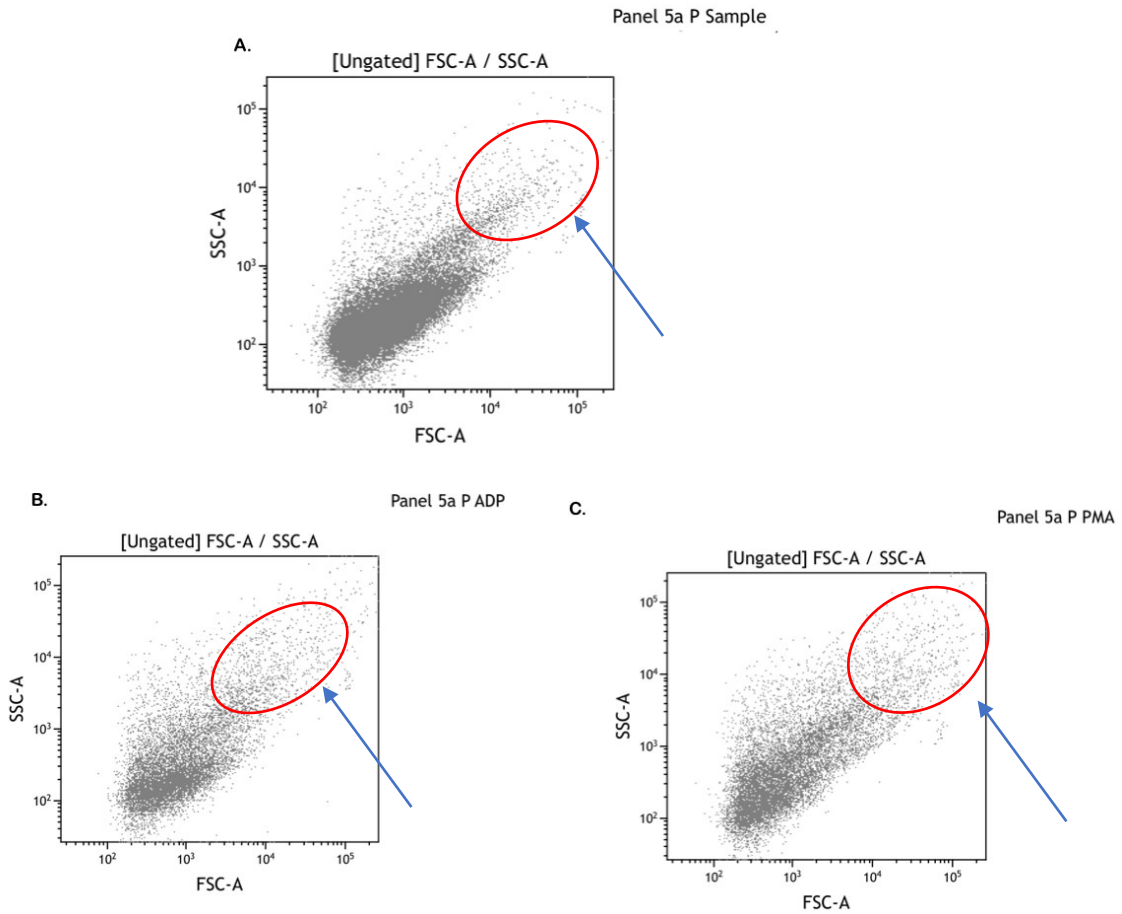
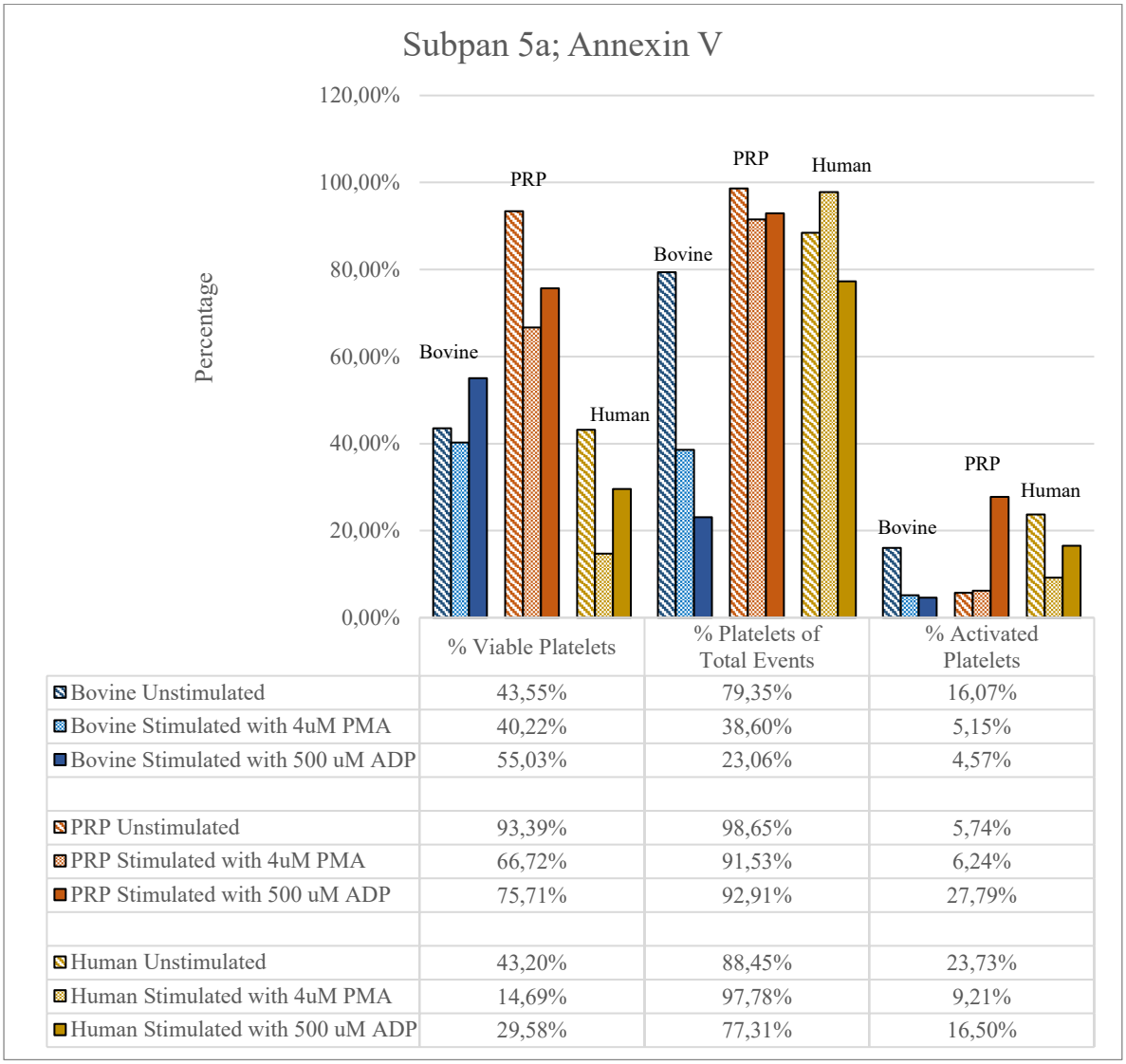


Figure 18. Forward and Sideward Scatter of Panel 5a after staining approach was altered. SSC-A: Sideward Scatter Area; FSC-A: Forward Scatter Area; PMA: Phorbol Myristate Acetate; ADP: Adenosine Diphosphate A: Unstimulated Sample of Panel 5a; B: Stimulated sample of Panel 4a with 500uM ADP; C: Stimulated sample of Panel with 4uM PMA.

Platelet rich plasma was used to eliminate the disturbance of other cells in the dot plots. The gating strategy is explained in section 4.3.2.2.

Figure 18 above, shows the forward and sideward scatter of the platelet rich plasma samples in Subpanel 5a. Due to Annexin's instability, samples of this Subpanel (Panel 5a), were measured on the same day as staining. The remainder scatter plots of whole bovine and human blood for Panel 5 can be found in the appendix in section 9.4.2.The single scatter events of platelet rich plasma samples are visible in a clearer manner than the samples with whole blood from

previous Panels (Panels 1-4). The presence of artifacts, background noise and other interfering cells is eliminated, when measuring. It is noticeably apparent in the dot plots of Panel 5a, that upon stimulation with ADP (Figure 18.B, blue arrow) and PMA (Figure 18.C, blue arrow) an upward shift of events occurs in comparison with the unstimulated platelet rich plasma sample (Figure 18.A, blue arrow).



	% Viable Platelets	% Platelets of Total Events	% Activated Platelets
Bovine Unstimulated	43,55%	79,35%	16,07%
Bovine Stimulated with 4uM PMA	40,22%	38,60%	5,15%
Bovine Stimulated with 500 uM ADP	55,03%	23,06%	4,57%
PRP Unstimulated	93,39%	98,65%	5,74%
PRP Stimulated with 4uM PMA	66,72%	91,53%	6,24%
PRP Stimulated with 500 uM ADP	75,71%	92,91%	27,79%
Human Unstimulated	43,20%	88,45%	23,73%
Human Stimulated with 4uM PMA	14,69%	97,78%	9,21%
Human Stimulated with 500 uM ADP	29,58%	77,31%	16,50%

Figure 19. Use of Annexin V to determine viable platelets, percent platelets and activated platelets of unstimulated and stimulated with ADP and PMA samples of Panel 5a after staining procedure was altered. PMA: Phorbol myristate acetate; ADP: Adenine Diphosphate; PRP: Platelet Rich Plasma; Blue: Bovine Whole Blood; Orange: Bovine Platelet Rich Plasma; Yellow: Human whole blood.

Figure 19 shows the percent platelets and activated/viable platelets in Panel 5a. In bovine and human blood samples a decrease in percent platelet activation detected by Annexin V, is present upon stimulation with both ADP and PMA. The stimulation of platelet rich plasma (PRP), however, showed an increase in percent activated platelets with both the addition of

PMA and ADP. The percent increase of activated platelets in the PRP sample after stimulation with PMA and ADP were, 0,50% and 22,05%, respectively. Viability decreased in PMA and ADP stimulated PRP samples. The percent decrease of viability in PRP samples was 26,67% and 17,68%, after stimulation with PMA and ADP, respectively. The percent platelets of all events detected from the PRP sample was above 90% in all 3 samples, with a standard deviation of 3,78%.

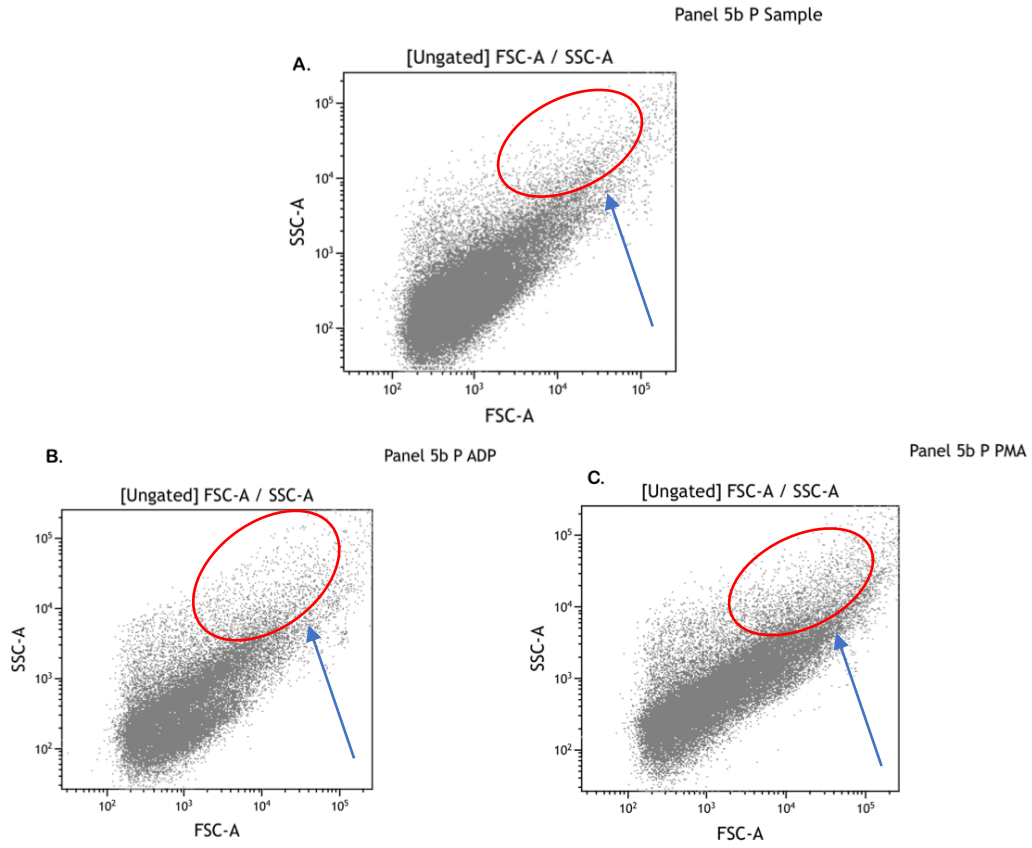
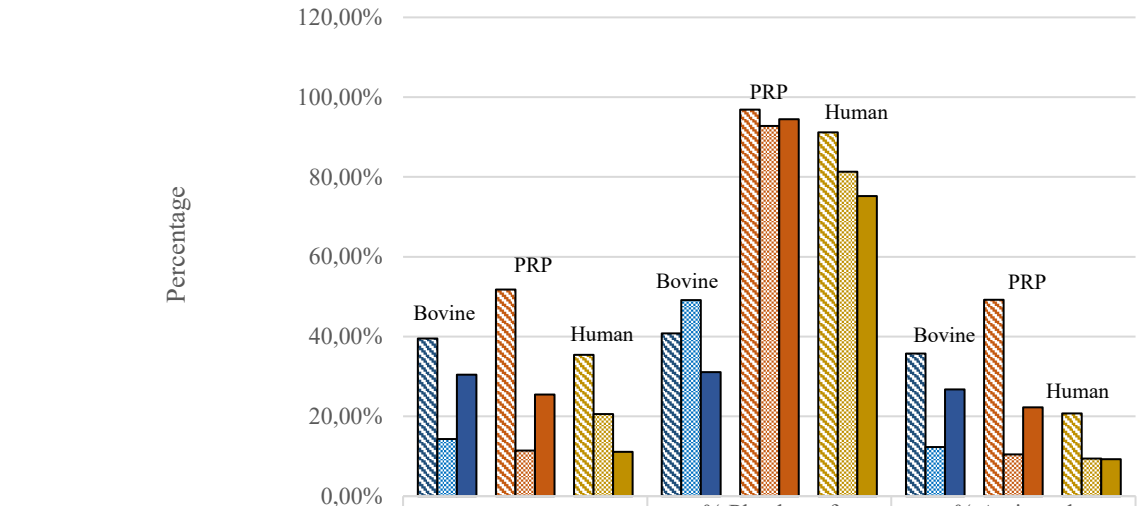


Figure 20. Forward and Sideward Scatter of Panel 5b after staining approach was altered. SSC-A: Sideward Scatter Area; FSC-A: Forward Scatter Area; PMA: Phorbol Myristate Acetate; ADP: Adenosine Diphosphate A: Unstimulated Sample of Panel 5a; B: Stimulated sample of Panel 4a with 500uM ADP; C: Stimulated sample of Panel with 4uM PMA.

Similarly, Figure 20, shows the SCC/FSC dot plots of Subpanel 5b in which Apotracker had been used instead of Annexin for the detection of activated platelets. Due to the feasibility of fixing and storing Apotracker stained samples, the measurement of samples of Subpanel 5b where measured the next day. In contrast to samples of Panel 5a, which were directly measured after staining with Annexin V. A slight upward shift of events can be seen upon stimulating samples with ADP and PMA (Figure 20. B. and C.).



	% Viable Platelets	% Platelets of Total Events	% Activated Platelets
Bovine Unstimulated	39,50%	40,78%	35,71%
Bovine Stimulated with 4uM PMA	14,33%	49,14%	12,32%
Bovine Stimulated with 500 uM ADP	30,44%	31,11%	26,77%
PRP Unstimulated	51,80%	96,91%	49,24%
PRP Stimulated with 4uM PMA	11,42%	92,81%	10,43%
PRP Stimulated with 500 uM ADP	25,44%	94,44%	22,27%
Human Unstimulated	35,39%	91,22%	20,71%
Human Stimulated with 4uM PMA	20,55%	81,35%	9,45%
Human Stimulated with 500 uM ADP	11,09%	75,25%	9,30%

Figure 21. Use of Apotracker to determine percent platelets, viable platelets and activated platelets of unstimulated and stimulated with ADP and PMA samples of Panel 5b after staining procedure was altered. PMA: Phorbol myristate acetate; ADP: Adenine Diphosphate; PRP: Platelet Rich Plasma; Blue: Bovine Whole Blood; Orange: Bovine Platelet Rich Plasma; Yellow: Human whole blood.

Figure 21, shows the data obtained after measuring the Apotracker stained samples with flow cytometry the next day and conducting the gating strategy described earlier (4.3.2.2). Both human and bovine whole blood as well as platelet rich plasma samples show a decrease in detected activated platelets upon stimulation with both ADP and PMA. The decrease in viability is also apparent in all samples, upon stimulation. The percent of platelet of total events is above 90% in all platelet rich plasma samples with a standard deviation of 2,06%.

Panel 5 CD41, CD61 and CD62P

As explained earlier (4.3.2.1), in Subpanel 5c and d, CD61/41 antibodies were used to stain platelets, and CD62P antibodies were used to detect activated platelets in bovine and human samples. Both Subpanels 5c and 5d have identical procedures. The difference lies with the

cross reactivity of the antibodies. In Subpanel 5c, human cross-reactive antibodies, CD61 and CD62P were tested for their ability to detect bovine platelets and activated platelets, respectively. In Subpanel 5d, antibodies with cross reactivity to murine platelets were used (CD41 and CD62P).

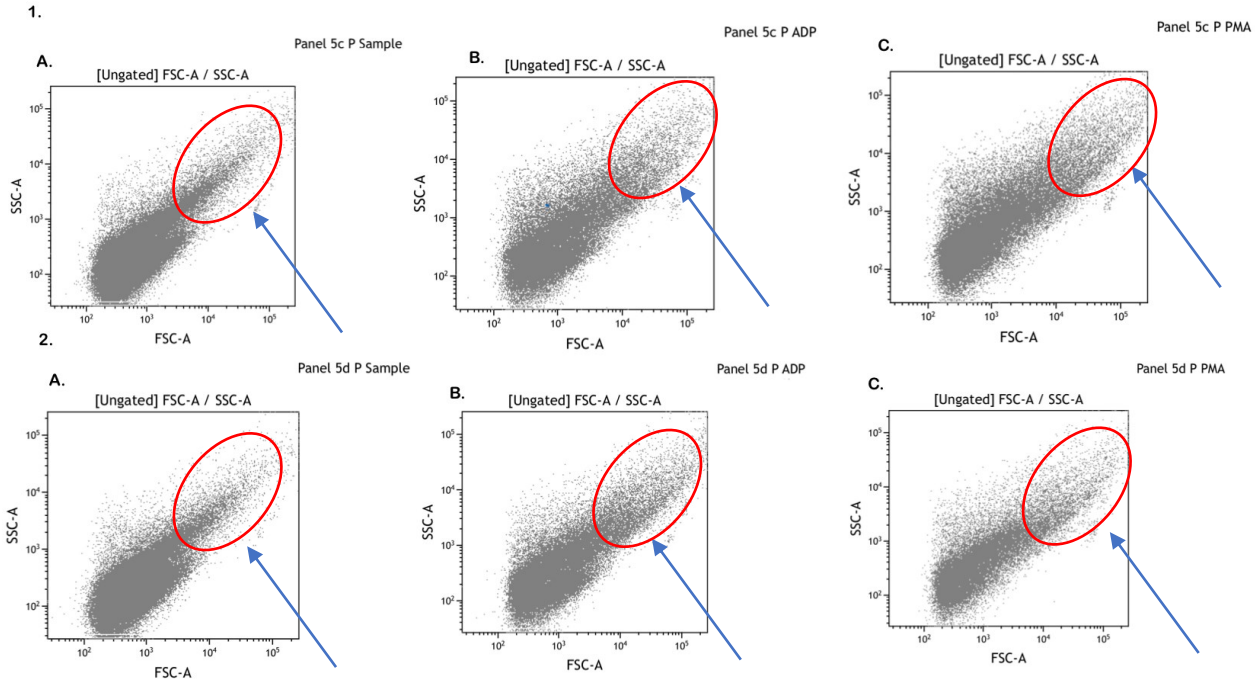
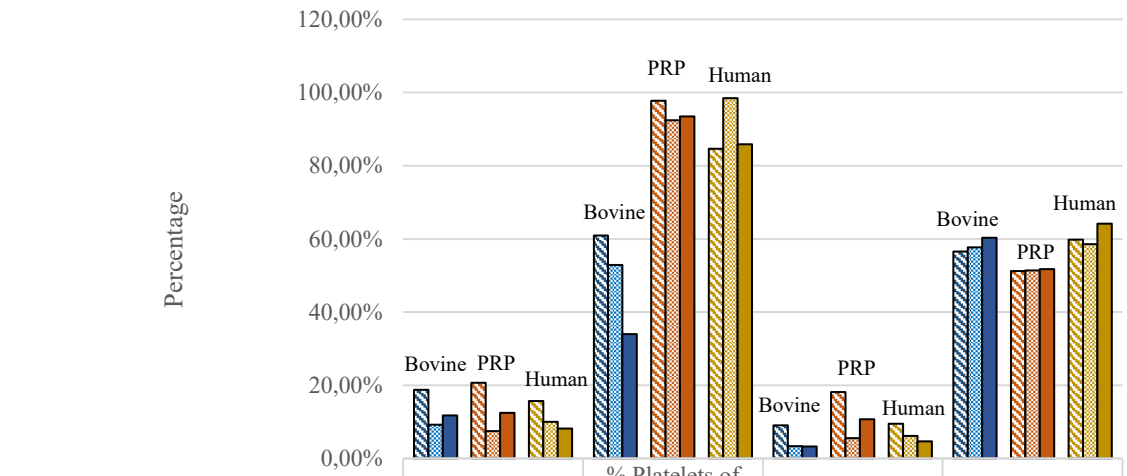


Figure 22. Forward and Sideward Scatter of Panels 5c and 5d after staining approach was altered. SSC-A: Sideward Scatter Area; FSC-A: Forward Scatter Area; PMA: Phorbol Myristate Acetate; ADP: Adenosine Diphosphate A: Unstimulated Sample of Panel 5a; B: Stimulated sample of Panel 4a with 500uM ADP; C: Stimulated sample of Panel with 4uM PMA.

Figure 22 above, shows a slight apparent shift of events in platelets in the PRP samples of Subpanels 5c and 5d upon stimulation with ADP and PMA. This is shown in the upper marked region of dot plots (circular red mark) as a higher population of events is expressed upon stimulation with ADP and PMA in this region (Figure 22.1.B/C and Figure 22.2.B/C). The scatter plots of bovine and human whole blood samples can be found in the appendix (9.4.2). Highly dense populations are present in lower parts of the plot in comparison to the events of the scatter plots of Subpanel 5a in Figure 18.

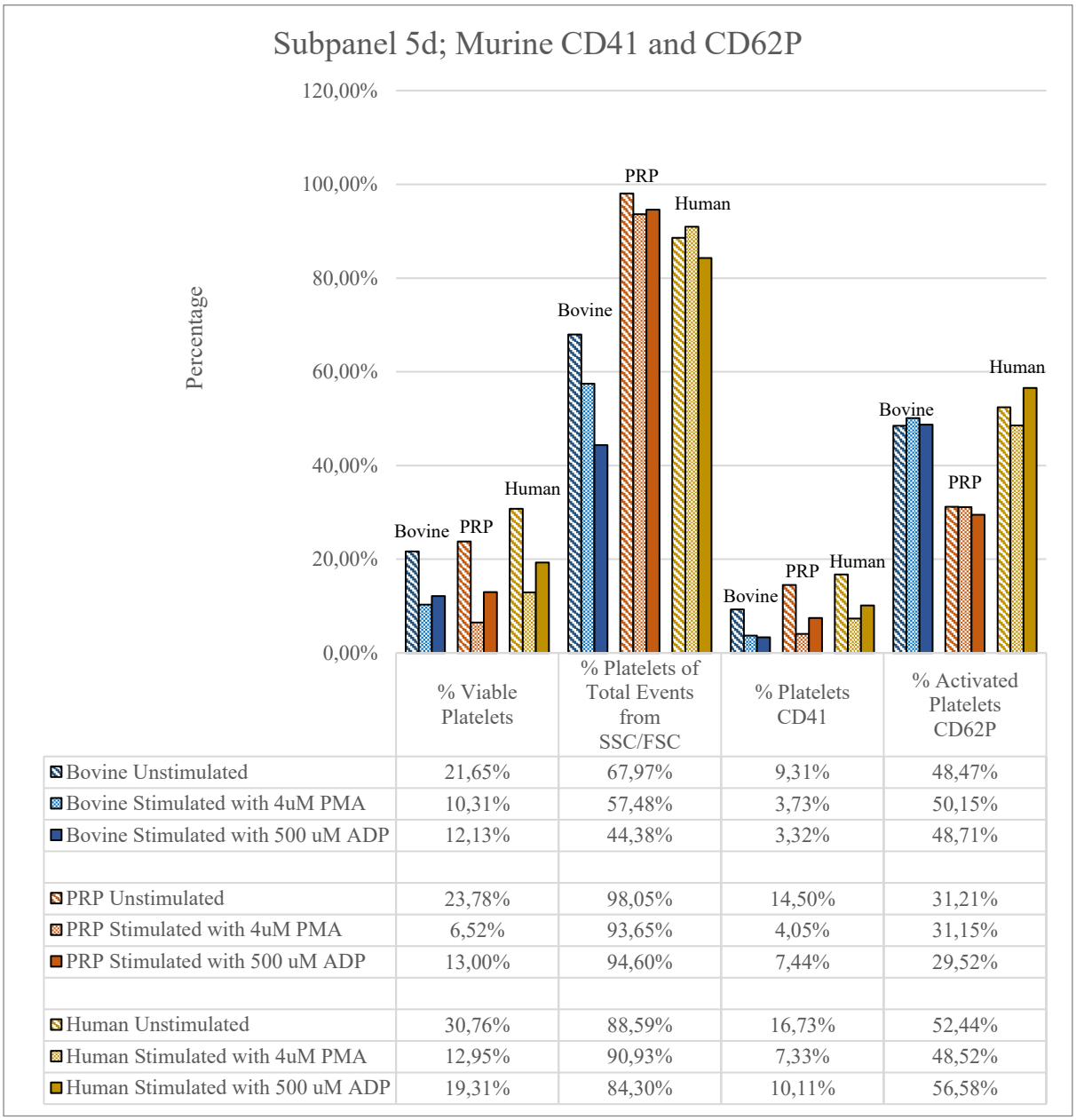


	% Viable Platelets	% Platelets of Total Events from SSC/FSC	% Platelets CD61	% Activated Platelets CD62P
Bovine Unstimulated	18,78%	60,92%	9,01%	56,56%
Bovine Stimulated with 4uM PMA	9,22%	52,90%	3,35%	57,70%
Bovine Stimulated with 500 uM ADP	11,78%	33,98%	3,31%	60,28%
PRP Unstimulated	20,70%	97,73%	18,18%	51,25%
PRP Stimulated with 4uM PMA	7,46%	92,40%	5,54%	51,35%
PRP Stimulated with 500 uM ADP	12,47%	93,49%	10,68%	51,76%
Human Unstimulated	15,68%	84,61%	9,45%	59,81%
Human Stimulated with 4uM PMA	10,03%	98,43%	6,16%	58,58%
Human Stimulated with 500 uM ADP	8,15%	85,90%	4,69%	64,20%

Figure 23. Use of flow cytometry to determine viable platelets, platelets from SSC/FSC, platelets expressing human cross-reactive antibodies, CD61 and CD62P. Unstimulated and stimulated with ADP and PMA samples of Panel 5c. PMA: Phorbol myristate acetate; ADP: Adenine Diphosphate; PRP: Platelet rich Plasma; SSC: Sideward Scatter; FSC: Forward Scatter.

Figure 23 above, shows the percent of activated, viable, SSC/FSC platelets as well as CD61+ platelets. Firstly, the percent of activated platelets in initial unstimulated samples is above 50% in all samples. An increase in percent activated platelets is observed upon stimulation with PMA and ADP in all types of blood. The increase of activated platelets upon stimulation with PMA and ADP determined by CD62P antibodies, in PRP samples is 0,1% and 0,51%, respectively. The percent change of activated platelets in human blood samples upon stimulation with PMA and ADP were, -1,23% and 4,39%, respectively. In this Panel a decrease of viable platelets is observed in all blood samples upon stimulation. CD61, a platelet-specific antibody was used to stain platelets. Platelets were also determined by gating in SSC/FSC plots. The mean standard deviation for the percent platelets determined by CD61-positive events in

comparison with platelets determined by SSC/FSC gating plots in PRP and human blood samples were: 58,74%, and 58,61% respectively.



	% Viable Platelets	% Platelets of Total Events from SSC/FSC	% Platelets CD41	% Activated Platelets CD62P
Bovine Unstimulated	21,65%	67,97%	9,31%	48,47%
Bovine Stimulated with 4uM PMA	10,31%	57,48%	3,73%	50,15%
Bovine Stimulated with 500 uM ADP	12,13%	44,38%	3,32%	48,71%
PRP Unstimulated	23,78%	98,05%	14,50%	31,21%
PRP Stimulated with 4uM PMA	6,52%	93,65%	4,05%	31,15%
PRP Stimulated with 500 uM ADP	13,00%	94,60%	7,44%	29,52%
Human Unstimulated	30,76%	88,59%	16,73%	52,44%
Human Stimulated with 4uM PMA	12,95%	90,93%	7,33%	48,52%
Human Stimulated with 500 uM ADP	19,31%	84,30%	10,11%	56,58%

Figure 24. Use of flow cytometry to determine viable platelets, platelets from SSC/FSC, platelets expressing murine cross-reactive antibodies, CD41 and CD62P. Unstimulated and stimulated with ADP and PMA samples of Panel 5d. PMA: Phorbol myristate acetate; ADP: Adenine Diphosphate; PRP: Platelet rich Plasma; SSC: Sideward Scatter; FSC: Forward Scatter.

In Figure 24, the flow cytometric results of Subpanel 5d in which murine-cross reactive antibodies were used are shown. There is no increase in activated platelets detected by CD62P upon stimulation with ADP or PMA in bovine platelet rich plasma samples. For human blood samples the change in percent of activated platelets is at -3,92% upon stimulation with PMA and 4,14% upon stimulation with ADP. In this Subpanel, a decrease in viability is also apparent

upon stimulation. The mean standard deviation for platelets determined by SSC/FSC plots and CD41 (murine platelet specific antibody) for PRP samples is 61,36% and 54,13% for human samples.

Panel 6 Anti-human CD61 and CD62P

As described earlier (4.3.2.1), due to the unsuccessful attempt of detecting activated platelets in both human and bovine blood with human antibodies in Panel 5c, the procedure was repeated with only human blood in Panel 6. In Panel 6, the sample volume was reduced and a change in stimulation procedure of active samples was applied.

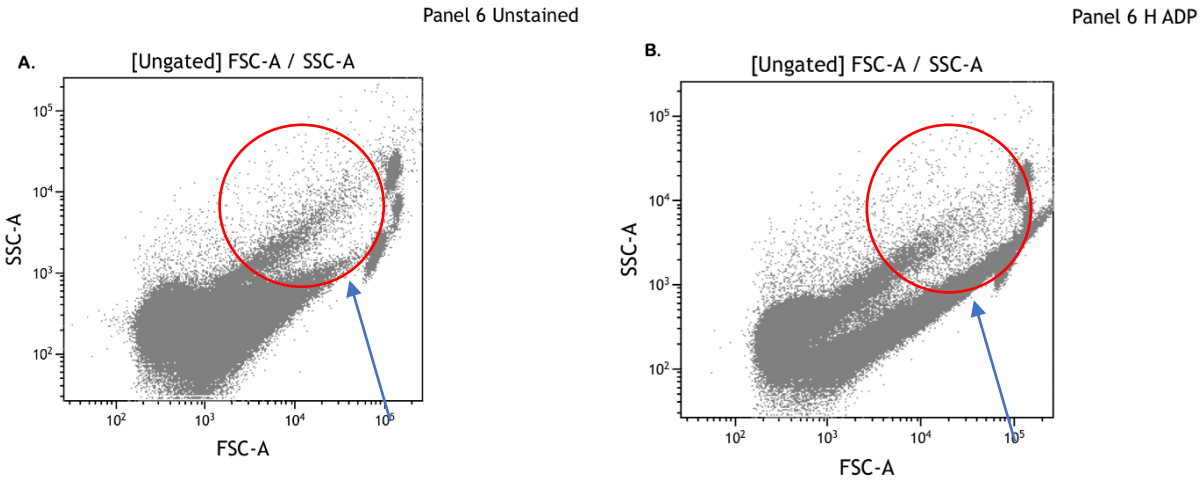


Figure 25. Forward and Sideward Scatter of Panel 6. SSC-A: Sideward Scatter Area; FSC-A: Forward Scatter Area; ADP: Adenosine Diphosphate A: Unstimulated Sample of Panel 6; B: Stimulated sample of Panel 4a with 10uM ADP for 1 minute.

A slight upward shift of SSC/FSC events upon stimulation with ADP can be seen in Figure 25. Upon stimulation the events of the sideward and forward scatter form two distinct populations. (Figure 25.B.).

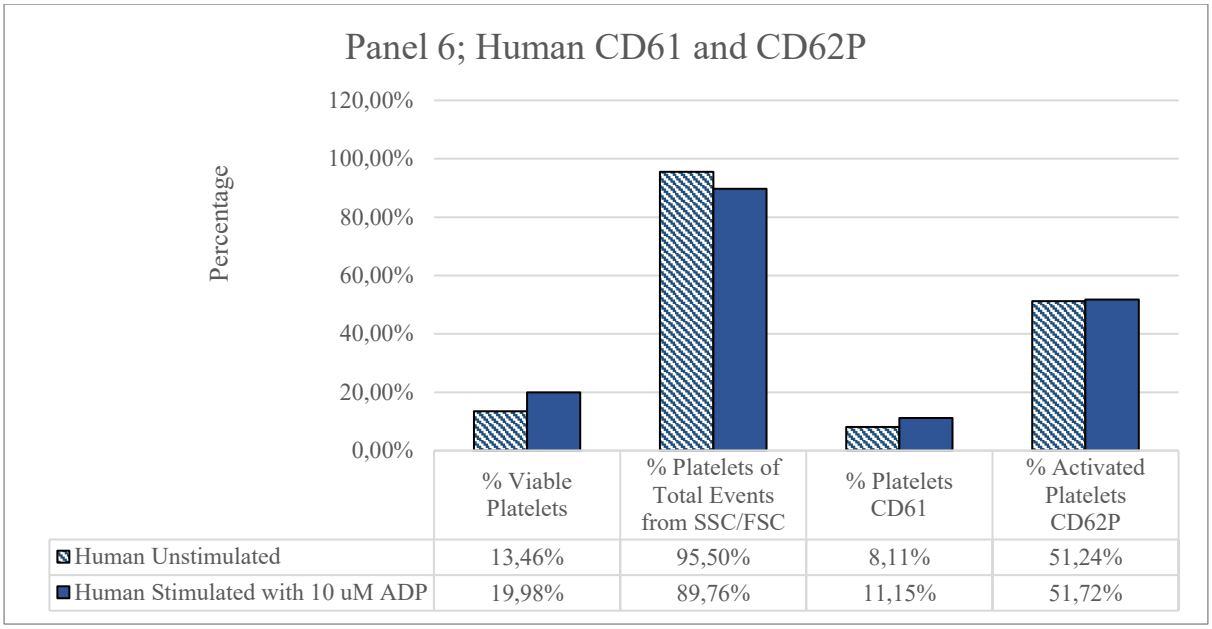


Figure 26. Use of flow cytometry to determine viable platelets, platelets from SSC/FSC, platelets expressing human cross-reactive antibodies, CD61 and CD62P. Unstimulated and stimulated with 10uM ADP human blood samples of Panel 6. ADP: Adenine Diphosphate; PRP: Platelet rich Plasma; SSC: Sideward Scatter; FSC: Forward Scatter.

Figure 26 above, shows the data obtained from measurements of stimulated and unstimulated human whole blood samples with flow cytometry. An 6,53% increase in viable platelets upon stimulation is observed. The percent increase of activation observed with CD62P and CD61 positive events is 0,48% upon stimulation. The standard deviation for determined platelets with CD61 antibody in comparison to determining activated platelets with SSC/FSC plot was 61,79% for unstimulated samples and 55,59% for stimulated samples.

6 Discussion

This study aimed to propose a reproducible protocol for testing the thrombogenicity potential of the HM III. The ACT results demonstrated the thrombogenic risk of the HM III while the results of the flow cytometric analysis of platelet activation revealed key aspects that should be avoided during the staining procedure. The findings also suggested that using PRP instead of whole blood, ADP instead of PMA as a positive control, and same day staining is pivotal to increase the efficiency of the detection of activated platelets in bovine blood.

6.1 Activated Clotting Time and Loop Experiment

Figure 10 shows the ACT measured in the first three loop experiments which correspond to the blood used in Panels 1-3. As mentioned earlier, a general decrease in ACT was observed in the hourly taken samples. Similar declines were reported by Brockhaus et al.[37]. General decrease of ACT over time is an indicator for the dissociation of the low molecular weight heparin that was used. The higher ACT values of the static control in comparison to the ACT values shown by samples extracted from the loops indicated the thrombogenic potential of HM III devices. The ACT, however, only assesses the function of the intrinsic pathway by its initial activation with Kaolin. The ACT does not provide information about the platelet activity and thus a platelet activation assay such as FACS is required to determine the platelet activating potential of LVAD devices.

6.2 Platelet Activation Assay

6.2.1 Positive Control

The stimulation of samples with PMA or ADP aimed to serve as a control for the gating and the detection of activated platelets. A successful positive control is established if the number of activated platelets in stimulated samples is higher in comparison with unstimulated samples. If this is not the case, the gating strategy and detection Panel cannot be established.

A large concern in this study was the sensitivity of platelets. The ineffectiveness of ADP or PMA in causing an upward shift of events in the SSC/FSC plots of several Panels (such as Panel 3, Figure 13), may be due to the excessive activation of platelets prior to the experiments. Many factors may contribute to the activation of platelets. Firstly, blood obtained from the slaughterhouse may have a high percent of activated platelets, caused by blood collection. The transportation of blood to the laboratory may have also contributed to an increase of activated platelets. Further, the filtering of blood may cause additional activation. Consequently, the addition of an activating reagent may not impose a high activation as with freshly extracted samples with a lower proportion of activated platelets. This is important to keep in mind in future experiments.

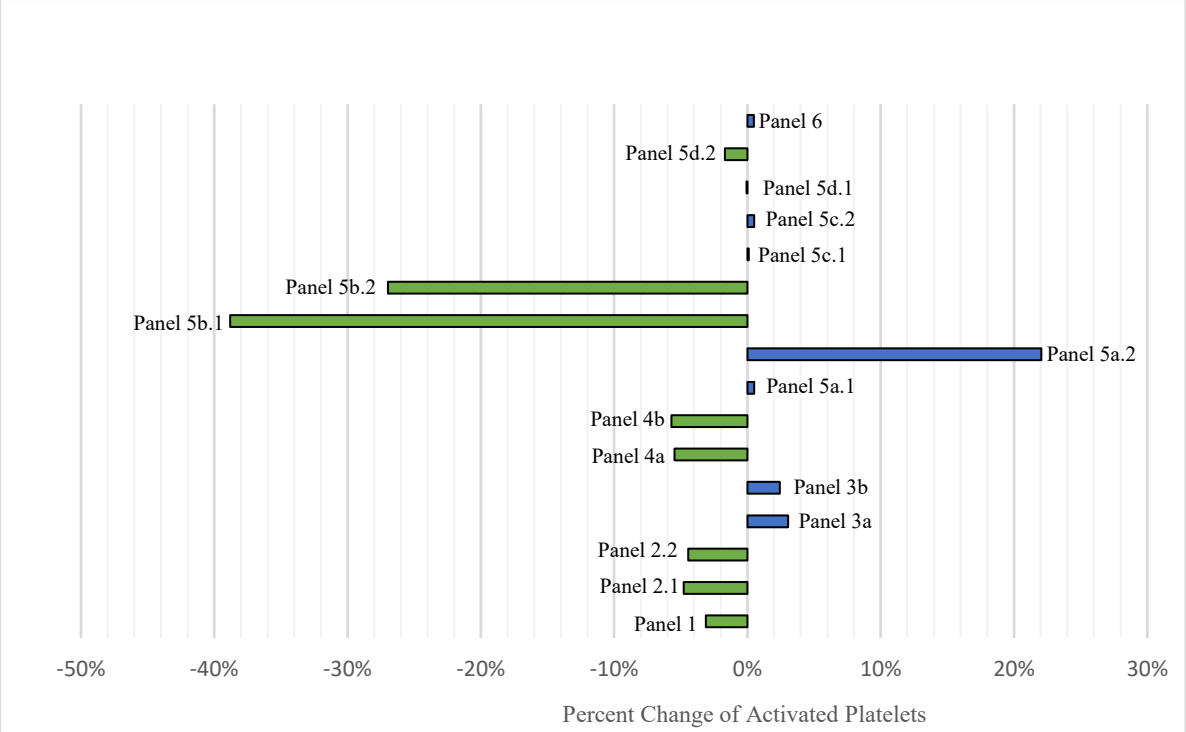


Figure 27. Percent difference of detected activated platelets upon stimulation of samples with either PMA or ADP in comparison to unstimulated samples. Panel 1: 4uM PMA detection using CAPP2A; Panel 2.1: 4uM PMA detection using CAPP2A; Panel 2.2: 12uM PMA detection using CAPP2A; Panel 3a: 4uM PMA detection using CAPP2A; Panel 3b: 4uM PMA detection using CAPP2A; Panel 4a: 4uM PMA detection using CAPP2A; Panel 4b: 4uM ADP detection using CAPP2A; Panel 5a.1: 4uM PMA detection using Annexin; Panel 5a.2: 500uM ADP detection using Annexin; Panel 5b.1: 4uM PMA detection using Apotracker; Panel 5b.2: 500uM ADP detection using Apotracker; Panel 5c.1: 4uM PMA detection using Antihuman CD62-P; Panel 5c.2: 500uM ADP detection using Antihuman CD62-P; Panel 5d.1: 4uM PMA detection using Antimouse CD62-P; Panel 5d.2: 500uM ADP detection using Antimouse CD62-P; Panel 6: 10uM ADP detection using Antihuman CD62-P.

Figure 27 above shows the percent difference in detected activated platelets between stimulated and unstimulated samples in all panels of this study. Generally, the results of Panel 1-4 showed the ineffectiveness of CAPP2A in the detection of activated platelets after stimulation with various concentrations of ADP and PMA. As displayed in Figure 27, neither the stimulation of samples with ADP nor the respective stimulation with PMA caused a significant increase in the expression of CAPP2A in Panels 1-4. The stimulation was able to cause an upward shift of events in the SSC/FSC plots (Figure 11) of Panels 1 and 2. Meaning that stimulation with 4uM PMA was successful ins panels 1 and 2. Subsequent detection with CAPP2A, however, in Panels 1,2 and 4 even showed decline in percent activated platelets upon stimulation. Panel 3 showed an increase of CAPP2A detected activated platelets of below 5%.

6.2.2 Measurement Day

Given these findings different staining approaches were tested in Panel 5. In Subpanel 5a, Annexin was used for the detection of activated platelets. Annexin binds to phosphatidylserine receptors expressed on the surface of activated platelets. In Panel 5a the increase in expression of Annexin in PRP samples upon stimulation with 4uM ADP for 1 hour was 22,05% (Panel 5a.2 in Figure 27). This is a considerable increase in comparison, to other Subpanels of Panel 5. The major underlying difference in Subpanel 5a, was that, due to the instability of Annexin, flow cytometric measurement of samples were conducted on the same day. Even though blood samples of all Panels were treated with gentamycin and antimycotic solution, the results displayed in Figure 27 showed that storage of samples seems to be problematic. This may either be due to contamination of samples but can also be due to the cold temperatures at which the samples are stored at. Furthermore, in Subpanel 5b, Apotracker green was used which, similar to Annexin, also binds to phosphatidylserine on the surface of activated platelets. Apotracker allows for the storage of samples and thus measurements the next day. In both Figure 21 and Figure 27 a decrease of Apotracker detected activated platelets is observed upon stimulation with both ADP and PMA in PRP samples. This further elucidates the importance of measuring the samples on the same day.

6.2.3 Whole Blood vs. Platelet Rich Plasma

In Subpanel 5a, Annexin V staining was also tested with human and bovine whole blood samples. According to the producer's supplemental information, Annexin V is cross reactive for all mammalian species and can thus be utilized for both human and bovine blood. Upon stimulation with both ADP and PMA, both whole blood sample types showed a decrease in Annexin detected activated platelets. Even though all the samples, (bovine whole blood, human whole blood and bovine platelet rich plasma) were measured the same day, the PRP samples were the only samples to show an excessive increase of activated platelets of 22% upon stimulation. This is an indication for the interfering cells presented in whole blood samples. These results show the potential benefit of using platelet rich plasma.

The results of Subpanel 5c further confirm this: In Subpanel 5c, human cross-reactive antibodies were used. CD61 was firstly used to stain platelets, and CD62P was then used to stain the portion of activated platelets. In Subpanel 5c the percent of platelets determined by

the SSC/FSC dot plots is higher than that determined by the platelet specific antibodies used in this Panel (CD61). As mentioned earlier, in the human whole blood samples of Subpanel 5c, the standard deviation between the detected platelets using CD61 and the platelets detected through gating of SSC/FSC plots was 58,61%. This goes to show that other cells present in whole blood samples may be expressed on the same region as the platelet gate potentially confounding the interpretation of the results. The use of whole blood may thus promote unspecific binding and falsification of corresponding results.

Unfortunately, the results of Subpanels 5c and 5d show the ineffectiveness for the use of human or murine cross-reactive antibodies in bovine blood. This is shown in Figures 23 and 24 in which the percent platelets determined by the SSC/FSC plots in bovine PRP samples is not identical to the percent platelets detected by the platelet specific antibodies CD41 and 61. And thus, the bovine PRP samples of Subpanels 5c and 5d do not further confirm this presumption (that PRP is more efficient than whole blood for the gating of platelets). A staining experiment with PRP human samples can be done to further confirm the use of platelet rich plasma as opposed to whole blood samples. The need for a platelet specific antibody is shown in Panel 5c. The percent of platelets detected through the forward sideward gate was 33,98 %. The percent of CD41 positive (platelet specific antibody) was only 3,31% in the bovine blood sample.

6.2.4 Number of Events

Another major confounder presented was that the number of events detected by the flow cytometer per unit time significantly differed from sample to sample. (9.4.3) This can be due to multiple reasons. The first reason may be that the samples are not homogenously mixed before measurement in the FACS machine. The second reason, which is probably the most apparent, would be that during the washing procedure, the supernatant is partially aspirated (Integra Vacusafe). During this step the chance for inaccuracies is high. Either the pellet, with the highest cell population may have partially been aspirated in some samples, or the supernatant and the pellet may have been mixed before the aspiration step. The settings in FACS measurement were not set to limit the number of cells that should be measured per sample. In our study, the limit was set per unit time. Several studies[52] suggest 10 000 as a set limit of detected events by FACS. Tscharre et al.[52] have also suggested to dilute whole blood with phosphate-buffered saline to obtain $20x10^3$ /µL platelets in 20µL samples. Additionally,

aspiration may be done manually with a pipette to gain more control over the aspirated supernatant and prevent the aspiration of the pellet.

6.2.5 Directly Conjugated Antibodies

Fixation of samples is essential to avoid activation of platelets during the staining procedure. Platelet activation may occur through pipetting, centrifugation, washing and aspirating. Hence, directly conjugated antibodies may reduce the complexity of the staining procedure and the amount of centrifugation steps. Thus, extensive platelets activation due to sample handling could be avoided.

6.2.6 Viability

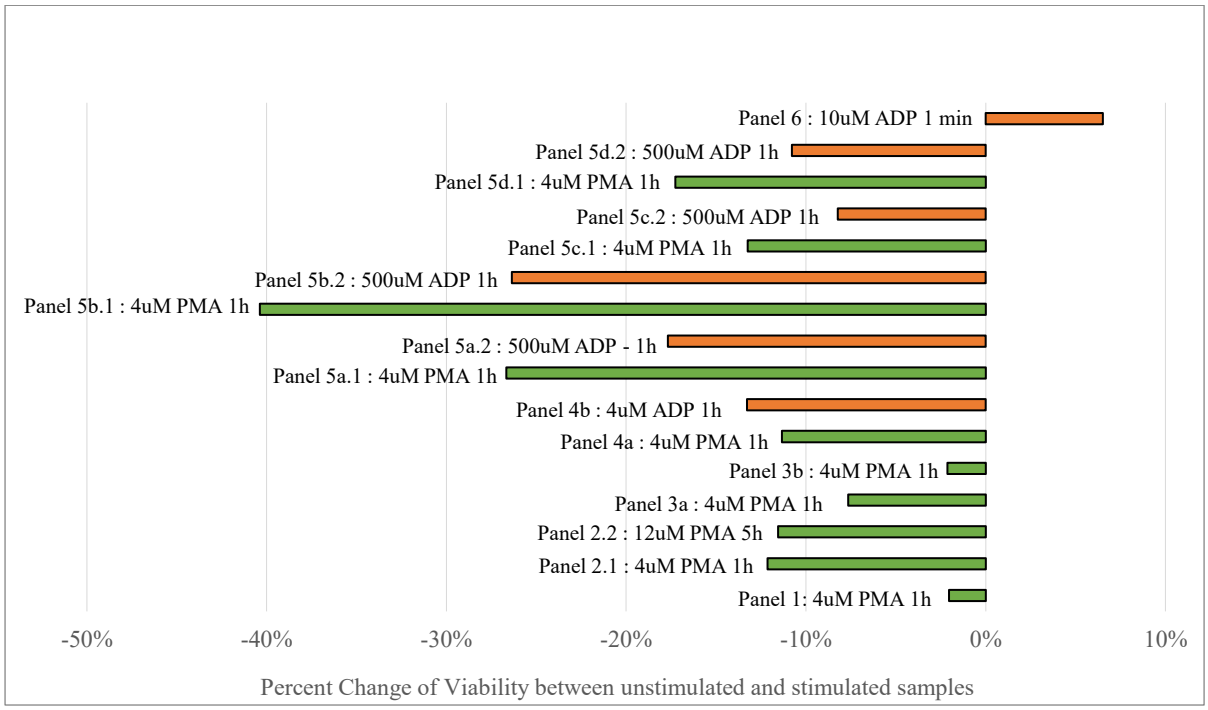


Figure 28. Percent change of viability of all cells determined by FVD500 between unstimulated and stimulated samples. Green; PMA, Orange; ADP. PMA: Phorbol myristate acetate; ADP: Adenosine Diphosphate; FVD500: Fixable Viability Dye.

Another important aspect was that a decrease in viability was almost always observed upon stimulation with ADP or PMA. Figure 28, above shows the percent change of viability of all Panels upon stimulation. Generally, PMA has caused a higher decrease of viability of cells than ADP. Moreover, in Panel 2 (2.1 vs 2.2, Figure 28) it is observed that an increase in concentration and incubation time of PMA causes no additional decrease in viability. Similarly, an increase of the concentration of ADP from 4uM to 500uM does not cause a significant

additional decrease in viability of samples (Panel 4b vs Panel 5a.2, Figure 28). This means that, using PMA for the stimulation of samples may decrease the sensitivity and proportion of detected activated platelets because the stimulant PMA causes the cells to undergo apoptosis (increased number of dead cells). Dead cells are not included in the gating of activated platelets as previously described in the gating strategy (4.3.2.2). The stimulation with ADP is proven to be beneficial as the decrease of viability in cells is much lower compared to that caused by PMA.

6.2.7 Lysing/Fixing Procedure

After large artefacts were presented in the SSC/FSC dot plots of Panel 1, the fixing and lysing order was altered (4.3.2.1). Panels 2 and 3 remained to show large partial artifacts. In Panel 4b significantly less artifacts were displayed in the SSC/FSC plots (Figure 15) than in Panel 1-4a. The difference between Subpanels 4a and 4b was that the lysing/fixing procedure was altered. In Subpanel 4a lysing occurred before fixing. This may be an indication that the lysing solution has an impact on cells other than red blood cells present in the samples. Fixing samples beforehand seems to be important to prevent artifacts.

An interesting observation is that the viability decrease presented upon the addition of the same concentration and incubation time of PMA in Panel 3a was higher than in 3b (Figure 28). The probable reason for this is that in 3a lysing of red blood cells occurred before fixing of samples. In 3b, lysing of red blood cells occurred after fixing. This is firstly a repeated indication of the interfering red blood cells present in the whole blood samples. This may also indicate that lysing, may decrease the viability of platelets. Panel 3 clearly shows that fixing samples first will interfere with lysing occurring afterwards. This problem is eliminated with the use of platelet rich plasma.

In the dot plots of Panels 4a and 4b (Figure 15), it is apparent that the procedure of Subpanel 4a yields to a higher proportion of artifacts presented in the dot plots than with Subpanel 4b. In Subpanel 4b fixing occurred with paraformaldehyde (PFA) which may be the ideal fixing reagent for this study in comparison to the “Streck cell preservative” reagent used in Subpanel 4a.

6.2.8 Staining Control

An important aspect that was disregarded in this study is the concept of unspecific binding. When antibodies are used it is possible that antibodies bind to receptors other than the target receptor. The study by Chan et al,[38] and Radley et al.[42] seemed to have also neglected this aspect while measuring platelet activation in bovine whole blood. Several studies [52] have used isotype antibodies to prevent unspecific binding of antibodies used to stain target receptors. Isotype antibodies are raised against antigens not found on the target cell. The isotype antibody should have the same fluorophore, host species and Ig subclass as the primary antibody intended for use. The isotype antibody allows to eliminate further background signal through unspecific binding, which will probably improve the visibility of events, reduce artefacts, and improve the accuracy of the staining results. In this study, compensation was calculated using the FACS Diva software to prevent overlapping of multiple fluorophores. Unstained samples were also used to calculate autofluorescence. Unspecific binding of antibodies, however, was completely disregarded and may be an important aspect to consider in future FACS staining studies.

6.2.9 Different Assay for Testing Platelet Activation Potential

Another assay that may be utilized for the quantification of the platelet activating potential of LVAD devices, is the ROTEM analysis suggested by Brockhaus et al. [37] The parameter this study primarily focused on, was thromboelastometry. This method makes use of a rotating pin inside a cuvette in which a blood sample is inserted. Coagulation parameters such as time taken to form a clot causing restriction, i.e. clotting time and maximum clot firmness can be utilized as an indicator for the formation of a blood clot. The standard EXTEM and FIBTEM tests were performed to gain more information about the present coagulation system. In both EXTEM and FIBTEM tests, thromboplastin an activator of the extrinsic pathway is added. Additionally, in only FIBTEM cytochalasin D a platelet inhibitor was added. By comparison of the results from EXTEM and FIBTEM tests, the clotting potential due to platelet activation can be determined.

7 Conclusion

The activated clotting time proved to be a good method to determine the thrombogenicity of blood. The use of flow cytometric platelet activation analysis for thrombogenicity testing of LVADs revealed to be a very complex process due to the sensitivity of platelets. The results of this study show several points that need to be considered when attempting to stain activated platelets in bovine blood. All samples should be measured the same day. Ideally, platelet rich plasma samples should be used to eliminate the interference of other blood cells, reduce artefacts, and simplify the procedure by skipping the lysing step which clearly interferes with the SCC/FSC dot plots. Furthermore, the use of platelet specific antibodies such as CD61 and CD41 would prove to be beneficial and increase accuracy of results. Isotype antibodies should also be used. Due to the complexity of the staining procedure, it is recommended to first establish a fixed staining protocol using static experiments. Once a staining protocol is established, hourly taken samples from loops can be used to test for the activation of platelets during operation of HM III over time. An interesting aspect would be the initial plan of this study, that is the comparison of two modes of action of the HM III in terms of platelets activation. Other methods such as the ROTEM analysis suggested by Brockhaus et al. may also be utilized with bovine blood for the detection of activated platelets in LVADs.

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9 Appendix

9.1 Extrinsic and Intrinsic Pathways of Secondary Hemostasis

Vascular smooth muscle cells express tissue factor (TF) which is the sole initiator for the extrinsic pathway.[21] Upon injury, vessel rupture causes extrinsic TF to come in contact with FVII forming the "extrinsic tenase". This further activates FX and leads towards inducing the common pathway, with the "intrinsic tenase".(Figure 29)

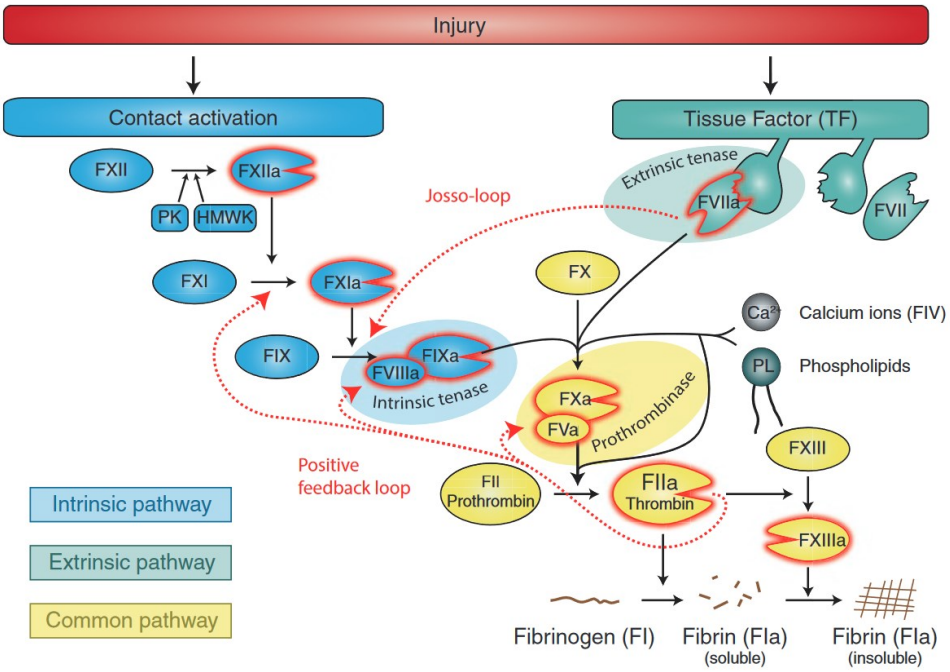

Figure 29. Overview of the coagulation cascade, Intrinsic and Extrinsic Pathways[21].

The extrinsic pathway initiates the coagulation cascade, thrombin formation however is solely dependent on the interaction of the intrinsic and extrinsic pathway. Concomitant activation of the coagulation cascade results in thrombin production which not only cleaves fibrinogen to fibrin, but also induces further platelet activation. [21] Hemostasis is a regulated process in which endothelial cells constantly release prostacyclin, thrombomodulin and nitric oxide to prevent platelet activation or thrombin binding.[21]

Physical contact towards negatively charged surfaces and/or other substances such as polyphosphates initiate the conformational change of FXII. FXII is the initiator of the intrinsic pathway. This activates FXI and thus activating FIX. FIX forms with FIIIa the intrinsic tenase. This interacts with FX from the extrinsic pathway to activate FV and form a "prothrombinase"

complex. This ultimately activates prothrombin to thrombin and cleaves fibrinogen into fibrin. Thrombin is also responsible for activating FXIII (transglutaminase) which stabilizes the fibrin mesh and ensures it becomes insoluble.

9.2 Overview of used Materials and Devices

Material	*Manufacturer*
INHIXA, low molecular weight heparin	*Techdow, Guangdong, China*
Gentamycin Solution, *G1397*	*Sigma Aldrich, Missouri, USA*
Antimycotic Solution, A5955	*Sigma Aldrich, Missouri, USA*
Sysmex XN 350	*Sysmex, Kobe, Japan*
pH Meter	*1000 L VWR, Pennsylvania, USA*
ACT Test Tubes, HRFTK-ACT	*Werfen, Barcelona, ESP*
Hemochron Response	*Accriva, California, USA*
Fixable Viability Dye eFluor506, *65-0866-14*	*Invitrogen, Massachusetts, USA*
FACS Buffer, *FC001*	*R&D Systems, Minnesota, USA*
FACS Lysing Solution, *349202*	*BD Bioscience, New Jersey, USA*
Streck cell preservative, *213358*	*Streck, Nebraska, USA*
Phorbol myristate acetate, *16561-29-8*	*Sigma Aldrich, Missouri, USA*
BD FACS Canto II	*Becton Dickinson, New Jersey, USA*
FACS Diva Software	*Becton Dickinson, New Jersey, USA*
Kaluza	*Beckman, California, USA*

Table 7. Overview of materials used and corresponding manufacturers.

Antibodies	*Manufacturer*
CAPP2A Antibody ***NBP2-60899***	*Novus Biologicals, Colorado, USA*
Alexa Fluor 448 goat anti-mouse, *A-11029*	*Life Technologies, California, USA*
Alexa Fluor 635	*Life Technologies, California, USA*
Annexin V, *640905*	*Biolegend, California, USA*
Apotracker Green, *427401*	*Biolegend, California, USA*
CD61-FITC, Anti-Human, *336403*	*Biolegend, California, USA*
CD62P-AF647, Anti-Human, *304918*	*Biolegend, California, USA*
CD41-FITC, Anti-Mouse, ***133903***	*Biolegend, California, USA*
CD62P-PECY7, Anti-Mouse, *148309*	*Biolegend, California, USA*

Table 8. Overview of antibodies used and corresponding manufacturers.

9.3 Recipes

For the preparation of 5L Binding Buffer
- 500ml 10x Phosphate buffered saline *diluted with* ***4500ml ddH2O***
- 5g *Sodium* ***azide (toxic)***
- 5g Bovine serum Albumin
- 1050µl 1M *Sodium Hydroxide*

Table 9. Recipe for the preparation of 5L binding buffer in Panel 5a. ddH20: double distilled water.

9.4 Flow Cytometric Findings

9.4.1 Panel 4 forward and sideward scatter

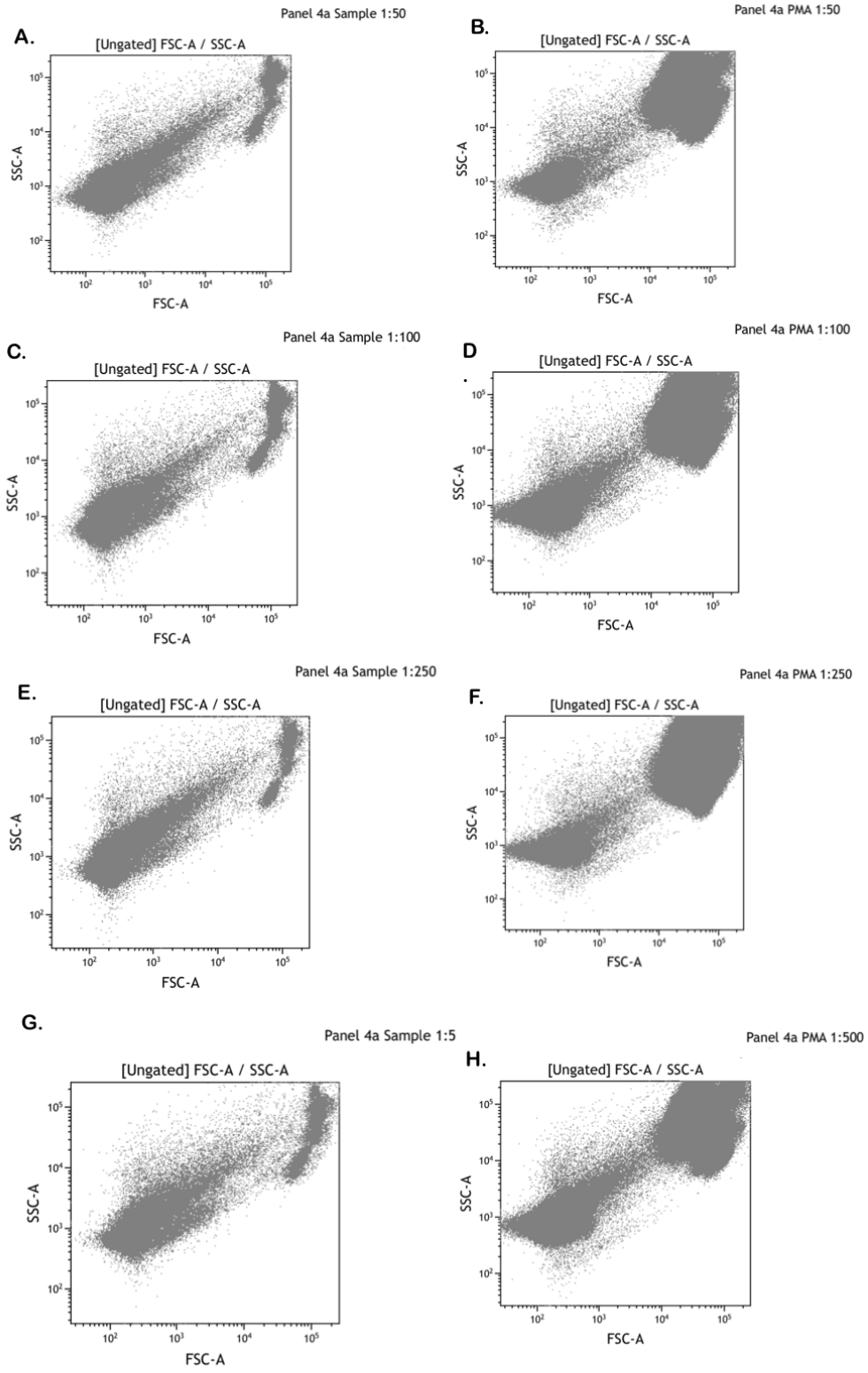

Figure 30. Forward and Sideward scatter of Subpanel 4a.

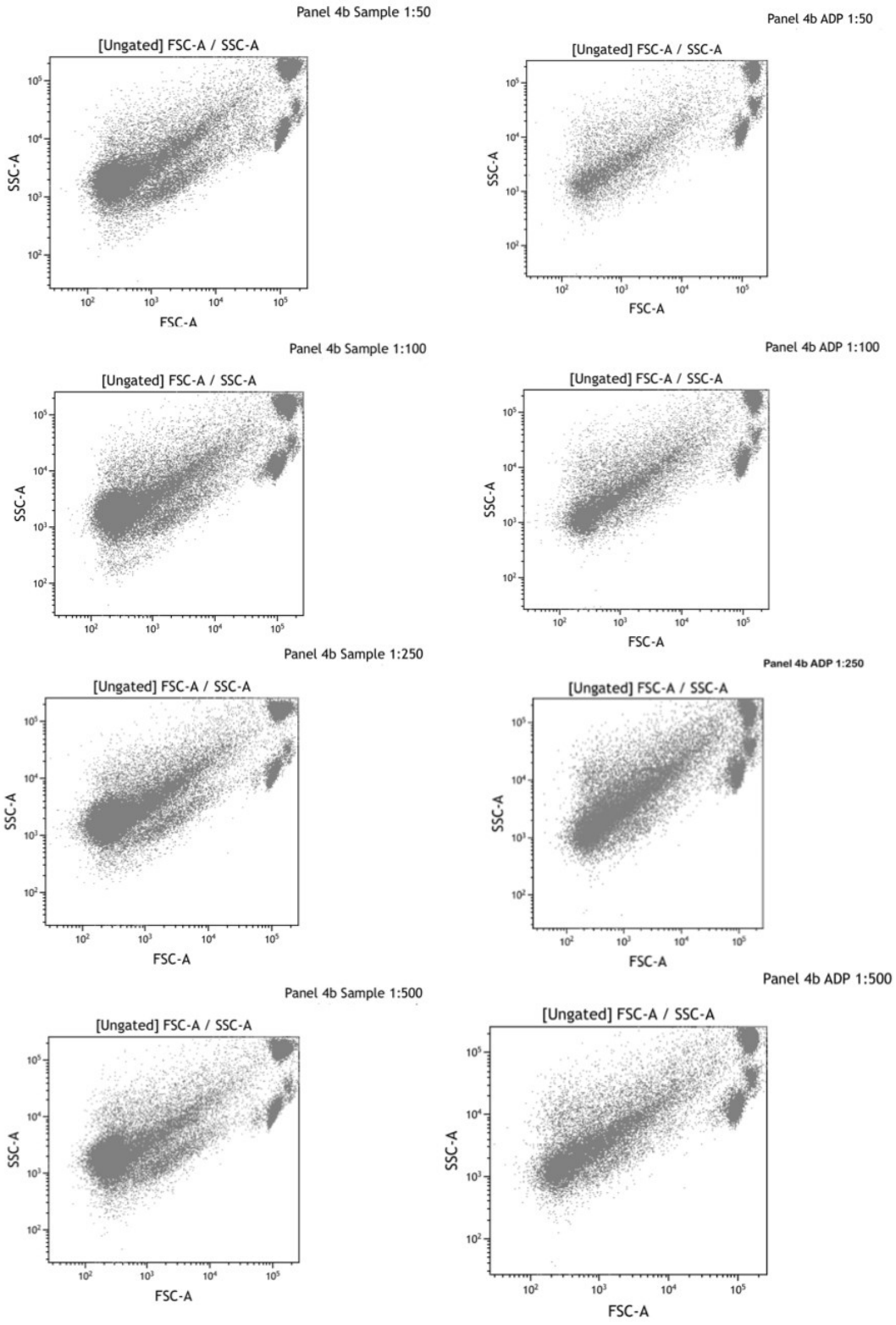

Figure 31. Forward and Sideward scatter of Subpanel 4b.

9.4.2 Panel 5 forward and sideward scatter

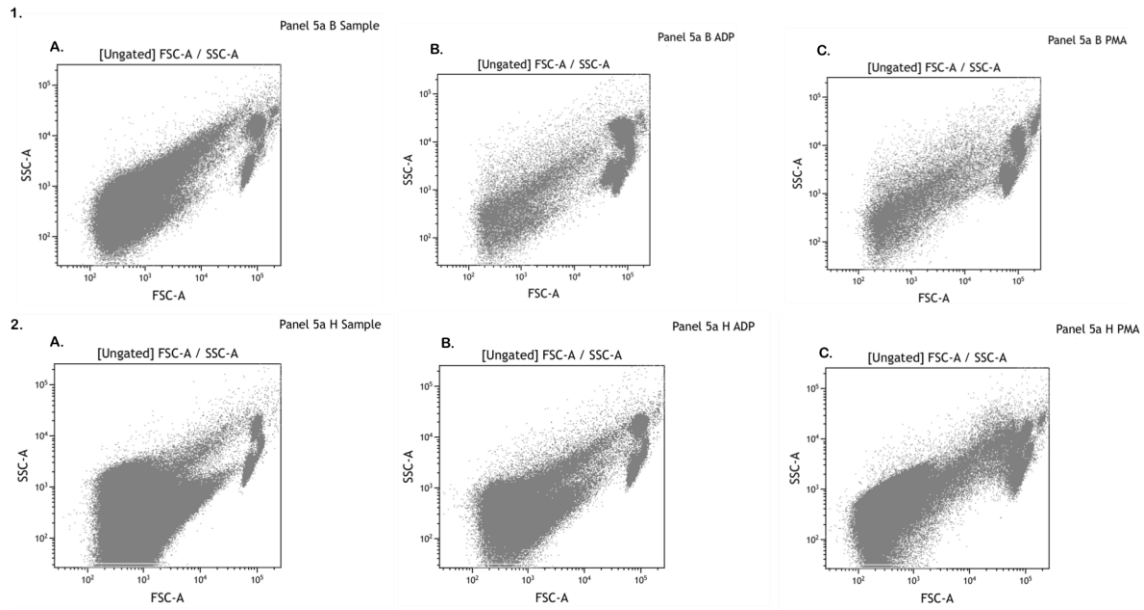

Figure 32. Forward and Sideward scatter of Subpanel 5a.

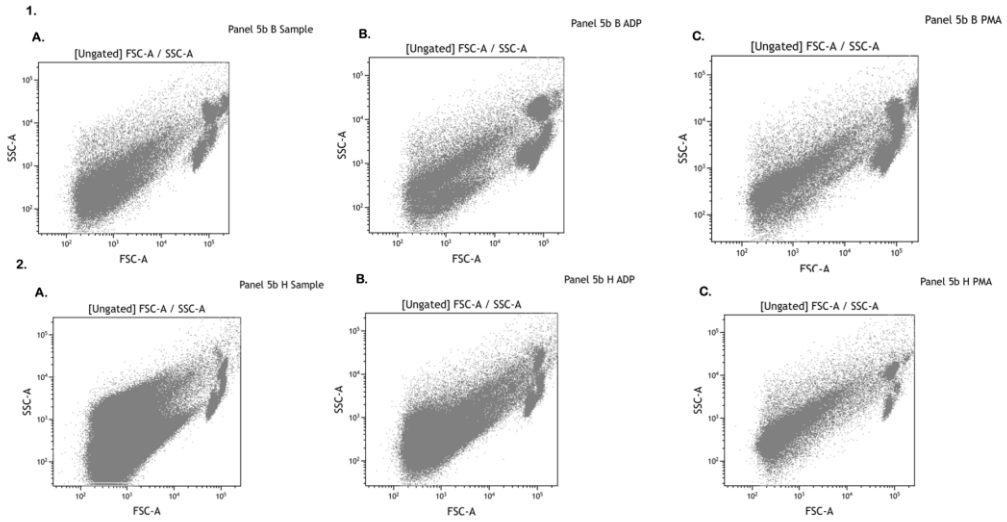

Figure 33. Forward and Sideward scatter of Subpanel 5b.

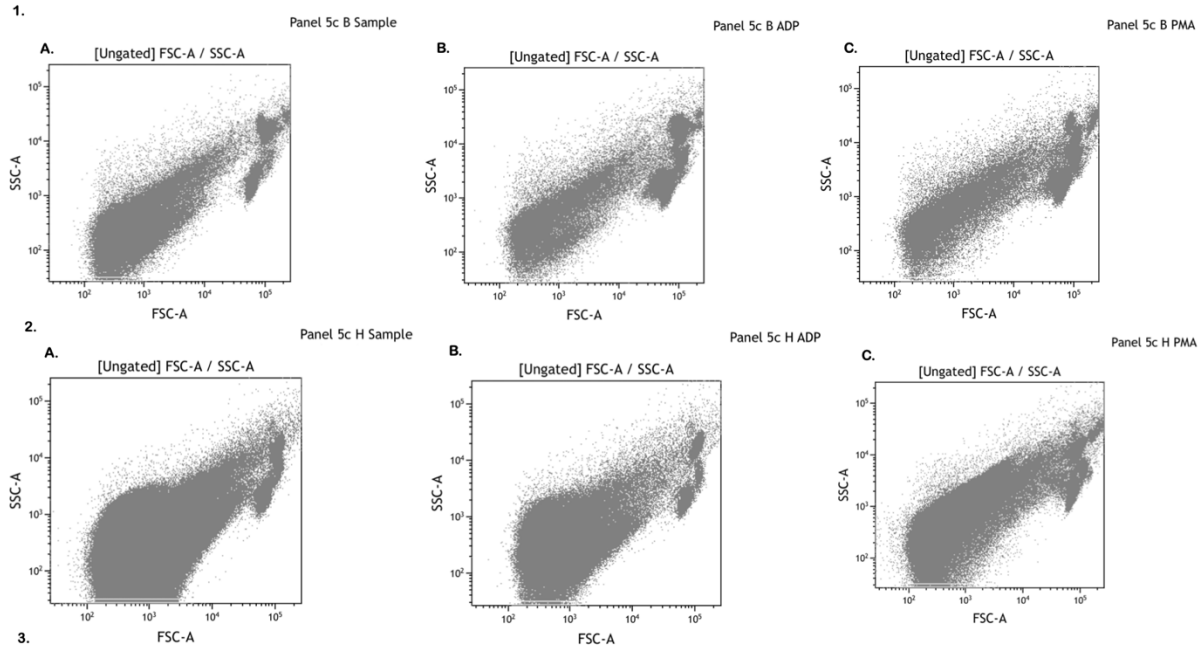

Figure 34 Forward and Sideward scatter of Subpanel 5c.

9.4.3 Total Platelet Activation Panel – Data

		Unstained	Unstimulated	Stimulated with PMA
Panel 1	% Activated Platelets	20.06 %	18.32 %	17.58 %
	% Viable Cells	40.26%	37.14 %	35.10 %
	% Platelets	63.39 %	60.50 %	90.45 %
	# Of Cells detected	557,982	246,726	287,145

Table 10 Flow Cytometric Results of Panel 1.

		Unstimulated	Stimulated with PMA
Panel 2	% Activated Platelets	16.37 %	11.59 % (12uM 11.93 %)
	% Viable Cells	39.98 %	27.84% (12uM 28,43%)
	% Platelets	81.70%	93.09% (12uM 95.51%)
	# Of Cells detected	1,598,481	160,164

Table 11 Flow Cytometric Results of Panel 2.

	Unstained	Unstimulated	Stimulated with PMA
Panel 3a	% Activated Platelets	12.37 %	15.43 %
	% Viable Cells	51.29 %	43,64 %
	% Platelets	80.41 %	91.54 %
	# Of Cells detected	634,025	295,325
Panel 3b	% Activated Platelets	12.67 %	15.10 %
	% Viable Cells	42.47 %	40.34 %
	% Platelets	94.08 %	87.08 %
	# Of Cells detected	726,775	355,875

Table 12 Flow cytometric results of Panel 3.

	Unstained	Unstimulated	Stimulated with PMA
Panel 4a 1:50	% Activated Platelets	17.30 %	14.54 %
	% Viable Cells	40.44 %	34.70 %
	% Platelets	91.04 %	47.75 %
	# Of Cells detected	248.225	481.875
Panel 4a 1:100	% Activated Platelets	16.44 %	16.36 %
	% Viable Cells	40.59 %	37.50 %
	% Platelets	85.20 %	54.60 %
	# Of Cells detected	186.350	1.202.825
Panel 4a 1:250	% Activated Platelets	17.79 %	12.32 %
	% Viable Cells	41.40 %	30.07 %
	% Platelets	94.98 %	21.41 %
	# Of Cells detected	276.200	1.986.675
Panel 4a 1:500	% Activated Platelets	13.98 %	16.69 %
	% Viable Cells	38.67 %	38.22 %
	% Platelets	80.26 %	83.85 %
	# Of Cells detected	105.200	1.584.650

Table 13 Flow cytometric results of Subpanel 4a.

		Unstained	Unstimulated	Stimulated with ADP
Panel 4b 1:50	% Activated Platelets	9.86 %	8.23 %	4.08 %
	% Viable Cells	25.00	29.84 %	21.81 %
	% Platelets	67.06 %	55.59 %	31.82 %
	# Of Cells detected	83.025	52.450	21.125
Panel 4b 1:100	% Activated Platelets	9.86 %	9.36 %	4.63 %
	% Viable Cells	25.00	30.05 %	17.59 %
	% Platelets	67.06 %	61.06 %	40.27 %
	# Of Cells detected	83.025	60.175	33.925
Panel 4b 1:250	% Activated Platelets	9.86 %	8.85 %	3.15 %
	% Viable Cells	25.00	28.95 %	15.67 %
	% Platelets	67.06 %	62.82 %	41.59 %
	# Of Cells detected	83.025	59.900	44.325
Panel 4b 1:500	% Activated Platelets	9.86 %	9.33 %	3.30 %
	% Viable Cells	25.00	31.48 %	17.08 %
	% Platelets	67.06 %	62.18 %	40.24 %
	# Of Cells detected	83.025	57.075	36700

Table 14 Flow cytometric results of Subpanel 4b.

		Unstained	Unstimulated	Stimulated with PMA	Stimulated with ADP
Panel 5a AxV PRP	% Activated Platelets	-	5.74 %	6.24 %	27,79%
	% Viable Cells	43.66 %	93.39 %	66.72 %	75.71 %
	% Platelets	98.35 %	98.65 %	91.53 %	92.91 %
	# Of Cells detected	37250	35050	10900	9750
Panel 5a AxV Human	% Activated Platelets	-	23.73 %	9.21 %	16.50 %
	% Viable Cells	39.72 %	43.20 %	14.69 %	29.58 %
	% Platelets	86.90 %	88.45 %	97.78 %	77.31 %
	# Of Cells detected	460.000	452.400	1.624.825	121.475

Table 15 Flow cytometric results of Subpanel 5a.

		Unstained	Unstimulated	Stimulated with PMA	Stimulated with ADP
Panel 5b Bovine	% Activated Platelets	3.22 %	35.71 %	12.32 %	26.77 %
	% Viable Cells	5.01 %	39.50 %	14.33 %	30.44 %
	% Platelets	40.17 %	40.78 %	49.14 %	31.11 %
	# Of Cells detected	101650	98975	65825	107900
Panel 5b PRP	% Activated Platelets	12.19 %	49.24 %	10.43 %	22.27 %
	% Viable Cells	19.88 %	51.80 %	11.42 %	25.44 %
	% Platelets	95.67 %	96.91 %	92.81 %	94.44 %
	# Of Cells detected	65500	57525	44200	31975
Panel 5b Human	% Activated Platelets	1.76 %	20.71 %	9.45 %	9.30 %
	% Viable Cells	2.25 %	35.39 %	20.55 %	11.09 %
	% Platelets	90.59 %	91.22 %	81.35 %	75.25 %
	# Of Cells detected	455575	467975	49125	139400

Table 16 Flow Cytometric results of Subpanel 5b.

		Unstimulated	Stimulated with PMA	Stimulated with ADP
Panel 5c Bovine	% Activated Platelets	56,56 %	57.70 %	60.28 %
	% Viable Cells	18.78 %	9.22 %	11.78 %
	% Platelets	60.92 %	52.90 %	33.98 %
	% Platelets (CD61)	9.01 %	3.35 %	3.31 %
	# Of Cells detected	124675	84250	111800

Panel 5c PRP	% Activated Platelets	51,25 %	51,35 %	51,76 %
	% Viable Cells	20.70 %	7.46 %	12.47 %
	% Platelets	97.73 %	92.40 %	93.49 %
	% Platelets (CD61)	18.18 %	5.54 %	10.68 %
	# Of Cells detected	96975	48325	40700
Panel 5c Human	% Activated Platelets	59,81 %	58,58 %	64,20 %
	% Viable Cells	15.68 %	10.03 %	8.15 %
	% Platelets	84.61 %	98.43 %	85.90 %
	% Platelets (CD61)	9.45 %	6.16 %	4.69 %
	# Of Cells detected	1170650	2363000	1961000

Table 17 Flow cytometric results in Subpanel 5c.

		Unstimulated	**Stimulated with PMA**	**Stimulated with ADP**
Panel 5d Bovine	% Activated Platelets	48.47 %	50.15 %	48.71 %
	% Viable Cells	21.65 %	10.31 %	12.13 %
	% Platelets	67.97 %	57.48 %	44.38 %
	% Platelets (CD41)	9.31 %	3.73 %	3.32 %
	# Of Cells detected	106750	78975	124600
Panel 5d PRP	% Activated Platelets	31.21 %	31.15 %	29.52 %
	% Viable Cells	23.78 %	6.52 %	13.00 %
	% Platelets	98.05 %	93.65 %	94.60 %
	% Platelets (CD41)	14.50 %	4.05%	7.44 %
	# Of Cells detected	87300	29675	47125
Panel 5d Human	% Activated Platelets	52.44 %	48.52 %	56.58 %
	% Viable Cells	30.76 %	12.95 %	19.31 %
	% Platelets	88.59 %	90.93 %	84.30 %

	% Platelets (CD41)	16.73 %	7.33 %	10.11 %
	# Of Cells detected	877175	396150	504125

Table 18 Flow cytometric results of Subpanel 5d.

		Unstained	**Unstimulated**	**Stimulated with ADP**
Panel 6	% Activated Platelets	50.59 %	51.24 %	51.72 %
	% Viable Cells	32.08 %	13.46 %	19.98 %
	% Platelets	95.11 %	95.50 %	89.76 %
	% Platelets (CD61)	60.68 %	8.11 %	11.15 %
	# Of Cells detected	372200	461100	482775

Table 19 *Flow cytometric results of Panel 6.*