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Characterisation of complement signalling pathways that lead to prothrombotic cell activation

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Abbreviations

(D)PBS	(Dulbecco's) Phosphate-buffered saline
(sh)hRBC	(Shattered) human red blood cells
(sh)rRBC	(Shattered) rabbit red blood cells
*	P value < 0.05 (statistically significant)
Abs.	Absorbance
ADP	Adenosine diphosphate
aHUS	atypical hemolytic uremic syndrome
AIHA	Autoimmune hemolytic anemia
ANOVA	Analysis of variance
AP	Alternative pathway
ASS	Acetylsalicylic acid
ATP	Adenosine triphosphate
C3G	C3 Glomerulopathy
CAD	Cold agglutinins disease
Cangr	Cangrelor
CAPS	Catastrophic antiphospholipid syndrome
CS-A	Chondroitin sulfate-A
CSA	Cyclosporine A
d	Days
DAMP's	Danger-associated molecular patterns
DNA	Deoxyribonucleic acid
Ecu	Eculizumab
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FB	Factor B
FBa	Factor Ba
FD	Factor D
FDPs	Fibrin degradation products
GPI	Glycosylphosphatidylinositol
GP-Ib	Glycoprotein Ib
GPIIb/IIIa	Glycoprotein IIb/IIIa
HMWK	High-molecular-weight-kininogen
IFN-gamma	Interferon-gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MASP-1, -2, -3	Mannan-binding lectin-associated serine proteases
MBL	Mannan-binding lectin
MFI	Median fluorescent intensity
Mg	Magnesium
MRS	MRS2179
NaCL	Sodium chloride
NaOH	Sodium hydroxide

NET’s	Neutrophil extracellular traps
OmCI	Ornithodoros moubata complement inhibitor
PAF	Platelet activating factor
PAI	Plasminogen activator inhibitor
PAMP’s	Pathogen-associated molecular patterns
PIG-A	Phosphatidylinositol glycan complementation class A
PNH	Paroxysmal nocturnal hemoglobinuria
Ravu	Ravulizumab
RBC	Red blood cells
TAFI	Thrombin activatable fibrinolysis inhibitor
TFPI	Tissue factor pathway inhibitor
TNF-alpha	Tumor necrosis factor-alpha
tPA	tissue type plasminogen activator
TxA2	Thromboxane A2
uPA	urokinase plasminogen activator
Vit	Vitamin
vWF	von Willebrand Factor
w	Weeks
wAIHA	Warm autoimmune hemolytic anemia

1 Introduction

1.1 Hemostasis from a clinical perspective with reference to complement diseases

Anticoagulants and antiplatelet agents are an integral part of clinical life. Their indication ranges from therapeutical use like the treatment of pulmonary embolism, phlebothrombosis, myocardial infarct or mechanical heart valve replacement to prophylactic use like the prevention of thromboembolism in atrial fibrillation or after operations. Of course, they are indispensable in many situations, but as every drug has its benefits their use also entails danger.

The risk of bleeding is always present and must be controlled permanently. For any therapy that interferes with hemostasis it should be assessed whether the benefits outweigh the risks. For several disease entities the clinical management of thrombotic complications still remains a challenge as anticoagulants and antiplatelets do not deliver their promised effects, though. This suggests that unknown or underappreciated mechanisms may drive the thrombotic complications in such diseases.

Two examples are paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS). PNH is a rare form of hemolytic anemia. People suffer from a somatic mutation of the X-linked PIG-A gene, which is responsible for a crucial step in the synthesis of GPI-anchors. This somatic mutation affects a proportion of hematopoietic stem cells. Consequently, the blood cells partially or completely lack GPI-linked proteins.[30] Two of these proteins, which therefore fail to be tethered to the cell surface are CD55 and CD59. These proteins are two crucial regulators of the complement system and their absence from host cell surfaces results in suboptimal complement control on the progenitor cells of the affected stem cells. The main clinical signs and complications are intracellular hemolysis and thromboembolism. [29, 30] Despite the use of anticoagulants/antiplatelets in PNH patients, the development of new thromboembolic events or progression of an existing thromboembolism have been observed. The use of Eculizumab has brought great success in the treatment of PNH. The antibody is an inhibitor of the complement protein C5 and thus it blocks the terminal and lytic complement pathway. Despite not directly affecting the networks in hemostasis, the treatment with Eculizumab led to a significant reduction of the thromboembolism rate next to reduced hemolysis, anemia and fatigue resulting in substantially improved quality of life. [29]

Examples of this are described below in section 1.6.1. The use of the C5 inhibitor was similarly successful in patients suffering from aHUS. This disease is also caused by misregulation of the complement system. In aHUS over-activation or under-regulation of the complement cascade leads to platelet, leucocyte and endothelial cell activation, eventually resulting in thrombotic microangiopathy. Eculizumab increased the platelet count and many patients ceased to suffer from thrombotic microangiopathy. Also the glomerular filtration rate improved under Eculizumab therapy as well as the health-related quality of life. Dialysis could be discontinued in four out of five patients in one trial. [43] Both diseases will be discussed later in more detail. Thromboembolic events are the main cause of organ failure and death in the two complement mediated diseases aHUS and PNH. That drugs interfering with coagulation or platelet aggregation failed to substantially prevent thromboembolism in both conditions, but inhibition of the terminal complement pathway with Eculizumab normalized the rate of thromboembolic events and death and provides an intriguing but compelling link between complement activation and thrombotic complications.

1.2 Complement system

The complement system is the major soluble component of the innate immune system. The main functions are the defense against infections, clearance of unwanted debris and turnover products like apoptotic bodies and guiding of adaptive immunity. It comprises more than 35 proteins. Complement opsonizes microbial intruders or particles by recognition of pathogen or danger-associated molecular patterns (PAMPs or DAMPs) to facilitate the killing/clearance directly or by the help of cells from the innate or adaptive arm of the immune system. Three initiation pathways called the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP) can trigger the complement cascade. [36, 70] A recently described pathway, the so-called extrinsic pathway, is discussed in 1.4. [47]

For the CP the immunoglobulins IgM and IgG are needed. They form immune complexes on pathogen surfaces, which are recognized by the first component of the complement system called C1q (in the blood stream attached to C1r and C1s). They consequently bind to these complexes which triggers the proteolytic activation of C1r, which cleaves C1s. Activated C1s cleaves C4 into C4a and C4b. C4b forms a complex with Mg2+ and C2, which

is cleaved again by C1s. C2b is split off and the complex C4b2a remains, also called the C3 convertase of the CP. This convertase has a central position in the complement cascade, all pathways lead to the formation of this complex. It cleaves C3 into C3a and C3b. C3a works as a mildly pro-inflammatory anaphylatoxin. C3b is highly reactive and opsonizes the target to mark it for phagocytosis. Additionally, an accumulation of C3b molecules on a surface enables that C5 is recruited to such a surface and becomes conformationally prepared (or 'primed') to be proteolytically activated by a complement convertase (*e.g.* C4b2a) which in combination with C3b dense deposition works as a C5 convertase. Furthermore, C3b is part of the AP amplification loop, which will be explained at the appropriate place. [22, 36, 47, 64, 70]

The LP is triggered *e.g.* by the recognition of particular saccharides (on the surface of bacteria or viruses) by Mannan-binding Lectin (MBL), Ficolins and collectins. In the blood stream these soluble pattern recognition molecules of the LP are attached to mannan binding lectin-associated serine proteases (MASP-1, -2, -3). In analogy to the CP, these LP complexes of pattern recognition and enzymatically active proteins cleave C4 and C2 resulting in the same products. [22, 36, 70]

The AP is activated at all time and explores both foreign and self-structures enabling constant immune surveillance. This phenomenon is due to the molecule C3, which contains a thioester in its center. Normally the position in the middle of the molecule shields the thioester from being activated, but in a small proportion of C3 molecules in blood of the thioesters are hydrolyzed spontaneously in a process called "tick-over" mechanism. The produced C3(H2O) has a similar conformation as the bona fide C3 activation product C3b. Hence, C3b and C3(H2O) have similar activities. The exposed thioester can react with nucleophiles on foreign or own surfaces. Next to acting as an initiation pathway the AP works also as an amplification loop. All three pathways are producing active C3b, which can form proteolytically active convertases if not inactivated by the plasma protease Factor I. C3b inactivation via Factor I into the opsonization factors iC3b and C3dg takes place as a part of the complement down-regulation. If this C3b procession does not happen fast enough, C3b self-amplifies via the so-called AP amplification loop. Factor B binds to C3b which is then susceptible to the cleavage by Factor D. This leads to the AP amplification C3 convertase C3bBb which further produces more C3b. Similar to the CP/LP described above,

accumulation of C3b molecules on surfaces lead to C5 recruitment and priming so that C3bBb consequently can also cleave and activate C5 (next to C3). Next to Factor I and cofactors there are also other natural regulators that decay the convertases to stop/limit their action (which, however, will not be discussed further here). [22, 36, 64, 70]

When activated strongly, all the pathways result in the formation of the membrane attack complex (C5b-9; MAC), which creates transmembrane pores and leads the attacked cell to lysis. The C5 cleavage product C5a also acts as a strong pro-inflammatory anaphylatoxin. [22, 36, 70]

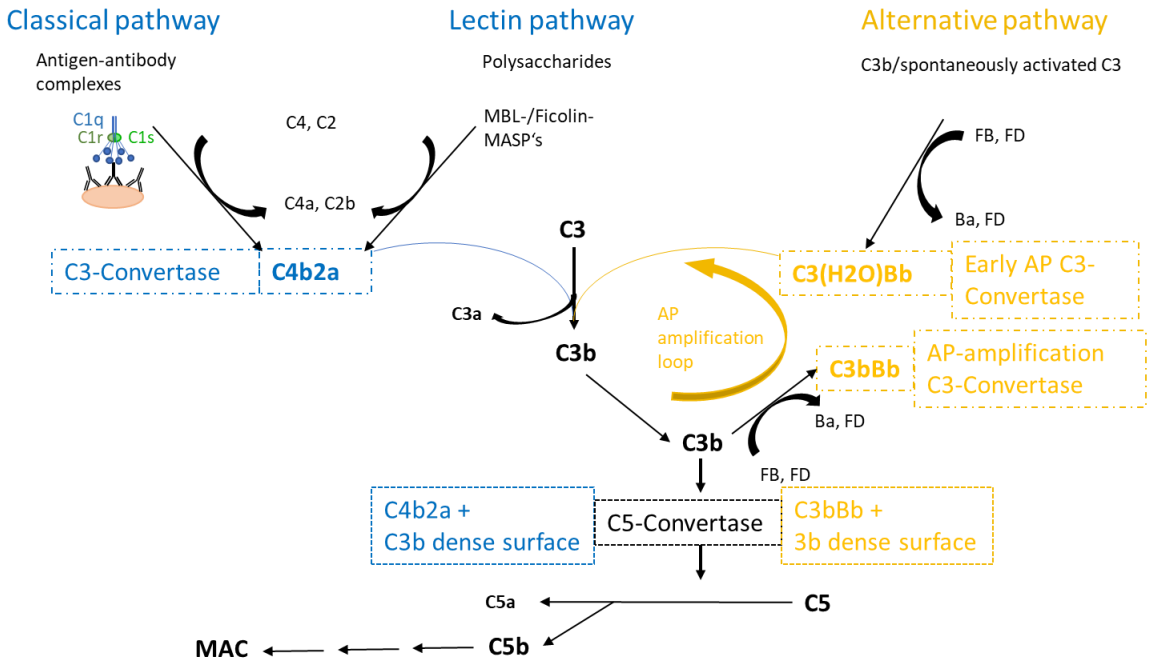

Figure 1: The complement cascade. *The complement system is triggered by three initiating pathways – the classical, the lectin and the alternative pathway. They all meet at the central component C3. Upon cleavage of C3, C3a as an anaphylatoxin is released as well as C3b, which opsonizes the cells and thus mark them for phagocytosis. The amount of firstly deposited C3b is increased by the amplification loop. The terminal pathway starts with the cleavage of C5 into C5a (as a further anaphylotoxin) and C5b, initiated by a C3b dense surface. C5b is part of the MAC which is formed consequently by binding C6, C7, C8 and C9.*
MBL: Mannan-binding Lectin; MASP`s: mannan binding lectin-associated serine proteases; FB: Factor B; FD: Factor D; AP: Alternative pathway; MAC: membrane attack complex

1.2.1 Therapeutic complement inhibitors and complement inhibitory agents

There are several agents that inhibit the complement system at different levels. Some of them are used only *in vitro*, others as drugs in clinical routine.

In this experiments the C3 inhibitor Compstatin (or more precisely the compstatin variant Cp40) is used. It binds C3 and prevents its cleavage by convertases. [49] To inhibit C5, Ornithodoros moubata complement inhibitor (OmCI), Ravulizumab and Eculizumab are used. OmCI binds C5 and inhibits the cleavage of it probably by preventing the interaction with the C5 convertase. [62] Ravulizumab and Eculizumab are both used in the clinic for the treatment of PNH and aHUS, as described above. Both are monoclonal antibodies and bind to C5 inhibiting its proteolytic activation. Ravulizumab is (so to say) a second generation antibody of Eculizumab. Some aminoacids have been exchanged for reaching a longer half-life of the drug in the body. [41, 50]

1.3 Coagulation system

The coagulation system can be split into the primary or cellular hemostasis, the secondary or plasmatic hemostasis and the fibrinolysis. As this is textbook knowledge only key features relevant for the work described here will be briefly mentioned.

1.3.1 Primary hemostasis

Primary hemostasis results from interaction between platelets, vessel walls and specific proteins. Vessel injury causes an alteration of the blood flow, reflex vasoconstriction, the release of local mediators like endothelin and platelet-derived thromboxane (TxA2) and the exposure of subendothelial tissue, which is highly thrombogenic. It contains collagen, von Willebrand Factor (vWF) and other proteins. Subsequently the platelets are activated and undergo a morphologic change. The platelet glycoprotein complex I (GP-Ib) is a receptor for platelet and plasma derived vWF, which thus stick the platelets to the collagen surface and promotes platelet adhesion. The platelets have two types of granules, which are degranulated after this adhesion. δ-Granules or dense granules contain adenosine triphosphate (ATP), adenosine diphosphate (ADP), calcium ions, serotonin, histamine and epinephrine. α-Granules contain P-selectin, fibrinogen and other procoagulant proteins. During platelet aggregation the following steps typically happen. ADP binds to the platelets which set in motion a conformational change of GpIIb/IIIa receptors. This receptor is now able to bind fibrinogen and thereby the platelets can be connected to each other. [12, 14, 31, 56]

1.3.1.1 P-selectin

Upon activation of thrombocytes different receptors are transposed to the outer membrane of the cells. One of them is P-selectin (also expressed by activated endothelial cells), which is stored in granules and brought to the cell surface after activation. One known task is the mediation of leukocyte adhesion. In our experiments it is used as a marker for the platelets to distinguish, whether they are activated or not. [3, 8] The role of the receptor and the possible link to the complement system will be discussed later.

1.3.1.2 Glycoprotein IIb-IIIa

This receptor is also called Integrin alpha IIb. All thrombocytes express this receptor on their cell surface. When the platelets are activated, the receptor exposes another binding site to connect with different molecules (Fibrinogen, vWF, fibronectin and vitronectin). Both the inactive and active forms can be color-coded with an antibody, even simultaneously. [9, 67, 69]

1.3.1.3 ADP and the P2Y12/P2Y1 receptors

One known and important agent that promotes platelet function is adenosin diphosphat (ADP). ADP (along with ATP) is, among other things, released from our cells when tissue is damaged. Via the P2Y1 receptor ADP is initiating the platelet response. The shape of the platelets changes from a disc to a spherical form. Interaction with the P2Y12 receptor leads to platelet aggregation. The P2Y12 receptor is currently a known antithrombotic target in the clinic (for example via the use of clopidogrel or prasugrel together with acetylsalicylic acid (ASS) in the treatment of acute coronary syndrome), which highlights the important role of this receptor for thrombus formation. [21, 74]

1.3.1.4 Excursus: Other agents influencing the thrombocyte activation

The platelet activating factor (PAF) is a proinflammatory lipid mediator that is produced by activated leukocytes, platelets and endothelial cells. It has many tasks, far more than only activating platelets to initiate aggregation and degranulation. It also stimulates neutrophils, monocytes and macrophages. PAF signalling may lead to activation of protein kinase C or processing of arachidonic acid and the molecule plays a role in blood pressure regulation and has many other biological activities. Some of them are not yet understood. [5, 33, 72]

Another activator of platelets is serotonin. It is a weak platelet activator and its activation properties have not been fully elucidated, yet. In an experiment reported by Li et. al,

serotonin alone failed to activate platelets, but when combined with ADP, it was able to potentiate activation as measured by P-selectin. [44]

1.3.2 Secondary hemostasis

Parallel to the primary also the plasmatic hemostasis is activated. This takes place via two different pathways that in the end lead to the formation of thrombin, which processes the water-soluble fibrinogen into insoluble fibrin 'threads'. [12, 14, 31, 56]

The extrinsic pathway starts with the exposition of tissue factor expressed in the subendothelial tissue that is now exposed because of the vessel injury. Tissue factor binds to factor VIIa and together with calcium ions and phospholipids of the cellular membrane the conversion of factor X to Xa is promoted. Prothrombin is activated to thrombin by the prothrombinase complex which is bound to the phospholipids of the membrane and consists of factor Xa, factor Va and calcium ions. The main task of thrombin is the conversion of fibrinogen to fibrin. However, thrombin also activates factor XIII, which is responsible for the cross-linking of fibrin, leading to a stable fibrin network. [12, 14, 31, 56]

The intrinsic pathway is a parallel pathway for thrombin activation, but its importance is not fully understood. The cascade involves different factors like factor XII, high-molecular-weight-kininogen (HMWK), prekallikrein, factors XII, XI and IX, together leading to the activation of factor X. The following steps are consistent with the extrinsic pathway and were described in the paragraph above. [12, 14, 31, 56]

In order to protect our body from excessive blood coagulation we have several different regulatory mechanisms like Antithrombin or the Protein C pathway. Furthermore, the development of a blood clot is accompanied by the task of removing it again. The responsible system is called fibrinolytic system. One example is plasmin, which plays an outstanding role. It dissolves fibrin into fibrin degradation products (FDPs). [12, 14, 31, 56]

1.4 Crosstalk between platelets, the coagulation system and complement system

When an injury occurs, ideally both the immune system and the hemostatic system are activated and must go hand in hand to be most effective. However, in a pathophysiological event of strong aberrant complement activation, such crosstalk might also result in complement triggered thrombosis or platelet activation vice versa: activation of the coagulation cascade might result in complement activation. Several studies have

investigated such crosstalk effects but their pathophysiological relevance remains poorly understood.

1.4.1 Evolution: hemocytes

Looking into the immune system – hemostasis crosstalk a closer look at the evolution of these systems might be helpful. Even in one´s lifetime the immune system is build up individually depending on the pathogens we are exposed to. In higher organisms the coagulation and immune system are complex with many different pathways and actions which for example eliminate pathogens. According to Arneth et al. the defense mechanisms have evolved from simpler ones and changed depending on the environment. Immunity and hemostasis are often considered separate, but in lower organisms these two systems are carried out by one cell called a hemocyte, which is a primordial immune cell. This would explain why the two systems partially overlap also in mammals. Hemocytes can undertake various tasks from both systems. They initiate the humoral and cellular immune response, recognize and eliminate foreign invaders and are even able to act as phagocytes and produce different substances to kill the pathogen. Additionally, hemocytes can release different pro-coagulatory factors influencing the process of hemostasis after a tissue injury by interacting with different hemolymph proteins to generate large cellular aggregates. Other molecules such as lipopolysaccharide (LPS) can also initiate the coagulation process which can be recognized by components of the hemolymph to trap and eventually kill an invading pathogen. All this leads to the assumption that both pathways have the same origin and were separated in the course of evolution. Therefore, many interactions and overlaps between immune surveillance cascade of the complement system and the hemostatic system appear logical. [4, 34]

This seems obvious with a look at the following example. In every wound of our body both the innate immune system and the coagulation system are activated in parallel. The former (which also includes the complement system) is responsible for the elimination of the pathogen. Platelet activation and the coagulation system leads to hemostasis, which prevents the spread of the pathogen in the body. All these findings lead me to assume that one should take a closer look to at the crosstalk between the complement system and hemostatic mechanisms.

1.4.2 Fluid phase

Many different links between the coagulation system and the complement system have been described in the literature.

1.4.2.1 Coagulation pathway of complement activation

Various proteins and factors of the coagulation system were studied and proposed to interact with complement, *e.g.* thrombin and plasmin. Huber-Lang *et al.* introduced the idea of an additional pathway. It was suggested that thrombin is acting as a potent C5 activating enzyme producing well-functioning C5a. [32] Amara *et al.* demonstrated that thrombin is capable of cleaving C3a off C3. [2] However, supra-physiological concentrations of thrombin were used in both experiments. Other studies using in vivo models did question a physiological role of such an extrinsic pathway. [2, 19, 20, 36] Additionally, several other coagulation factors were found to effectively cleave C3 and C5. [36] *E.g.* FXa and plasmin were most effective in cleaving C3 and C5. Thus, according to this study plasmin and FXa are even more potent in cleaving the complement factors than thrombin. Also, Foley *et al.* do not see thrombin as the major activator of C3 and C5. In their experiments, plasmin was much more effective than thrombin or even FXa, especially regarding C5 activation. They created the concept, that thrombin contributes to C5a-generation via plasmin-mediated events and not because it is a potent complement activating protease itself. Because of its ability to generate fibrin and be a co-factor for tPA-mediated plasmin formation, it is important for the initiation of fibrinolysis. [20] C5a and C3a created in these experiments were found to be chemotactically effective towards human mast cells and neutrophils, despite being cleaved by none-complement proteases. This illustrates the ability of coagulation factors to initiate pro-inflammatory pathways, but whether or not such complement activation by extrinsic pathway plays a role under physiological conditions is not clear. [19, 20, 36]

1.4.2.2 C3a/C5a

C3a and C5a are proinflammatory and immunoregulatory mediators of the complement cascade. [22] Their target are various immune and non-immune cells. They function as vasodilatator, increase of permeability of small blood vessels, induce contraction of smooth muscles and activate macrophages, neutrophils, eosinophils, basophils and mast cells. [38] Beside the pro-inflammatory properties both anaphylatoxins were also described to have

pro-coagulatory tasks. C3a is capable of directly triggering platelet activation and aggregation in some experiments. [55] C5a is able to activate platelets, leucocytes and endothelial cells, upregulate the expression of adhesion cells, induce the secretion of pro-inflammatory and procoagulant cytokines, promote tissue factor expression by neutrophils and endothelial cells and induce formation of neutrophil extracellular traps (NET's; discussed later). [20, 55] It is certainly not yet fully understood, how strong these effects are in vivo and what impact anaphylatoxins have on the course of some diseases, in which the complement system is involved. This calls for a thorough testing to determine whether or not anaphylatoxins do induce a pro-thrombotic phenotype on *e.g.* thrombocytes. Landsem *et al.,* for example, found no effects of C3a on the activation of platelets. [42]

1.4.2.3 MAC

The membrane attack complex is composed from C5b, C6, C7, C8 and multiple C9 molecules. It is the final product of the complement pathway. The complex was described to induce a change in the phospholipid membranes of endothelial cells and platelets and thus promoting the exposure of prothrombinase assembly sites (Factor Va and Xa) which leads to the generation of thrombin. Furthermore, MAC inserted in platelets, endothelial or other human cells induces vWF and P-selectin expression and platelet microparticle release. [13, 20, 36]

1.4.2.4 Other examples

There are still more interactions between the complement system and hemostasis that have been described today. For all the pathophysiological relevance is not really clear. Examples include neutrophil extracellular traps (NET's). These traps contribute to both coagulation pathways by activating *e.g.* FXII and tissue factor. [16, 36, 68] Factor XII by itself is known to be able to initiate the intrinsic coagulation cascade. The mechanisms of FXII activation in vivo are not yet known and the pathophysiologic significance of the intrinsic pathway is not well understood, partly because FXII deficiency is not associated with abnormal bleeding, indeed questioning the physiological relevance of this crosstalk. [36, 52, 59]

1.4.3 Thrombocytes

Thrombocytes are the essential part of the primary hemostasis and their activation results in a fast-growing thrombus. Apart from purely hemostatic roles they also influence and interact with the immune system by various mechanisms. [16, 36]

E.g. platelets can bind bacteria and present them to the appropriate immune cells, which helps to induce an adaptive immune response. [16, 36] Additionally, the complement cascade can be activated directly by platelet components. During activation, the platelets release the content of the alpha granules. These contains Chondroitin sulfate A (CS-A) among other things, which can activate the complement system. [25, 26, 36] In the course of the complement cascade, C3 can also bind to platelets and thus is able to initiate a connection between platelets and leukocytes by being a ligand for them. [25, 36]

Del Conde *et al.* attribute an important role to P-selectin. As described above P-selectin is a receptor, which is brought to the outer membrane upon activation. According to Del Conde *et al.* P-selectin is a receptor for C3b. Binding of C3b to P-selectin may enable formation of C3 convertase complexes with subsequent increased production of more C3b and C3a and initiating the further formation of C5b-9. This would mean that activated platelets are able to elicit an inflammatory response via activation of the complement system. [13, 15] While fascinating, it is not clear again if these findings bear physiological/clinical relevance or rather are observable due to specific experimental conditions with no or little physiological relevance.

1.5 Crosstalk between the complement system and other cell types

1.5.1 Leukocytes

As part of the immune system, leukocytes are also connected with the complement system. When the complement system is activated, C3a and C5a are released. Especially, C5a is well-known to activate leucocytes. [38] As mentioned above, neutrophil released NET´s can activate both the complement and coagulation system (already described in 1.4.2.4). Interestingly, activation of the complement system by resting neutrophils is not possible. Only after activation by chemoattractants or cytokines, the neutrophils are able to activate the complement system. [11] Furthermore, leukocytes and platelets are building complexes via different receptors (like P-selectin). They are markers of thrombo-inflammatory diseases like systemic lupus erythematodes, acute ischemic stroke or

hepatitis. Ståhl *et al.* described that the complement system is also activated on the surface of these complexes and helps to eliminate cell debris or, conversely, to activate these cells. [17, 73]

1.5.2 Endothelium

An intact endothelial layer is important to prevent blood clotting by controlling prothrombotic pathways. Endothelial cells express receptors for the anaphylatoxins C3a and C5a. Binding of these leads, among other things, to an expression of P-selectin and a release of vWF (as a link between the complement and coagulation pathway). The membrane attack complex can also activate endothelial cells by inducing the expression of P-selectin and the release of IL-1, triggering coagulation processes (such as the expression of tissue factor) and contributing to release proteins that alter the vascular tone. In complement diseases, such as aHUS or PNH, the excessive/uncontrolled complement activation leads to more C5a in the bloodstream, which activates the endothelial cells. Via P-selectin and other receptors, the endothelium becomes susceptible to thrombosis (systemic or glomerular thrombotic microangiopathy is the leading symptom of aHUS). This could be another explanation for the effect of C5 inhibitors in preventing thrombosis in people with aHUS or PNH. The formation of C5a and thus the effect of binding to the endothelium is prevented. [10, 18, 28, 35, 43]

Endothelial cells, like other cells, have regulatory proteins such as CD59 or CD55 on their surface, to protect themselves as host cells from undue complement attack. However, activation of complement in the vicinity of endothelial cells may still be sensed by these cells as anaphylatoxins diffuse away with the blood stream and engage anaphylatoxin receptors on nearby cells like leukocytes or endothelial cells. The anaphylatoxins C3a and C5a become quickly inactivated by carbopeptidases in plasma and hence their proinflammatory effects do not last too ‘long and far’. [10, 18]

Given these data it is likely that in principle the endothelium next to platelets are able to respond by a prothrombotic phenotype upon exposure to complement activation products. Yet, it is important to extract, from available data, how and when such interactions have pathophysiological consequences.

1.6 Examples of a dysfunctional complement system

1.6.1 PNH

Paroxysmal nocturnal hemoglobinuria is a rare form of hemolytic anemia resulting from a clonal disorder of hematopoietic stem cells. The abnormal cells have somatic mutations in the X-linked gene PIG-A (phosphatidylinositol glycan complementation class A). This gene is crucial for the synthesis of glycosylphosphatidylinositol anchors (GPI-anchors), its mutation causes an early block in the synthesis of these anchors leading to their absence. GPI-anchors normally tether many different proteins to the cell surface. Consequently, cells not able to produce these linkers lack all GPI-anchored cell surface proteins. Two of them are CD55 and CD59, both complement regulatory proteins on the surface of blood cells such as erythrocytes, leukocytes or platelets. Their absence renders these cells susceptible to complement attack including the terminal and lytic pathway. CD59 normally blocks the formation of MAC of the complement system thus protecting the blood cells from complement-mediated lysis. PNH cells can be classified in either type II or type III, depending on whether the cells lack the GPI-linked proteins partially (type II) or completely (type III). [28–30, 35, 46, 78]

The uncontrolled complement activation leads to intravascular hemolysis and platelet activation. [28, 35] This causes anemia, hemoglobinuria and the subsequent presence of free hemoglobin. The related complications include thrombosis, abdominal pain, dysphagia, erectile dysfunction, pulmonary hypertension, renal impairment, fatigue and shortness of breath. [27, 30, 35, 66] The increased risk of thromboembolism may lead to organ damage and death and is indeed the leading cause of mortality in these patients prior anti-complement therapy. [29, 68] On the other hand a proportion of these patients may suffer from bone marrow failure possibly leading to pancytopenia and aplastic anemia and then the clinical picture is dominated by hematopoietic insufficiency. [46, 66] Thromboembolism (which can occur everywhere in the body) is responsible for approximately 40% to 67% of deaths (of all death with known reason) of patients with PNH before complement therapy was available. Patients who presented themselves with thrombosis had a 40% survival rate at 4 years. [28] The pathophysiology of thrombosis is very complex and not fully understood. There are many factors that contribute to the prothrombotic state, such as platelet-activation, complement mediated hemolysis, inflammatory mediators or nitric oxide. [28, 35]

While anticoagulation and/or antiplatelet therapies had limited effects, the C5 complement inhibitor Eculizumab dramatically changed patient outcomes. Hemoglobin levels stabilized (49% of patients compared to 0% in those without Eculizumab) and intravascular hemolysis was dramatically reduced (shown by LDH level). [30] Furthermore, the thromboembolic event rate decreased from 7,37 events /100 patient years before treatment to 1,07 events/100 patient years with the introduction of Eculizumab therapy. [29] Over the years, the efficacy of C5 inhibition in PNH has been confirmed and a second generation C5-inhibitor with better pharmacokinetic properties is available in the clinic. [46, 65]. The use of anticoagulants is still indicated in a certain constellation (*e.g.* increased episodes of thrombosis). [46] It is known that the complement activation leads to platelet activation, but regularly PNH patients were put on anticoagulation therapy and only rare cases are described when anti-platelet therapy was used. Given the number of studies describing a link between complement and platelet activation this is perhaps a bit surprising. [6, 28, 65]

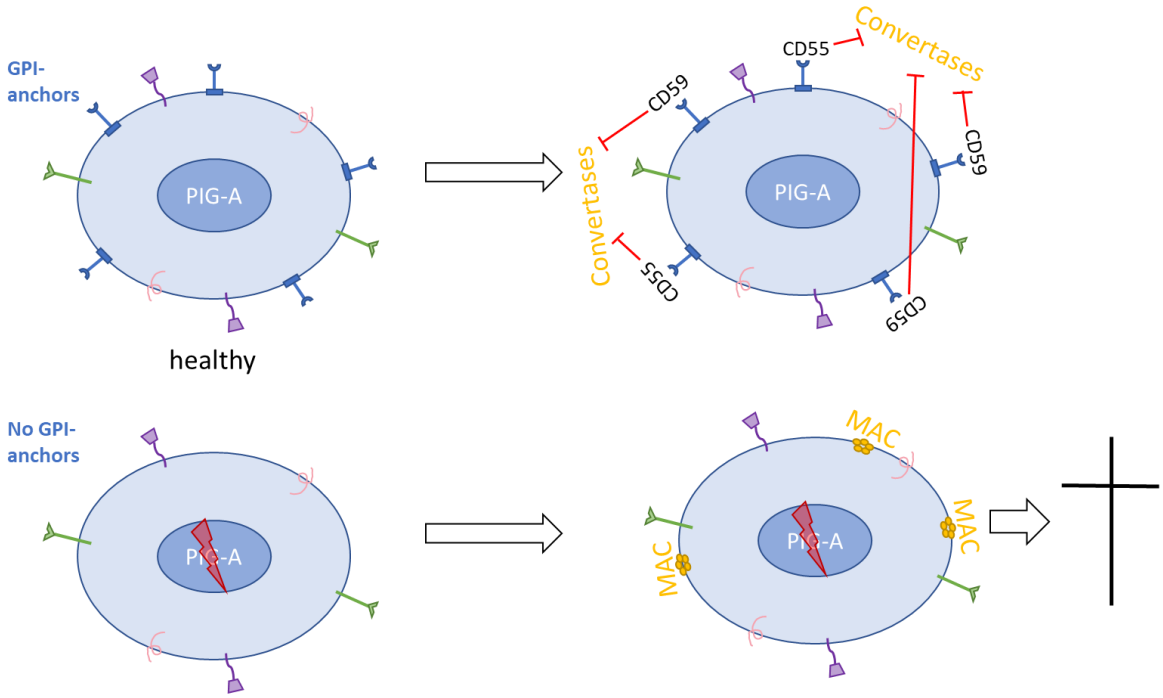

Figure 2: PNH. *With a functioning PIG-A gene, CD55 and CD59 can bind to the membrane of the cells to prevent MAC formation. However, in PNH, CD55 and CD59 cannot attach to the surface and the MAC leads to lysis of the cells.*
PNH: paroxysmal nocturnal hemoglobinuria; GPI-anchors: glycosylphosphatidylinositol-anchors; MAC: membrane attack complex

1.6.2 Other selected complement mediated diseases

A well-known disease in children with a complement link is hemolytic uremic syndrome (aHUS). The disease is usually triggered by infection with Shiga-toxin producing bacteria. However, in about 10% of the cases, the disease is caused by germline mutations concerning the complement system and is denominated atypical HUS. The main features of aHUS are nonimmune microangiopathic hemolytic anemia, thrombocytopenia and acute kidney injury, often with glomerular thrombotic microangiopathy. The mutations lead to uncontrolled activation of the complement system, activating platelets, leucocytes and endothelial cells, resulting in systemic thrombotic microangiopathy. In particular, the alternative pathway dysregulation with subsequent MAC formation leads to thrombotic microagiopathy on endothelial cells. [65] Patients always live with the risk of worsening symptoms. As in PNH, Eculizumab has led to significant improvement in patient health in aHUS patients. It resulted in significant inhibition of thrombotic microangiopathy (increase in platelet count and thrombotic microangiopathic event free status) and it is associated with an improvement in renal function and the health-related quality of life. [43, 53, 54, 65]

Yet, another kidney disease with a proven pathophysiology due to aberrant complement activation is C3 glomerulopathy (C3G). C3G represents a group of rare kidney diseases caused by dysregulation of the alternative pathway. Patients suffer from heamaturia, proteinuria and hypertension to glomerulonephritis. [71] Thrombosis (as in aHUS and PNH) is not typical here. [65] Dysregulation of mainly the alternative complement pathway (like autoantibodies against C3 or C5 convertases unnaturally stabilize the convertases) occurs in the fluid phase and glomerular microenvironment and leads to C3 deposition in the kidneys. Unlike other complement diseases, the formation of the membrane attack complex and subsequent lysis is prevented by soluble regulators that quench the MAC before it becomes cytolytic. Symptoms in C3G appear to be primarily due to the deposition of C3 in the kidneys and the release of C5a, which further drives the inflammation. In contrast to PNH and aHUS, where Eculizumab significantly improves patients' outcome, there is as yet no disease specific treatment. [57, 65, 71]

There are many other diseases, in which the complement system plays an important role. Some of them are already known, such as autoimmune hemolytic anemia (AIHA) or

(Catastrophic) antiphosholipid syndrome (CAPS), but their exact role is not yet clear. Complement activation, hemolysis and thrombosis occur in both diseases, but the exact connection has not yet been explored. AIHA can be divided further into cold (CAD) or warm (wAIHA) agglutinins disease depending on the temperature at which the autoantibodies occur. In CAD, the CP is activated, which leads to the deposition of C3. A monoclonal antibody targeting C1s, which inhibits the initiation of the CP, is bringing good results to patients, *e.g.* stopped hemolysis. [61, 65]

For sure, there are many more diseases where the complement system plays a role and anti-complement treatment would improve the clinical situation and outlook.

1.7 Pathophysiological significance of the crosstalk between the complement system and hemostasis

As described above, there are a lot of studies that discuss the crosstalk between complement and hemostasis. They are all not fully conclusive and sometimes even contradictory. This raises the question of the pathophysiological significance of all these results. To come closer to an answer to this question, three selected complement diseases are compared. The main features of PNH, aHUS and C3G are shown in the following table:

Table 1: Main features and respective therapies of PNH, aHUS and C3G

Disease	Main features	Therapy	
PNH	• Uncontrolled complement activation • **Thrombosis** • **MAC** formation	• Resistant to anticoagulation/anti-platelet therapy • Terminal complement inhibition >	Stop of fatal thrombotic events
aHUS	• Uncontrolled complement activation • **Thrombosis** • **MAC** formation	• Terminal complement inhibition >	Stop of fatal thrombotic events
C3G	• Dysregulation of the complement system • Hematuria, glomerulonephri-tis,… but **no thrombosis** • **No MAC** formation	• No specific treatment >	Terminal complement inhibition does not help

The disease C3G is a prominent example for a disease with widespread complement activation (strong C3 and C5 activation) that does not lead to thrombotic complications. However, C5 activation in C3G does not lead to cytolytic events as the developing C5b-9 complexes become quickly quenched by soluble regulators resulting in non-lytic sC5b-9. Mechanistically, the crosstalk between complement activation products and prothrombotic cell activation has been pinpointed to proximal and terminal complement activation, *i.e.* C3 and C5 activation, respectively. In contrast to this, therapeutic inhibition of the terminal complement cascade, at the level of C5, is sufficient to almost completely

prevent thrombotic complications in PNH and aHUS patients. While in these clinical situations with C5 inhibition, the proximal activation fragments C3a and C3b and all complement components are actively generated up to the cleavage of C5, thrombotic complications have largely disappeared. [65]

1.8 Aim of the thesis

Activation (or dysregulation) of the complement system can lead to thrombosis as has been confirmed several times in the clinic and the laboratory. However, a discrepancy between in vitro results and in vivo experience must be noted as there are diseases with complement activation without thrombotic complications, like C3G. The combined clinical evidence is in contrast to many mechanistic in vitro studies and argues that proximal complement activation does not act strongly prothrombotic in vivo. The aim of this thesis is to mechanistically test the hypothesis, that complement activation only leads to substantial prothrombotic cell activation when it induces cytolysis. Thus, this thesis aims to determine the level of complement activation that is responsible for prothrombotic cell activation. Therefore, a novel whole blood model is developed to test the crosstalk between complement activation and prothrombotic cell activation of platelets, and to prove or disprove the proposed hypothesis.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

The equipment used in the laboratory and the corresponding applications are listed in Table 2.

Table 2: Equipment

Equipment	Company	Application
Bandelin Sonopuls GM70	BANDELIN electronic GmbH & Co. KG	Ultrasound stick
BD FACSVerse™ flow cytometer	BD Biosciences, USA	Flow cytometer
Certomat® HK	B. Braun Biotech International GmbH	Incubator
IEC Micromax	International Equipment company	Microcentrifuge
Infinite M1000 Pro	Tecan Group AG, Switzerland	Microplate reader
MilliQ Plus	Millipore Cooperation, USA	Water purification system
NanoDrop 1000 Spectrometer	Thermo Fisher Scientific Inc., USA	Determination of protein/DNA concentration
pHenomenal™	VWR International, Germany	pH meter
Pipetboy	INTEGRA Biosciences AG	Pipetboy
RCTbasic IKA Labortechnik	Janke & Kunkel GmbH & Co. KG	Heating plate and magnetic stirrer
ROTEM® delta	Tem Innovations GmbH	Thromboelastometry
Sigma 2K15	Sigma Laborzentrifugen GmbH, Germany	Microcentrifuge
Sigma 4K15C	Sigma Laborzentrifugen GmbH, Germany	Centrifuge

Vortex-2 Genie	Scientific Industries, USA	Vortex mixer
VWR Water Bath 5L; Cat. No: 462-0556	VWR International, LLC.	Water bath
Ziegra Eis Maschine	ZIEGRA Eismaschinen GmbH, Germany	Ice machine

2.1.2 Patients and probands

The experiments with the healthy thrombocytes or monocytes were performed with volunteers older than 18 who had not taken any anticoagulant therapy including ASS and Ibuprofen for the previous two weeks. The experiments with blood from donors with PNH were performed with voluntary patients older than 18 and who were receiving appropriate medication for their disease.

The blood was collected under the ethical approval by the ethics committee of Ulm University (Number 188/16).

2.1.3 Consumable material

The consumable material is listed in Table 3.

Table 3: Consumable material

Consumables	**Company**
BRANDplates® microtitration plates, 96-well, pureGrade™	BRAND GmbH & Co. KG, Germany
Butterfly	Sarstedt AG & Co. KG, Germany
Falcon™ tubes (15ml, 50ml)	Thermo Fisher Scientific Inc., USA
MicroVue™ Complement C3a Plus EIA (A032)	Quidel Corporation, USA
Reaction tubes (1.5 ml, 2 ml)	Eppendorf AG, Germany
Sample Tube 5ml, 75x12mm, PS	Sarstedt AG & Co. KG, Germany
S-Monovette® 1.6ml Hirudin, 75x13 mm	Sarstedt AG & Co. KG, Germany
S-Monovette® 3 ml, Citrate 3.2% (1:10), 75x13 mm	Sarstedt AG & Co. KG,Germany

S-Monovette® 9 ml, Lithium Heparin, 92x16 mm	Sarstedt AG & Co. KG, Germany
S-Monovette® 7.5 ml, K3 EDTA, 92x15 mm	Sarstedt AG & Co. KG, Germany
S-Monovette® 10ml, Citrate 3.2% (1:10), 92x16 mm	Sarstedt AG & Co. KG, Germany

2.1.4 Reagents and antibodies

All used antibodies are shown in Table 4:

Table 4: Reagents and antibodies

Reagents/antibodies	**Company**
Adenosin 5'-Diphosphat Lot: 46H7836	Sigma Chemical Co., USA
Anti-Human CD59, APC; Cat. 17-0596-42; Lot: 4339508/2213390; Clone: OV9A2	Thermo Fisher Scientific Inc., USA
Anti-P-Selectin: CD62P, Biotin; Cat. ab239233; Lot: GR3293634-2; Clone: AK4	Abcam plc., UK
APC-Mouse IgG1 k Isotype Control; Cat. 555751; Lot: 9282592; Clone: MOPC-21	BD Bioscience, Germany
BD FACS™ Lysing Solution 100 mL— Cat. 349202	BD Bioscience, Germany
C3a, Cat. A118; Lot: 22	Complement Technology, Inc., USA
C5a, Cat. A144; Lot: 18	Complement Technology, Inc., USA
C5, Cat. A120; Lot: 23c	Complement Technology, Inc., USA
Cangrelor tetrasodium salt, SML 2004	Sigma-Aldrich Corporation, USA
Compstatin (Cp40)	a gift from Prof. Dr. John D. Lambris of the University of Pennsylvania, Philadelphia, USA
Dulbecco's Phosphate Buffered Saline (DPBS) 14190-094	Gibco™ ,Thermofisher Scientific, Germany
Eculizumab	Ecu, Soliris™, Alexion, USA (remnants from infusion bags)

FITC Mouse Anti-Human PAC-1; Cat. 340507; Clone: PAC-1	BD Bioscience, Germany
FITC Mouse IgG1, k Isotype control; Cat. 555909; Clone: MOPC-21	BD Bioscience, Germany
FITC Mouse IgM, k isotype control; Cat. 553474; Lot: 0037147; Clone: G155-228	BD Bioscience, Germany
Integrin alpha IIb: Anti-Human CD41, FITC; MCA467F; Lot: 151411; Clone: PM6/248	Bio-Rad Laboratories, Inc., USA
Mouse anti Human CD41, Alexa Fluor® 647; MCA467A647T; Clone: PM6/248	Bio-Rad Laboratories, Inc., USA
Mouse IgG1 negative control, Alexa Fluor® 647; MCA928A647	Bio-Rad Laboratories, Inc., USA
MRS 2179 ammonium salt hydrate, M3808	Sigma-Aldrich Corporation, USA
OmCi	In house production by collegues
Paraformaldehyd (P6148)	Sigma-Aldrich Corporation, USA
Rabbit-Erythrozyten in Alsevers (AN/0010/032015)	Fiebig Nährstofftechnik, Germany
Ravulizumab	Ravu, Ultomiris®, Alexion, USA (remnants from infusion bags)
Serotonin creatinine sulfate monohydrate (H7752)	Sigma-Aldrich Corporation, USA
Streptavidin AlexaFluor™ 405 conjugate, S32351, LOT: 2160388	Thermo Fisher Scientific Inc., USA
Streptavidin APC Conjugate; SA 1005; Lot: 2110694/2085685	Thermo Fisher Scientific Inc., USA
Zymosan; Cat. Z4250	Sigma-Aldrich Corporation, USA

2.1.5 Preparation of reagents

2.1.5.1 Preparation of rRBC

1 ml of rabbit erythrocytes (or the required amount) was put into a 15ml Falcon and centrifuged for 3 minutes at 2000g at 4°C. Afterwards the supernatant was discarded and the pellet was mixed with 15ml of PBS. The mixture was centrifuged again under the same conditions and the washing step was repeated until the supernatant was clear. Then the clear supernatant was discarded again. The remaining pellet contained the packed rabbit red blood cells (rRBC) to be used in the assays.

2.1.5.2 Preparation of shattered rRBC

Packed rRBC were filled in a microtube and disintegrated by applying ultrasound pulses using an ultrasound stick. The resultant mixture was centrifuged at 10 000g for 20 seconds at room temperature. The supernatant was used for the experiments.

2.1.5.3 Preparation of shattered hRBC

Hirudin whole blood was transferred from a blood collection tube into a 15ml falcon tube (from the same blood tube used for the respective assay). The preparation of shattered human red blood cells (hRBC) is the same as the preparation of shattered rRBC (please refer 2.1.5.2).

2.1.5.4 Preparation of 4% paraformaldehyd

To prepare a 4% solution of paraformaldehyd, 0.4g of crystalline paraformaldehyd were mixed with 9.6g of PBS in a 50ml falcon tube. The falcon tube was put into a water bath of 55-60°C for 10 minutes. Three drops of 1M NaOH were added and after another 5 minutes the solution was clear (and the paraformaldehyde was depolymerized). To obtain a neutral solution, the pH was measured and depending on the initial pH either some drops of HCl and NaOh were added to reach a pH of 7.4. This stock was stored in the fridge.

2.1.5.5 Preparation of an ADP stock solution

To obtain a stock of 1mM ADP, 1mg of ADP (or 1.6mg) were mixed with 2.341ml (or 3.746ml) of PBS, respectively. The suspension was aliquoted (100µl each), put into liquid nitrogen and then stored at -20°C. Each aliquot was used only once.

2.1.5.6 Preparation of zymosan

0.2g of zymosan were diluted in 5ml of H2O in a falcon tube. The tube was placed in a beaker filled with water and a magnetic stirrer. The water was previously boiled on a hot plate at 100°C. The falcon tube was left in the boiling water for 30 minutes. Afterwards, the suspension was transferred to a 15 ml falcon tube and centrifuged at 300g for 5 minutes at room temperature. The supernatant was removed and PBS added to the suspension at the bottom of the tube. This wash of the zymosan suspension was repeated. In the end the supernatant was removed again and the pellet was resuspended in 20ml of PBS. This stock was stored in the fridge. Before the immediate use in the experiments, 20µl of the well-mixed suspension were added to a 1.5ml microtube, centrifuged for 5 minutes at room temperature at 350g. The supernatant was discarded. Then all the other ingredients were added as described below.

2.1.5.7 Preparation of a Cangrelor stock solution

Cangrelor is a reversible inhibitor of the ADP receptor P2Y12 on platelets. [76] A solution of 0.5mg of Cangrelor dissolved in 5.785ml of PBS was produced to reach a concentration of 100µM. It was aliquoted into microtubes at volume of 100µl each. The microtubes were put in liquid nitrogen for shock-freezing and then placed in the freezer at -80°C. Each aliquot was used only once.

2.1.5.8 Preparation of a stock solution of MRS2179

MRS2179 is an antagonist of P2Y1 receptor, another ADP receptor on platelets. [7] 1mg of MRS2179 was dissolved in 2.352ml of PBS to reach a stock concentration of 1mM. This solution was aliquoted into microtubes of a volume of 100µl each. After being shock-frozen in liquid nitrogen, the aliquotes were put in the freezer at -80°C. Each aliquot was used only once.

2.1.5.9 Preparation of AlexaFluor 405 Streptavidin conjugate

1ml of PBS was added to the tube containing antibody powder, which then had a concentration of 1mg/ml. 50µl of this stock were diluted in 950µl of PBS in a non-transparent microtube (to protect the solution from light) to achieve a concentration of 0.05mg/ml. This stock was used for the experiments. The 1mg/ml stock was stored at -80°C and the 0.05mg/ml stock was stored at -20°C.

2.2 Methods

2.2.1 Whole blood assay for measuring the activation of thrombocytes of healthy donors and preparation for flow cytometry

Whole blood was drawn from an antecubital vein. The first 2.5ml of blood were discarded afterwards and the blood used for the experiments was slowly collected in a hirudin tube. The blood was stored at room temperature for about 40 minutes while the other reagents were prepared. Microtubes of a maximal volume of 1.5ml were used. To reach a final volume of 100µl, the tubes contained 80µl of reagents mixed with PBS (see below). To this mix 20µl of whole blood in the hirudin-tube was added. The reagents (controls, stimulators or inhibitors of complement activation, depending on the assay (see result section and labeling of each graph)) and PBS were added to reach final concentrations of the reagents as listed in Table 5.

Table 5: Final concentrations of reagents

Reagent	**Final concentration/amount**
ADP	5µM
C3a	1.8µM
C5a	0.18µM
C5	0.5µM
Cangrelor	100µM
Compstatin (Cp40)	4µM
Eculizumab	0.4µM
MRS2179	1mM
OmCI	1.2µM
PAF	0.95µM
Ravulizumab	0.4µM
rRBC	5µl
Serotonin	10µM
shhRBC	Dependent on the assay
shrRBC	5µl
Zymosan	Pellet of 20µl (see description above)

After adding all the reagents, 20µl of the whole blood were put into the tubes to activate and start the reaction. The microtubes were incubated at 37°C for 20 minutes. The next steps depended on what staining procedure was used. One duplicate was used for isotype staining while the second was used for the staining with the actual detection antibody.

If just CD62P (to detect P-selectin) was used as an activation marker, 100µl of a fixation solution were added immediately (0.5% paraformaldehyd) after the incubation of the reaction was finished. The samples were then centrifuged for 5 minutes at 300g at room temperature. To measure the level of hemolysis in the reaction, where applicable, 50µl of the supernatant of the centrifuged reaction were added to a 96-well plate to be evaluated spectrophotometrically in the TECAN plate reader at 405nm. Then 500µl of PBS were added. Next the samples were stained with antibody-cocktails that were prepared up front. Each cocktail contained PBS and so much of the desired antibody that final volumes of the antibodies were as listed in Table 6. After addition of the antibody mixtures, the samples were incubated in the dark at room temperature for 20 minutes while shaking.

If PAC-1 (to detect CD41 of activated platelets) was used as a staining antibody, the fixation step was performed after the staining step. In this case, the antibody cocktails were added and the samples were incubated at room temperature for 20 minutes, before the fixation solution was added. Centrifugation and levels of hemolysis were done as described above.

500µl of PBS were added. The antibody CD62P is not visible per se in the flow cytometer. So its requires a marker that binds to CD62P to make it visible. Therefore, 25µl of another staining cocktail containing the corresponding agent was applied to the samples and incubated at room temperature in the dark, but this time for 30 minutes. As soon as the 30 minutes were over, the samples were centrifuged under the same conditions as before and 500µl of PBS were added again. Finally, 50µl of the sample were diluted in a FACS tube with 450µl of PBS and platelet activation was measured.

2.2.2 Activation of PNH thrombocytes and preparation for flow cytometry

Since the CD59 antibody binds to all CD59 receptors of all cells and not only to platelets, a large amount of this reagent would be required if staining whole blood. In order to achieve a concentration of the antibody high enough to stain all CD59 receptors and therefore all platelets (and not just some of them) the samples were diluted more before adding the antibodies (to have fewer cells in total). The initial sample preparation steps were the same

as described above, but before adding the antibodies, 100µl of the samples were mixed with 400µl of PBS and stained with the appropriate antibodies. After completion of the entire assay, the dilution step at the end became unnecessary before measuring the samples in the FACS.

Table 6: Final dilution of the antibody/staining molecule

Antibody	**Final sample volume**
AlexaFluor® 405-Streptavidin	1µl
APC-Streptavidin	1µl
CD41 (AlexaFluor®647)	2µl
CD41 (FITC)	2µl
CD59 (APC)	2µl
CD62P (Biotin)	0.5µl
IgM isotype control (FITC)	4µl (predilution of 1:20)
Mouse IgG1 k Isotype Control (APC)	2µl
Mouse IgG1, k Isotype control (FITC)	2µl
Mouse IgG1 negative control (Alexa Fluor® 647)	2µl
PAC-1 (FITC)	4µl

2.2.3 Flow cytometry based analysis of platelets

The fluorescence signals and intensities of the stained cells were measured by flow cytometry. To gate platelets, FACS plots of the harvested whole blood reactions were shown in plots with the dimensions' sideward scatter versus FITC-A signal. The latter corresponds to the CD41 (GPIIb)-expression on cells (which is a marker of non-activated and activated platelets). Using a positive control, a manual gate was set around the CD41 positive cells, what can be seen in Figure 3. The actual measurements were performed by counting 10 000 within the set gate or until the sample tube was empty (this happened only with some PNH samples). The flow rate was set medium. Only the CD41+ cells (meaning all platelets) were then analyzed for CD62P and PAC-1 surface expression by changing the FITC-A signal into the respective signal of the antibody used (APC or AlexaFluor) to measure the amount of active platelets. For this the median fluorescent

intensity (MFI) of each sample was calculated using FlowJo (version 7.6.5.). Examples are shown in Figure 4.

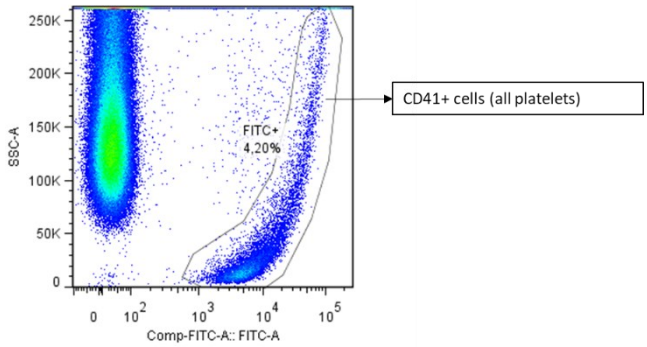

Figure 3: Gating strategy of thrombocytes. *For the x-axis, FITC was set to detect all platelets. A gate was set around the CD41 positive cells.*

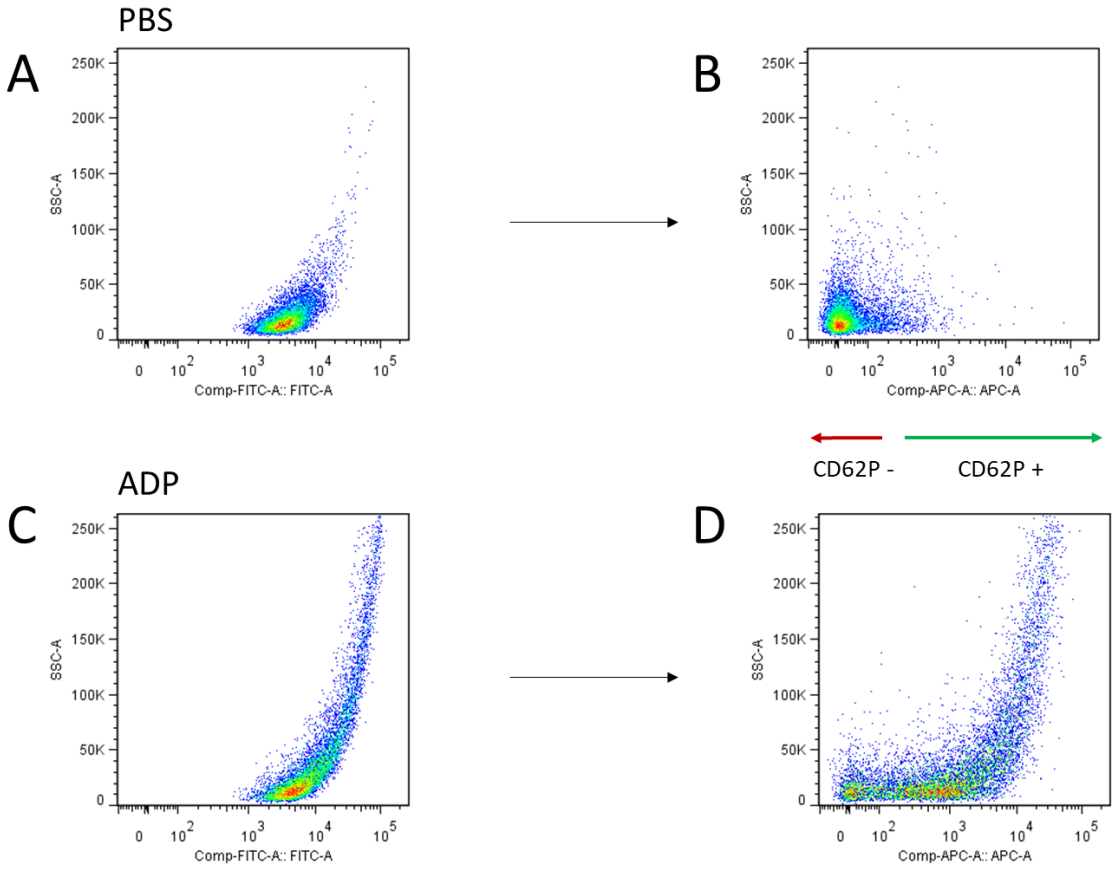

Figure 4: Stimulation of the diluted whole blood with PBS or ADP and analysis of platelet activation by flow cytometry (plots created with FlowJo).

A *shows the general gating strategy for platelets from diluted whole blood samples. In this example the platelets were not activated and the blood was only diluted in PBS. The gate is set around all CD41+ cells (all platelets). Further measurements are only carried out with these cells.* ***B****: The dimension x-axis changes from FITC-A to APC-A, which represents CD62P, the activation marker. As most cells are on the left side (nearly no CD62P can be detected), the platelets are not activated.* ***C*** *shows the same than A, but ADP (activation marker of thrombocytes) was added to the whole blood instead of PBS. The set gate is the same.* ***D****: The dimension is again changed to APC to detect the CD62P positive cells. More positive (and therefor activated) platelets can be measured.*

PBS: Phosphate-buffered saline; ADP: Adenosine diphosphate

2.2.4 Sample preparation for C3a-ELISA

In preparation for the C3a-ELISA, the complete thrombocyte activation assay (see section 2.2.1) was performed again (samples: PBS, rRBC, rRBC+Cp40, rRBC+Ravulizumab+OmCI, Zymosan, Zymosan+Cp40), but this time the assay was scaled up by the factor two (twofold amounts) yielding a final volume of 100µl. Subsequently, the samples were divided into two 100µl aliquots. One half was used to perform the flow cytometry analysis for platelet activation as described above. The other half was used to determine the C3a concentration in the assay using a commercial C3a-ELISA kit. For this, 10µl of a 50mM EDTA solution (instead of paraformaldehyde as described in 2.2.1, since the C3a-ELISA could not be performed when molecules are fixed with paraformaldehyde) were added to each tube before centrifugation at 300g for 5 minutes at room temperature. The supernatant was transferred to a new tube, frozen in liquid nitrogen and stored at -20°C freezer prior of the C3a-ELISA assay being performed.

2.2.5 C3a-ELISA

The preparation of all the reagents for the ELISA was performed as described in the manual. As recommended, the samples were diluted in two steps at a ratio of 1:200 (10µl of test specimen with 90µl of diluent specimen; 25µl of the resultant solution were added to 475µl of the diluent specimen). 100µl of each (such diluted) sample and the supplied standard were added to the microtiter plate (samples as duplicates). The plate was incubated at room temperature for 60 minutes. Then the liquid was removed with a pipette and 300µl of wash solution were added to each well. This washing step was performed for a total of four times. After that, the liquid was completely removed by tapping the plate upside down on an absorbent paper. Then, 100µl of the C3a plus conjugate were dispensed into each well and the plate was incubated for 60 minutes at room temperature. After another four washing steps (as above), 100µl of the substrate solution were added and the plate was developed by detecting the colour change of the wells that contained the standards curve. 100µl of the stop solution were added when the well containing the standard had developed a clearly visible colour. The absorbance at 450nm was measured with the TECAN plate reader.

2.2.6 Calculation of the results

All results were first processed using Microsoft Excel. For descriptive statistics, statistical testing, and graphical representation, Prism 9.5.1 (GraphPad Software) was used. Results were considered statistically significant for $P < 0.05$ (marked with "*" when statistically significant, with "ns" when statistically non-significant). For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown.

3 Results

3.1 In vitro whole blood model using healthy donor blood reveals that hemolysis is sufficient and necessary for activating platelets

3.1.1 ADP as a positive control and PBS as a negative control

Since the aim of the thesis is to characterise complement signalling pathways that lead to prothrombotic cell activation, a way had to be found to visualize the activation of the cells (in this case thrombocytes). Two different antibodies were used for this purpose. A non-conformational epitope on CD41 (glycoprotein IIb-IIIa) was used to recognize all thrombocytes (whether active or not). An appropriate commercial antibody against this epitope was used to gate platelets in the whole blood assay. The well-known platelet activation marker CD62P (P-selectin) was used to evaluate platelet activation. A commercial antibody recognizing CD62P was used in flow cytometry to detect activated platelets. In experimental conditions in PBS-diluted whole blood, without addition of a known "platelet activator" almost no P-selectin was detected in the flow cytometer (Figure 5). This condition served as negative control in this setup. Since ADP is a known activator of platelets, it was used as positive control. By adding 5µM ADP to the whole blood sample, the thrombocytes showed a much higher median fluorescence intensity (MFI) of P-selectin in the flow cytometer than when adding PBS. Consequently, more P-selectin was translocated to the outer membrane of the platelets, indicating that the activation with ADP worked and can be used as positive control. After performing the assay four times and obtaining the same results each time, the negative and positive controls could be set this way.

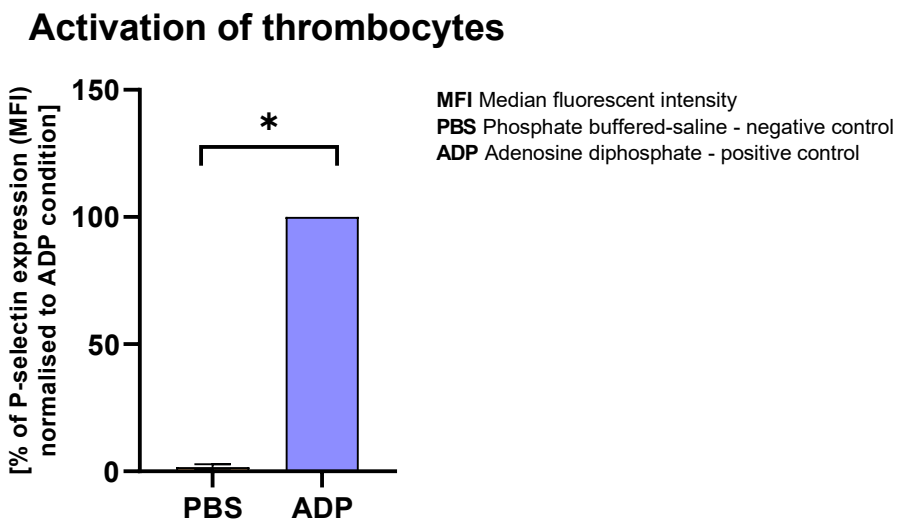

Figure 5: Positive and negative control. *The figure shows the activation of thrombocytes after the addition of either PBS or 5µM ADP to the whole blood sample. The P-selectin expression in the ADP condition was defined as 100% activation level. The average of four independent experiments with standard deviation is shown. Data were tested for statistical significance with unpaired t-test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: p < 0.05*

3.1.2 Rabbit red blood cells as activator of the complement system

The next step was to investigate the effect of an activator of the complement system on thrombocyte activation in my diluted whole blood setup. Red blood cells of rabbits (rRBC) are a known activator of the alternative pathway of the complement system. To test the different effects of complement activation on platelet activation, rRBC were added to the diluted human whole blood. Measuring the occurring hemolysis in the reaction (or specific complement activation products like *e.g.* the C3a) allowed in infer to what level complement activation had occurred in the reactions. By employing different well-known complement inhibitors, the reactions could be controlled to exclusively allow proximal or terminal complement activation. When adding PBS, as negative control, or ADP, as positive control, no hemolysis had occurred indicating that under these conditions no erythrocytes lysed. This was expected as only human erythrocytes were present under these conditions which are not expected to be lysed by the complement AP. However, when rRBC were added, the supernatant of these reaction turned red due to the released hemoglobin (unless the reactions were supplemented with reagents that inhibit the activation of the complement system).

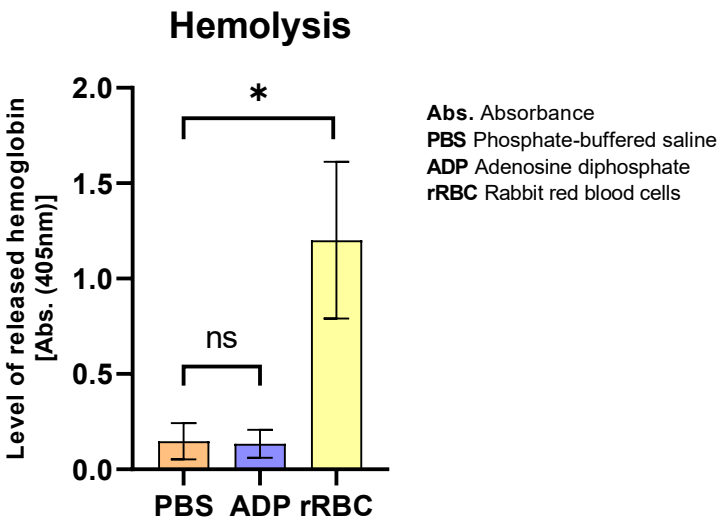

Figure 6: Hemolysis of rRBC in the diluted whole blood assay using control reagents. *After having incubated the blood samples with PBS, 5µM ADP or 5µl of rRBC and after fixation of the cells, the samples have been centrifuged and the absorbance of the supernatant was measured at 405nm. The figure shows the average of five independent experiments with standard deviation. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant*

To determine the effects of complement activation on thrombocytes, their activation was measured as described above. Figure 7 shows that the addition of rRBC into complement active whole blood results in activation of platelets, as measured by the translocation of P-selectin to the outer membrane. The rabbit cells activate the complement system, become opsonized with C3 and eventually lysed by establishing the membrane attack complex, as evidenced by the hemolysis. This rise in P-selectin is not as high as with the potent activator ADP, but it is clearly increased from the levels of P-selectin expression in the negative control condition (PBS).

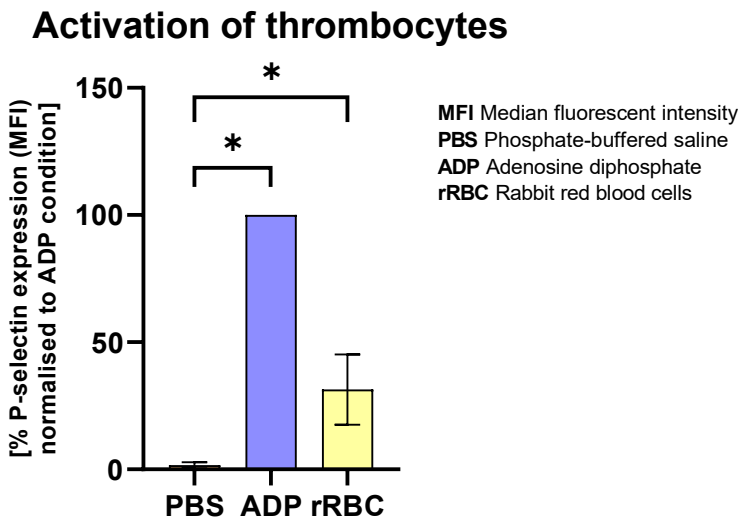

Figure 7: Activation of thrombocytes in the diluted whole blood assay using control reagents. *After the addition of either PBS, ADP or rRBC to the samples, the MFI of P-selectin was determined. P-selectin expression after simulation with ADP was defined as 100% activation and used as a reference. The average of five independent experiments with standard deviation is shown. Data sets were tested for outliers using ROUT outlier test (Q=5%). 1 outlier was removed for PBS before statistical testing. The cleaned data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$*

3.1.3 Effects of complement inhibition by Cp40 and eculizumab on platelet activation in the whole blood model

Various inhibitors of the complement system were added to the samples to determine if and in case how the complement system impacts on the activation statues of thrombocytes. Two well-established drugs that inhibit at different levels of the cascade were used for this purpose. Cp40 is an inhibitor of C3 by preventing its activation. C3 activation happened within the proximal complement cascade and represents the central step of the whole cascade. Eculizumab is a clinically approved C5-inhibitor and is used as a therapeutic in clinical practice. Addition of rRBC in the whole blood assay resulted in their lysis. However, when either Cp40 or Eculizumab was added, no hemoglobin release could be detected. Thus, inhibition of the complement system with Cp40 or eculizumab inhibited the lysis of rRBC in diluted human whole blood demonstrating the rRBC lysis was depended on an active complement cascade in the sample. The addition of rRBC was the only sample that induced significantly more hemolysis than the other reactions.

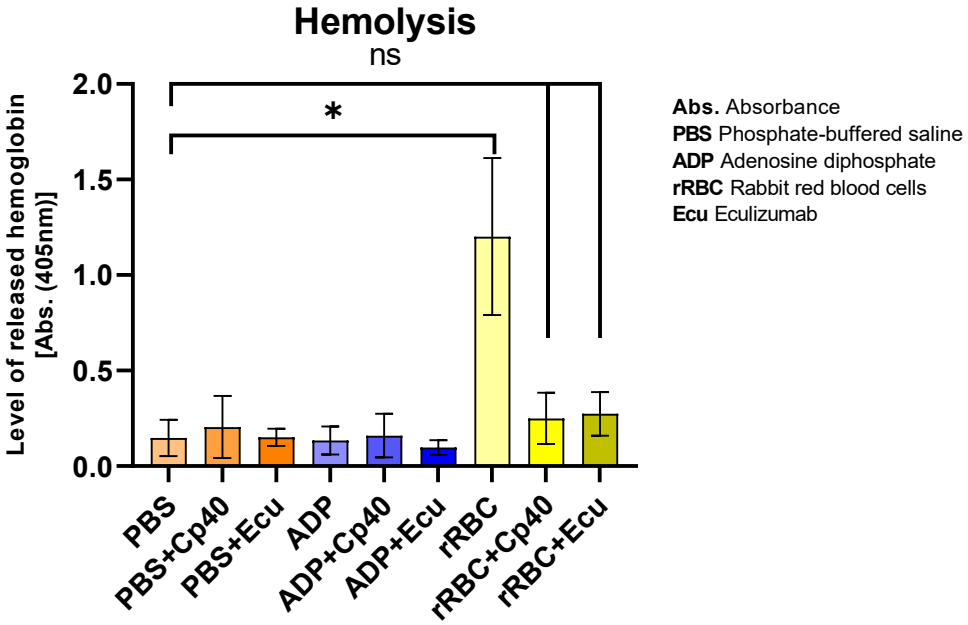

Figure 8: Hemolysis inhibited by complement inhibition. *After treatment of the blood samples with PBS, 5µM ADP, 5µl of rRBC with or without Cp40 or Eculizumab as described, the absorbance of the supernatant was measured at 405nm. The average of five independent experiments with standard deviation is shown. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant*

After demonstrating that the complement system could be properly inhibited by the used inhibitors, thrombocyte activation in these various conditions was measured by flow cytometry. Addition of ADP or rRBC (in absence of complement inhibitors) activated platelets. In contrast, addition of the complement inhibitors and rRBC prevented hemolysis and platelet activation although not completely. There was no difference between those two conditions (Eculizumab or Cp40). Under Eculizumab inhibition only the terminal complement pathway was blocked, the proximal part of the complement system was fully expected to be active. However, the thrombocytes were not more activated when compared to the Cp40 condition, where the proximal and terminal cascade were inhibited. Both times, the MAC was not formed. This means that initiating the complement cascade and letting it run until the MACs are formed results in the release of CD62P on platelet surfaces. To compare the effect with the other samples, the inhibitors were also added to control reactions containing PBS or ADP but no rRBC. With or without Cp40 or Eculizumab, activation in these controls remained comparable, indicating that the effect of ADP is not affected (largely) by complement activation products of the proximal and/or terminal pathway.

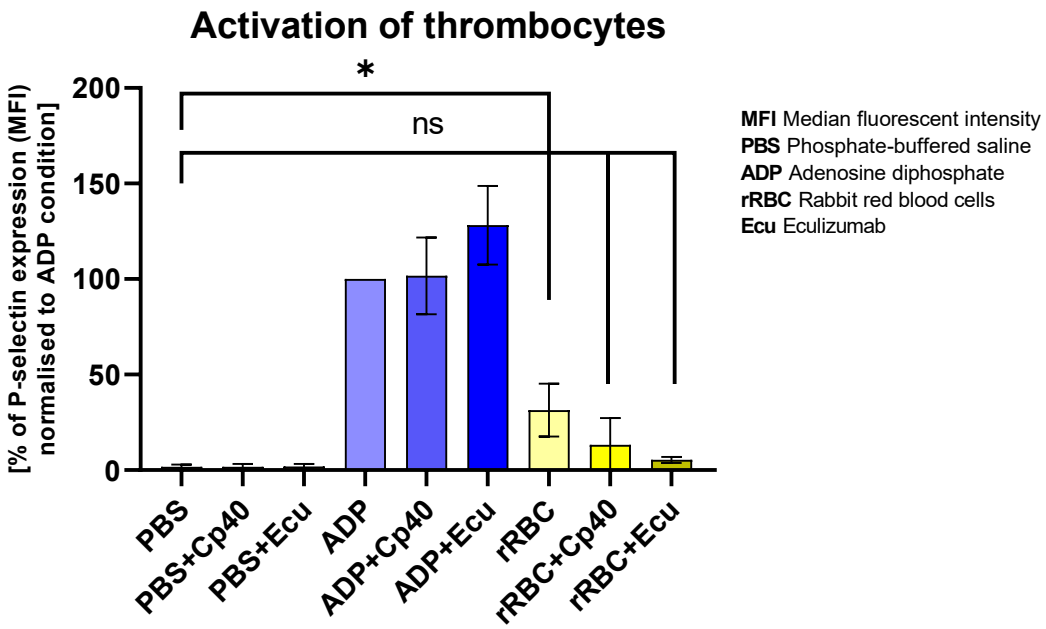

Figure 9: Activation of thrombocytes in the whole blood assay in presence of complement inhibitors. *After the addition of either PBS, ADP or rRBC to the samples and after combining them with Cp40 or Eculizumab, the MFI of P-selectin was determined. P-selectin expression after simulation with ADP was defined as 100% activation and used as a reference. The average of five independent experiments with standard deviation is shown. Data sets were tested for outliers using ROUT outlier test (Q=5%). 1 outlier each for PBS, PBS+Cp40, PBS+Ecu and rRBC+Ecu was removed before statistical testing. The cleaned data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant*

3.1.4 Platelet activation in whole blood after addition of mechanically shattered rRBC

Since in the previous results activation of thrombocytes was only seen in the hemolytic samples, a method had to be found to discriminate whether complement activation (leading to lysis) or the release of the intracellular content of the destroyed erythrocytes are responsible for platelet activation (or indeed both). The rRBC were therefore destroyed mechanically to release the content immediately before adding these shattered rRBC to the samples. The process of shattering the rabbit erythrocytes is described in 2.1.5.2. Instead of "normal" intact rRBC, the same corresponding number of shattered cells was added to the samples. Addition of shattered rRBC led to the detection of released hemoglobin, as expected. The levels of released hemoglobin were similar to those when intact rRBCs had been added to complement active whole blood resulting in complement mediated lysis of rRBCs.

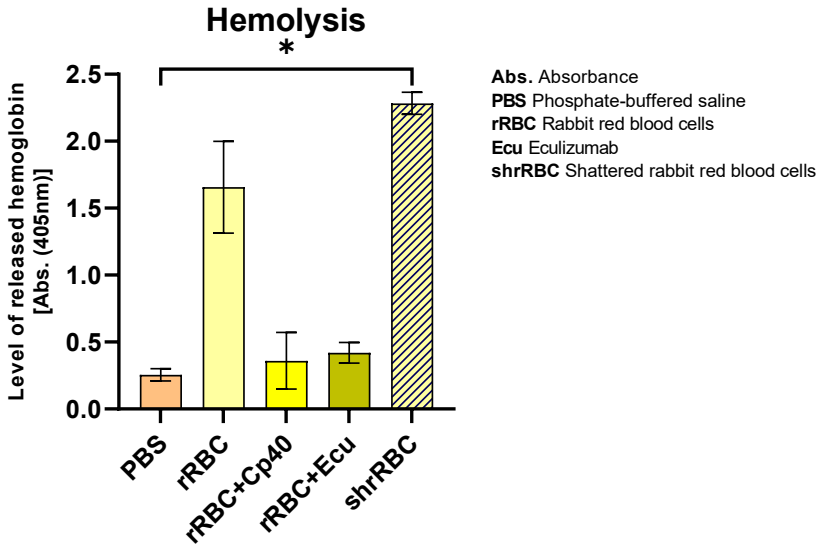

Figure 10: Hemolysis in the diluted whole blood assay after addition of shattered or intact rRBCs. *After treatment of the blood samples with PBS, 5µl of rRBC with or without Cp40 or Eculizumab or 5µl of shrRBC as described, the absorbance of the supernatant was measured at 405nm. The average of four independent experiments with standard deviation is shown. Data sets were tested for outliers using ROUT outlier test (Q=5%). 1 outlier for shrRBC was removed before statistical testing. The cleaned data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$*

Addition of the shattered rRBCs strongly activate the platelets.

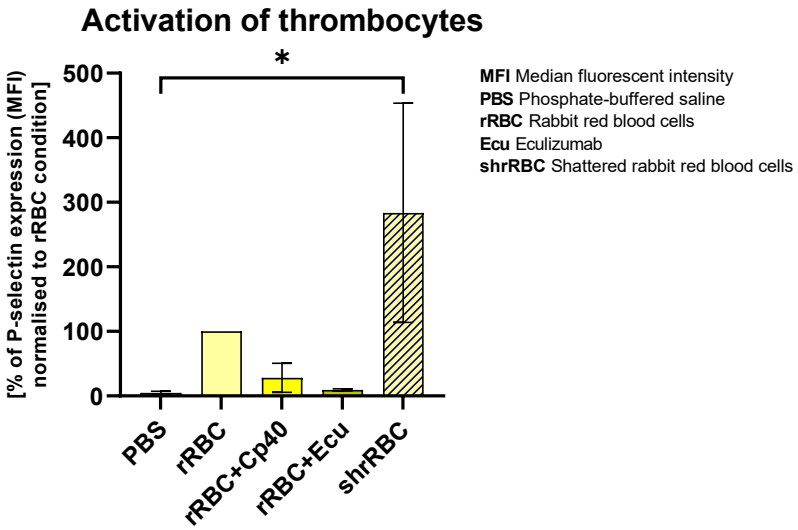

Figure 11: Platelet activation in whole blood after addition of shattered or intact rRBCs. *After the addition of either PBS, rRBC or shrRBC to the samples, the MFI of P-selectin was determined. P-selectin expression after simulation with rRBCs was defined as 100% activation and used as a reference. The average of seven independent experiments with standard deviation is shown. Eculizumab was only added in 4 experiments. Data sets were tested for outliers using ROUT outlier test (Q=5%). 1 outlier for rRBC+Ecu was removed before statistical testing. The cleaned data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$*

3.1.5 Inhibition of ADP-signalling by adding the ADP-receptor blockers Cangrelor/MRS2179

Since an erythrocyte contains many components, the responsible agent that activates the platelets upon release of intracellular substances in rRBCs upon mechanically shattering of rRBCs or their complement-mediated lysis must be singled out. One known activator of thrombocytes that may be released during lysis is ADP. The next step was then to find out, if this can be the responsible element for the strong platelet activation observed so far. ADP interacts with two different purinergic receptors on platelets, termed P2Y1 and P2Y12. Numerous inhibitors exist for P2Y12, and Cangrelor was used for the in vitro experiments here. To prevent any effect of ADP, the second purinergic receptor, P2Y1 was also inhibited. This was achieved with the agent MRS2179. Before the reagents were used, the ideal amount was determined by titrating the respective concentrations of these two receptor antagonists. To see if the drugs worked and what concentration was needed, the experiments were performed with ADP as appropriate activator. The ADP-receptor antagonists were added to the whole blood reactions alongside with ADP as shown in the figures below. The inhibition of platelet activation by both ADP-receptor antagonists is concentration dependent (Figure 12/13). With Cangrelor, a well-functioning (but not complete) inhibition of the platelets was achieved at 1µM, with MRS2179 at 10µM. These concentrations were used in the following, as they are sufficient in combination for a complete platelet inhibition, which can be seen in the following experiments.

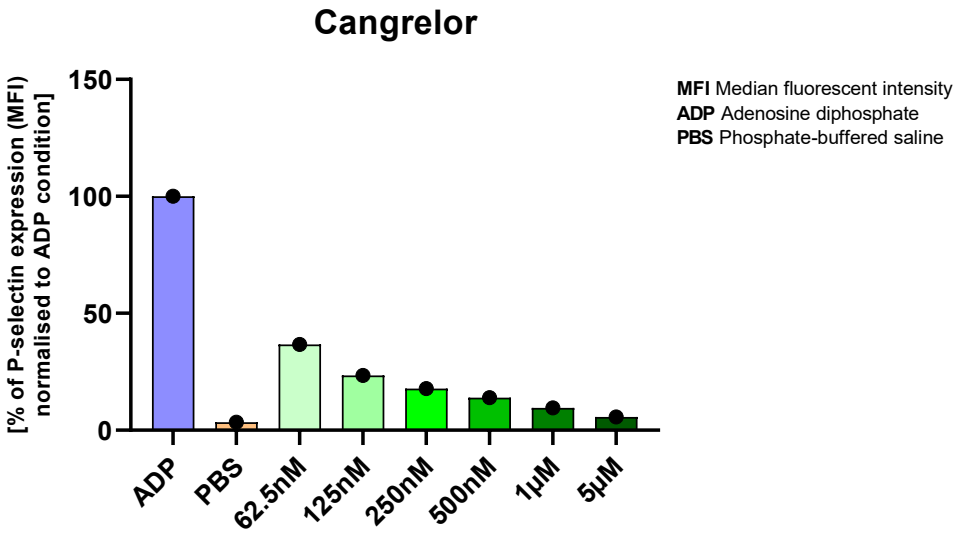

Figure 12: Activation of thrombocytes when using different concentrations of Cangrelor. *ADP, PBS and ADP plus different concentrations of Cangrelor were given to the samples. The MFI of P-selectin was determined and shows the platelet activation.*

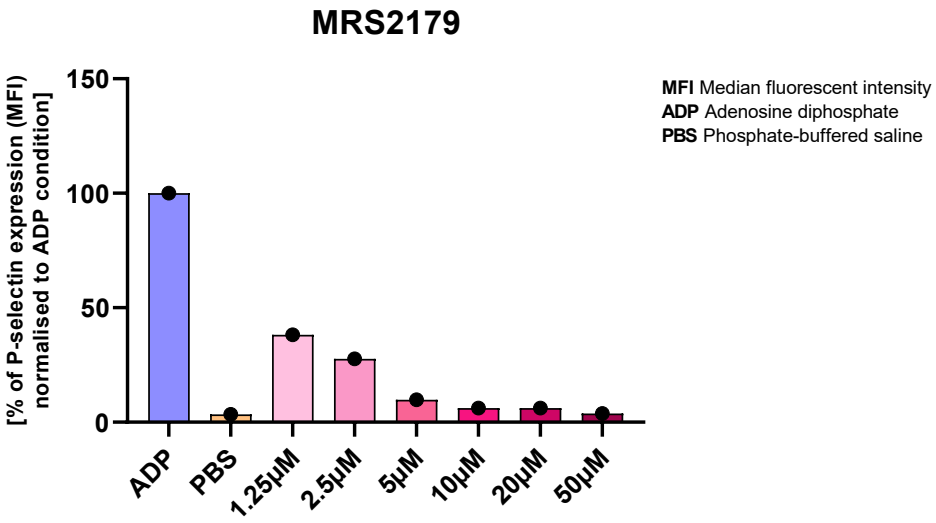

Figure 13: Activation of thrombocytes when using different concentrations of MRS2179. *ADP, PBS and ADP plus different concentrations of MRS2179 were given to the samples. The MFI of P-selectin was determined and shows the platelet activation.*

Now, that it is to know that the ADP-receptor antagonists are able to inhibit ADP-triggered platelet activation, the same experimental setup was performed with the same concentration series of the receptor antagonists, but this time rRBCs were used instead of ADP. The inhibitors were able to substantially inhibit the activation of the platelets even though no ADP had been added before. However, a full inhibition of platelet activation was not achieved by a single ADP-receptor antagonist when rRBCs were added to complement active whole blood.

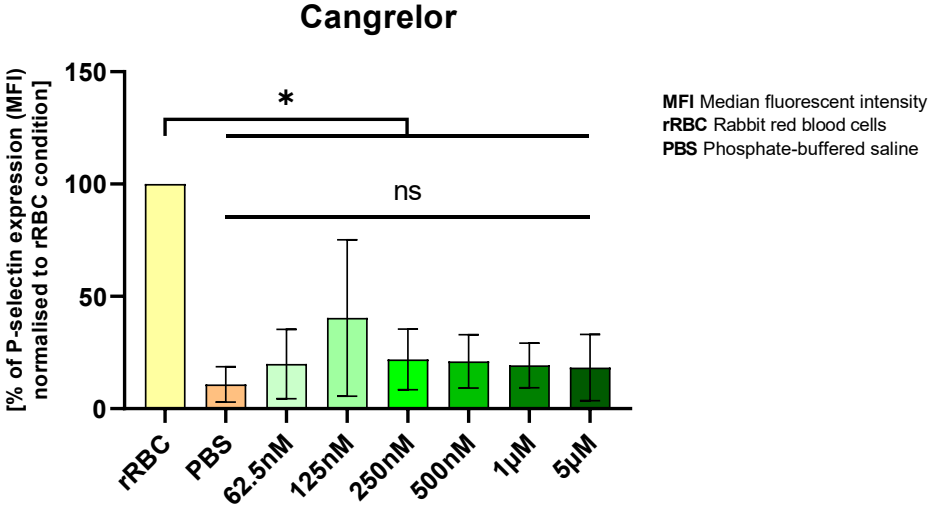

Figure 14: Activation of thrombocytes when using different concentrations of cangrelor. *RRBC, PBS and rRBC plus different concentrations of Cangrelor were added to the samples. The MFI of P-selectin shows the platelet activation. RRBC was defined as 100% activation and used as a reference. The average of three independent experiments with standard deviation is shown. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant*

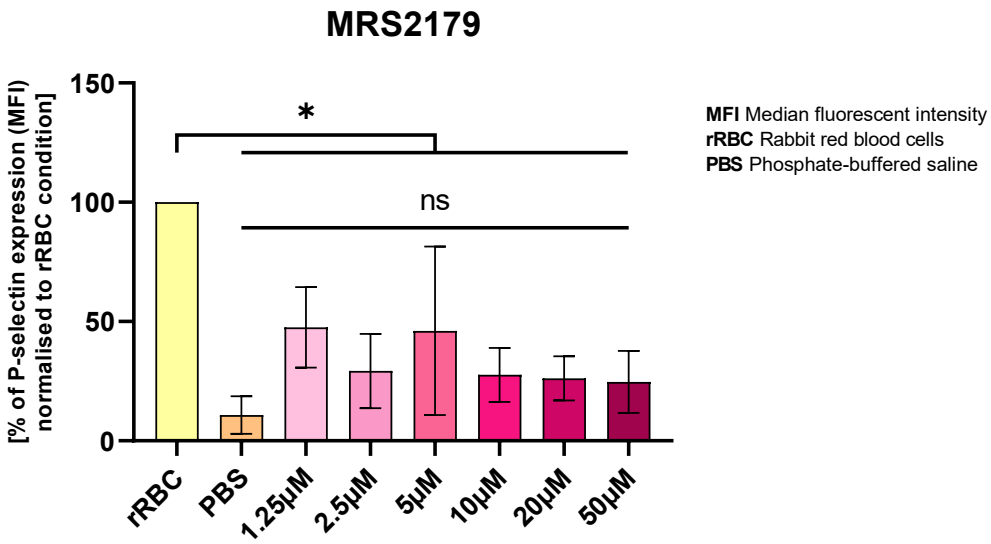

Figure 15: Activation of thrombocytes when using different concentrations of MRS2179. *RRBC, PBS and rRBC plus different concentrations of MRS2179 were added to the samples. The MFI of P-selectin shows the platelet activation. RRBC was defined as 100% activation and used as a reference. The average of three independent experiments with standard deviation is shown. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant*

Therefore, in the next experiments, the ADP-inhibitors were added simultaneously together to the samples to guarantee complete inhibition of the ADP-receptor responses. A concentration of 1µM of cangrelor and 10µM of MRS2179 was chosen to largely exclude effects of ADP signalling.

The different activators and inhibitors were added to the samples in the following way: PBS as negative control, ADP as positive control and also mixed with the ADP-receptor antagonists and rRBCs and shrRBCs were added alone or mixed with either Cp40 as inhibitor of the complement system or the ADP-receptor antagonists.

In the first step, hemolysis was measured to identify in which samples complement-mediated lysis of rRBCs occurs. RRBC did induce the cascade, as expected. Cp40 was able to inhibit C3 and thus complement-mediated lysis of rRBCs. Cangrelor and MRS2179 had no effect on inducing or stopping rRBCs lysis.

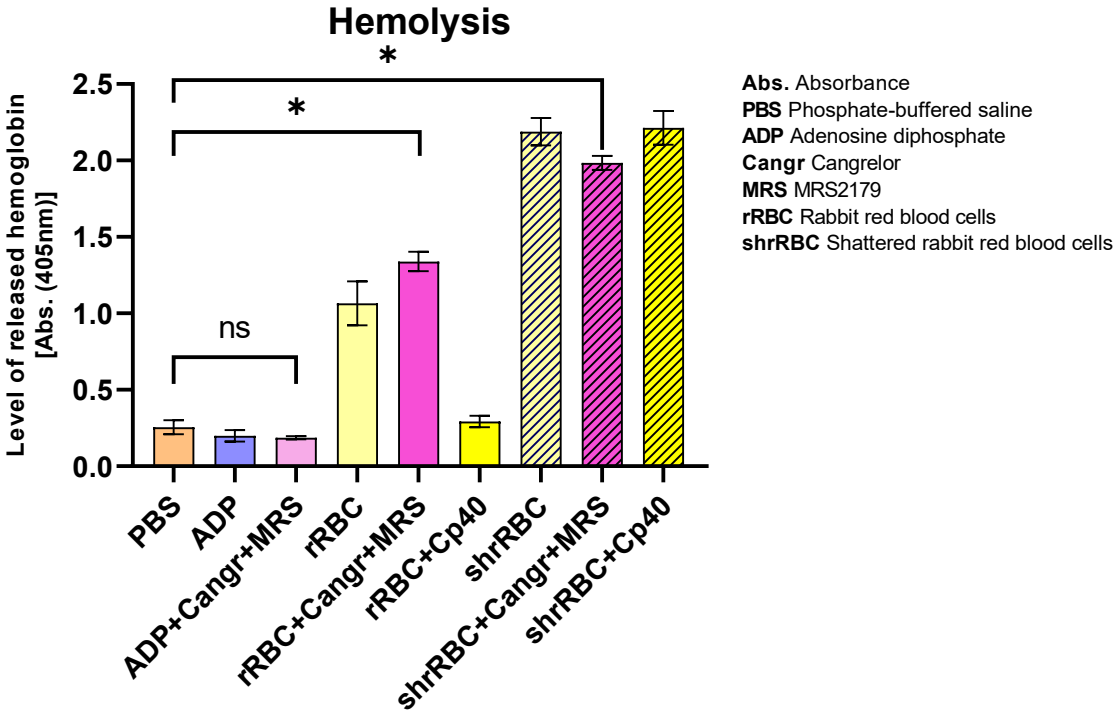

Figure 16: Hemolysis in the diluted whole blood assay after using Cangrelor/MRS2179. *After treatment of the blood samples with different activators and inhibitors, the absorbance of the supernatant was measured at 405nm. The average of three independent experiments with standard deviation is shown. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant*

Samples with added rRBC exhibited hemoglobin release, indicating that the complement system was forming MAC. Cangrelor and MRS2179 had no effect on complement-mediated hemolysis. If the complement system were the activating factor of the thrombocytes, increased P-selectin expression on platelets should be measurable by flow cytometry in all samples where hemolysis occurred. This would be the samples rRBC and rRBC + Cangr +MRS. ShrRBC +/- inhibitors, of course, shows hemoglobin release as the erythrocytes are shattered mechanically before adding them to the assay. The results show that the ADP-receptor antagonists completely prevented platelet activation, regardless of which platelet activator was added (ADP, or rRBCs in conditions of active complement). Cangrelor and MRS2179 inhibited the activation by ADP. They also inhibited the activation by complement-mediated or mechanically-mediated lysis of rRBCs. As a control Cp40 was analyzed also in conjunction with shrRBCs, but the C3 inhibitor had no effect on preventing platelet activation when rRBCs were added that had already been lysed mechanically prior addition into the assay. The results from this series of experiments imply that ADP, released

from erythrocytes, after they have been lysed by the MAC, represents an important danger signal that leads to platelet activation.

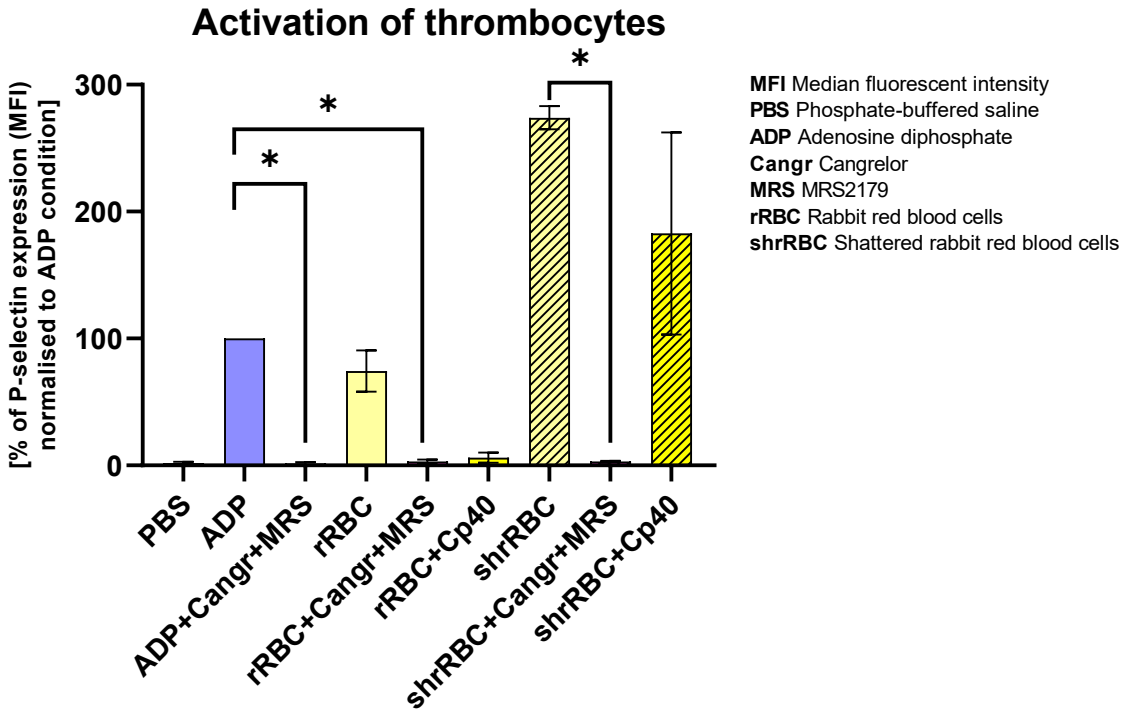

Figure 17: Activation of thrombocytes in the diluted whole blood assay after addition of Cangrelor/MRS2179. *After the addition of either PBS, ADP, rRBC or shrRBC to the samples combined with either Cp40 or Cangr/MRS, the MFI of the activation marker P-selectin was determined and shows the platelet activation. RRBC was defined as 100% activation and used as a reference. The average of three independent experiments with standard deviation is shown. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$*

3.1.6 Human system: shattered human RBC

The result above show that Cangrelor and MRS2179 are able to prevent platelet activation due to addition of ADP or release of ADP from lysed/shattered rRBCs. The erythrocytes used in the previous experiments were from a foreign source, *i.e.* rabbits. RRBCs were used because they are known to be strong activators of the complement alternative pathway whereas human erythrocytes are not. Nonetheless, by adding rRBCs, a source of foreign cells was added to the whole blood assay and in the next step I wanted to control for this and remain within a completely human setting using shattered (mechanically disintegrated human RBCs). In the next experiments, a proportion of the erythrocytes from the same donor who had donated the blood for the whole blood assay samples were washed and shattered and added to the whole blood assay. Indeed, this way I simulated what effect the lysis of the donor's own erythrocytes would have on the thrombocytes and I observed

whether ADP inhibitors could prevent the thrombocyte activation as in the heterologous system with the rRBCs.

In the following experiments, donor erythrocytes were shattered like the rabbit cells (described above) to release the content and imitate hemolysis before being added to the whole blood assay at different concentrations. To a sample with the highest concentration of shhRBC used, Cangrelor and MRS2179 were also added. Of course, the supernatant is stained with released hemoglobin when shattered erythrocytes are given to the samples. As can be seen in figure 18 below, the assay worked well, the color of the supernatant is dependent of the amount of shhRBC added.

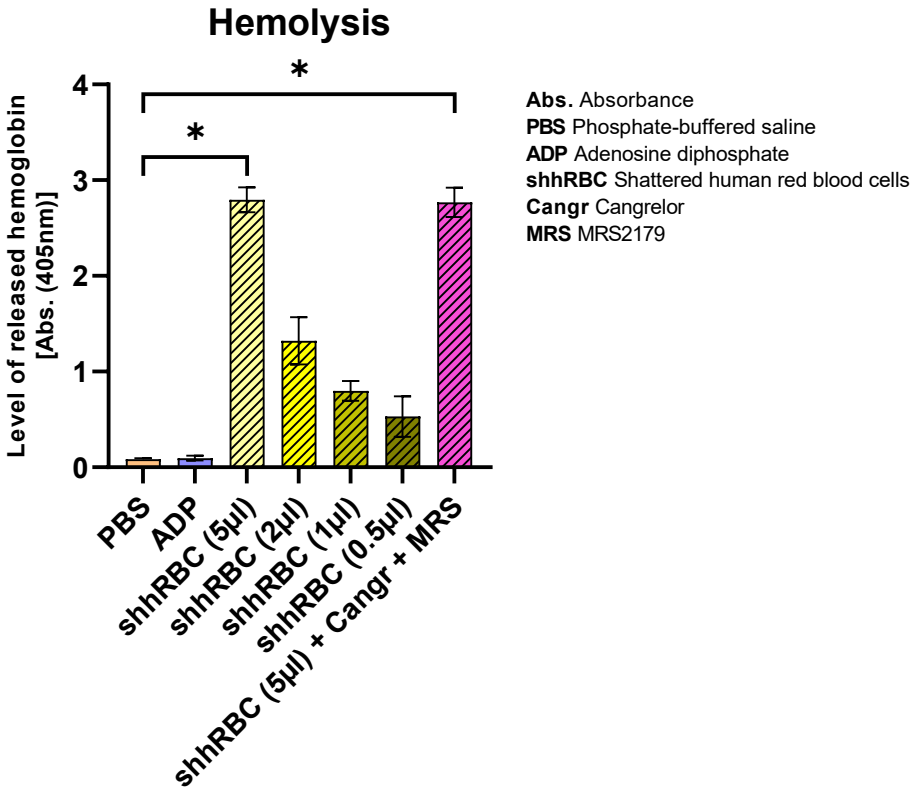

Figure 18: Hemolysis and the addition of shhRBC and Cangr/MRS. *After treatment of the blood samples with shhRBC in different concentrations and Cangr/MRS, the absorbance of the supernatant was measured at 405nm. The average of four independent experiments with standard deviation is shown. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$*

Platelets were strongly activated by the shattered human erythrocytes, correlating with the amount of intracellular danger signals. Cangrelor and MRS2179 were able to inhibit the activation completely to the levels of the negative control PBS.

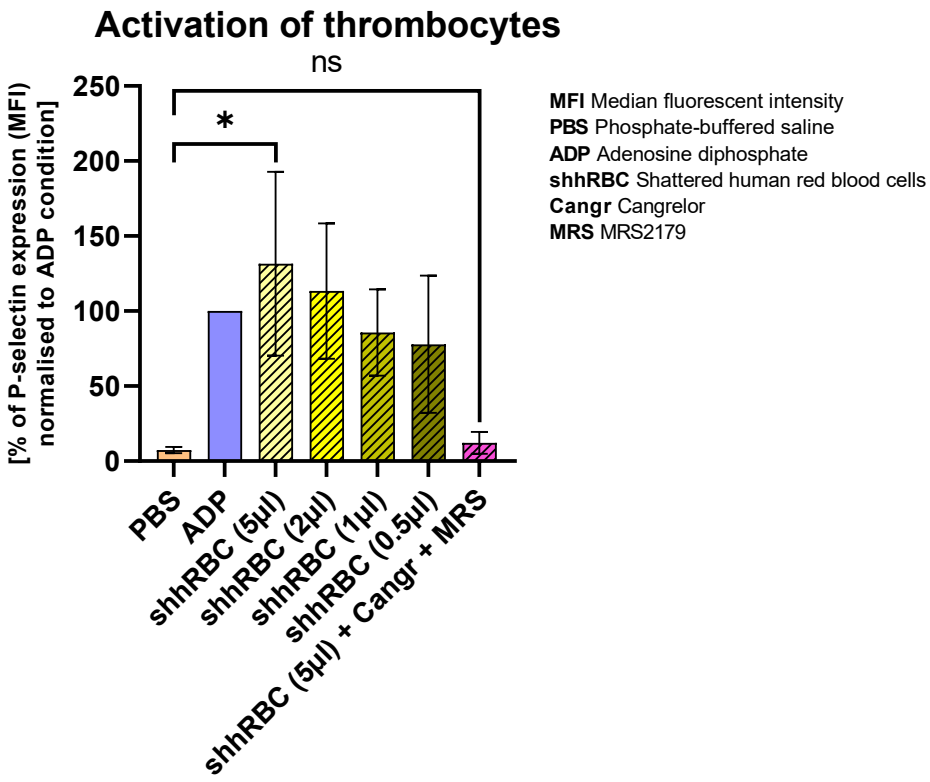

Figure 19: Activation of thrombocytes with shhRBC and Cangr/MRS. *After the addition of either PBS, ADP or shhRBC in different concentrations and in combination with Cangr/MRS, the MFI of P-selectin was determined and shows the platelet activation. The average of four independent experiments with standard deviation is shown. Data sets were tested for outliers using ROUT outlier test (Q=5%). No outliers were removed. The data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant.*

3.1.7 C3a and C5a

To confirm the hypothesis that the anaphylatoxins C3a and C5a of the complement system do not have a major influence on activating platelets, C3a and C5a and a mixture of both were added to the samples at a much higher concentration than would occur naturally. The anaphylatoxins were also added to already moderately preactivated platelets (with 0.5µM of ADP instead of the 5µM normally used) to see if they may induce platelet activation upon pre-stimulated cells. C3a and C5a individually or in combination, failed to induce thrombocyte activation. No P-selectin could be measured by FACS. Additionally, the anaphylatoxins were not able to enhance already preactivated platelets, and the MFI level was the same as without C3a and/or C5a.

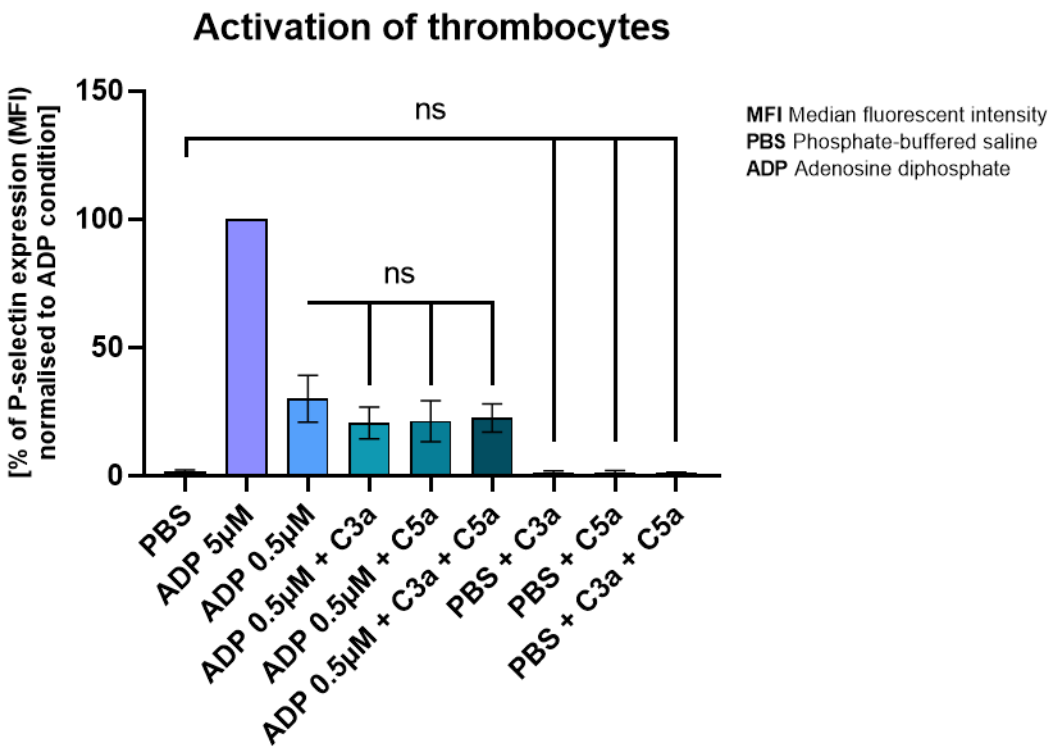

Figure 20: Activation of thrombocytes with ADP, C3a and C5a. *After the addition of PBS or ADP in different concentrations and each of them combined with C3a and C5a, the MFI of P-selectin was determined and shows the platelet activation. ADP was defined as 100% activation and used as a reference. The average of four independent experiments with standard deviation is shown. All data sets except PBS, ADP 5µM and ADP o.5µM was only used three times. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. ns: non-significant*

3.1.8 Zymosan

In the experiments shown so far, the complement system was activated with rRBC only. To support our hypothesis more broadly, another activator of the complement cascade was investigated in the whole blood assay. For this purpose, the well-known activator of the alternative complement pathway, zymosan, was used. For comparison PBS, rRBC with or without complement inhibitors and shattered rRBC are also used in this experiment. After incubation of the whole blood samples and centrifugation like before, hemolysis was measured. As previously observed, the samples with rRBC show hemoglobin in the supernatant. PBS serves as a negative control and Cp40 and Eculizumab inhibited lysis when added to the conditions with rRBC. Zymosan is unable to induce red cells to lysis but is expected to activate the complement system.

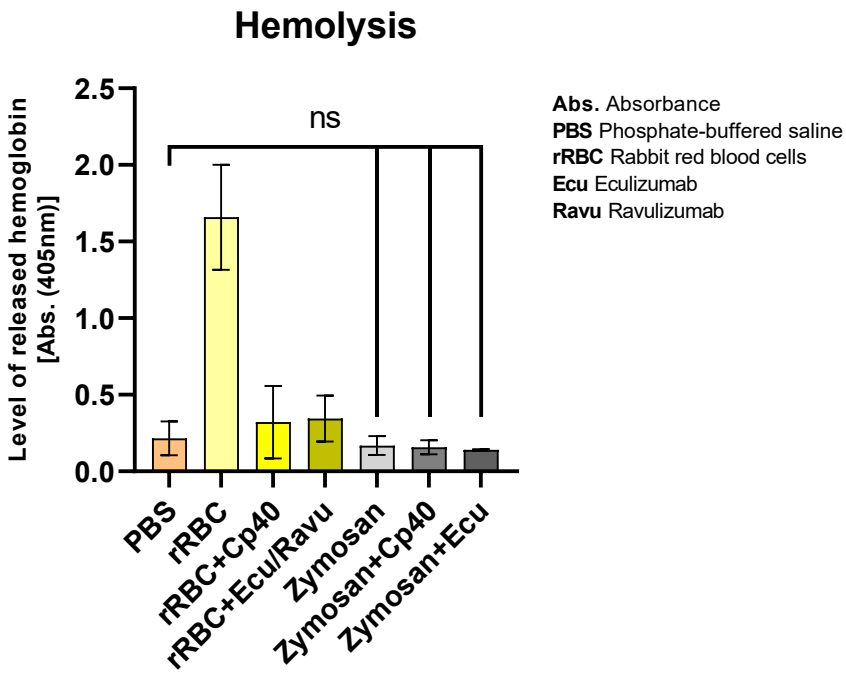

Figure 21: Hemolysis after the addition of zymosan. *After treatment of the blood samples with zymosan and rRBC and each of them in combination with either Cp40 or Ecu/Ravu, the absorbance of the supernatant was measured at 405nm. The average of four independent experiments with standard deviation is shown. Zymosan+Ecu was only used three times. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. ns: non-significant*

The next step was to find out whether the platelets were activated. As before, rRBC and shrRBC induced a flip of P-selectin to the outer membrane of the cells, which is seen as higher MFI in FACS. Cp40 and Eculizumab were able to inhibit the complement activation induced by rRBC and subsequent platelet activation. In the samples with zymosan, either alone or together with Cp40 or Eculizumab, the platelets showed no activation of platelets. Consequently, in these experiments, an active complement system is unable to properly activate platelets, mirroring the previous results. However, as a proof that complement activation had occurred, the generation of the anaphylatoxin C3a in the supernatant of the reactions was measured (Figure 25).

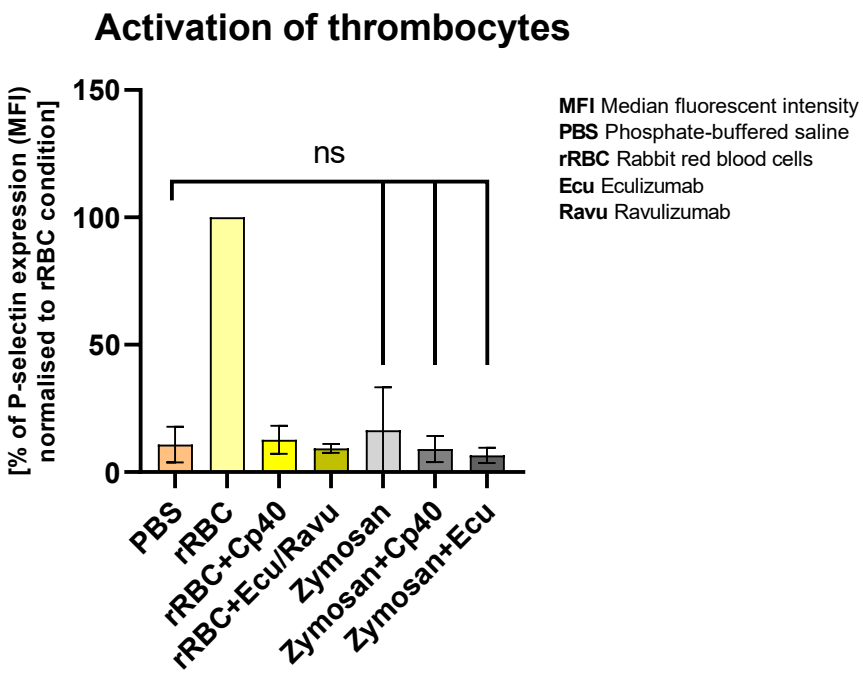

Figure 22: Activation of thrombocytes by zymosan. *After the addition of zymosan and and rRBC and each of them in combination with either Cp40 or Ecu/Ravu, the MFI of P-selectin was determined and shows the platelet activation. RRBC was defined as 100% activation and used as a reference. The average of four independent experiments with standard deviation is shown. Zymosan+Ecu was only used three times. Data sets were tested for outliers using ROUT outlier test (Q=5%). 1 outlier for rRBC+Ecu/Ravu was removed before statistical testing. The cleaned data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. ns: non-significant*

3.1.9 C3a-ELISA

The next experiment was performed to prove that the complement system was activated by the addition of zymosan. Platelets were incubated with PBS, rRBC, rRBC + Cp40, rRBC + Ravulizumab + OmCI, zymosan and zymosan + Cp40. The hemolysis and P-selectin assays were performed as before to ensure that the samples and agents were working as desired. The results were the same than described in 3.1.8, which can be seen in the following figures. The rRBCs were the only ones that induced hemolysis and activated the platelets. All other samples showed neither hemolysis nor activation of thrombocytes.

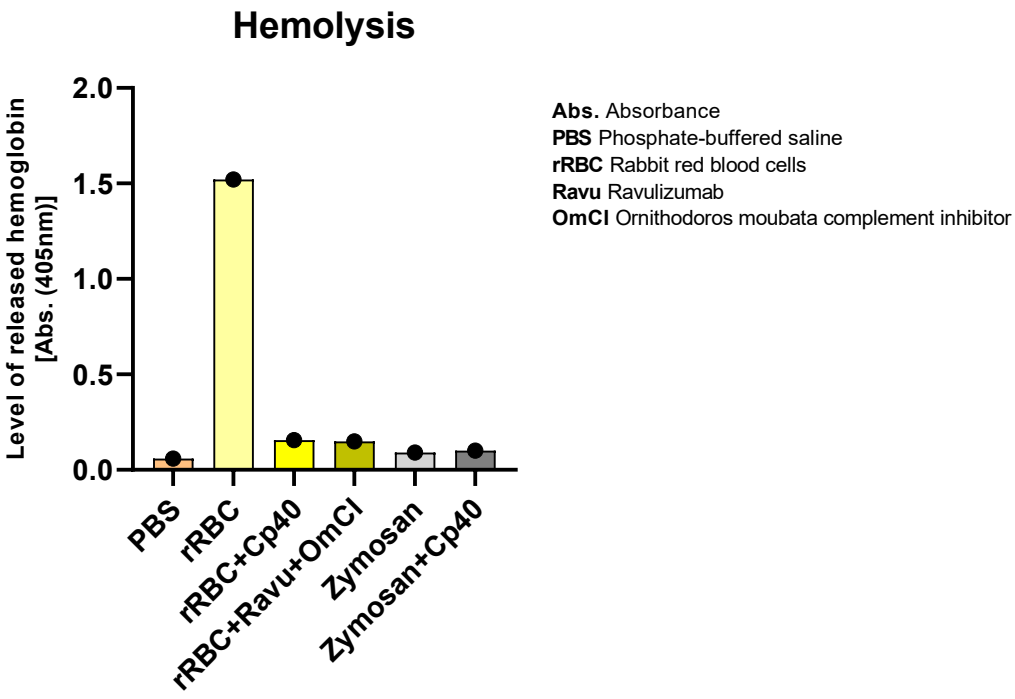

Figure 23: Hemolysis assay for C3a ELISA. *After treatment of the blood samples with PBS, rRBC, rRBC+CP40, rRBC+Ecu+OmCI, zymosan and zymosan+Cp40, the absorbance of the supernatant was measured at 405nm.*

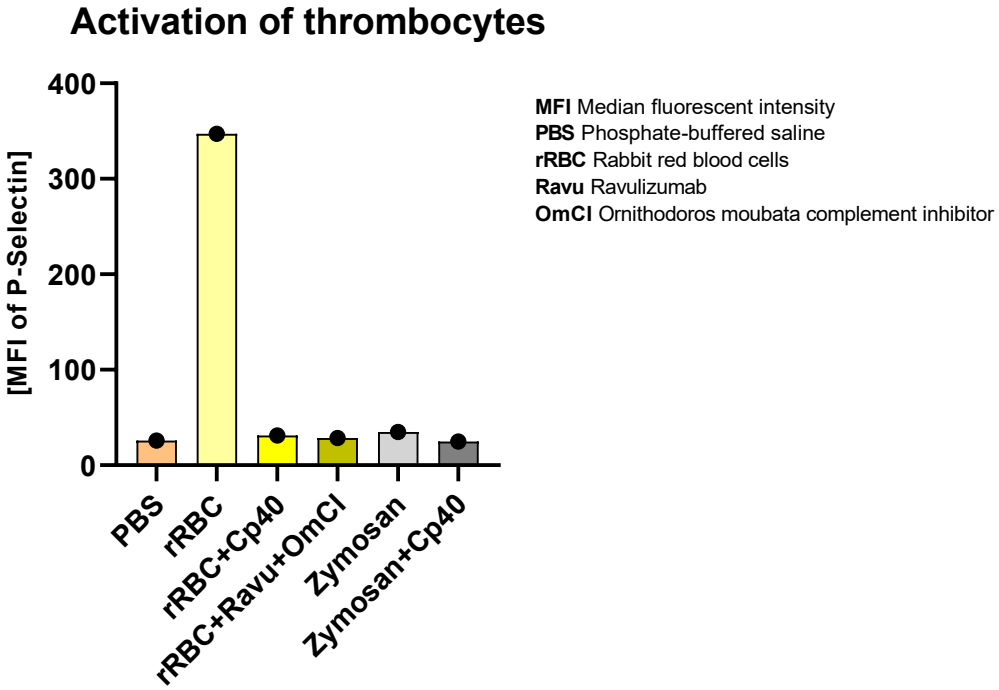

Figure 24: Activation of thrombocytes, assay for C3a ELISA. *After the addition of PBS, rRBC, rRBC+CP40, rRBC+Ecu+OmCI, zymosan and zymosan+Cp40, the MFI of P-selectin was determined and shows the platelet activation.*

An ELISA was then performed to detect C3a in the supernatant of the samples. The supernatant was frozen immediately after the incubation step and used the next day as described in 2.2.7 and 2.2.8. No C3a was detected in the negative control with PBS; rRBC showed an active complement system, as C3a was released. Cp40 as a C3 inhibitor was able to prevent the cleavage of C3, so no C3a could be measured. Conditions with Eculizumab and OmCI, as C5 inhibitors, showed production of C3a. Zymosan containing conditions indeed showed complement activation, as indicated in the positive C3a signal in the sample. This assay proves, that zymosan and rRBC induce the complement cascade leading to at least proteolytic C3 activation. These results show, that an active complement system that does not lead to hemolysis, as is the case with zymosan, is not able to induce platelet activation.

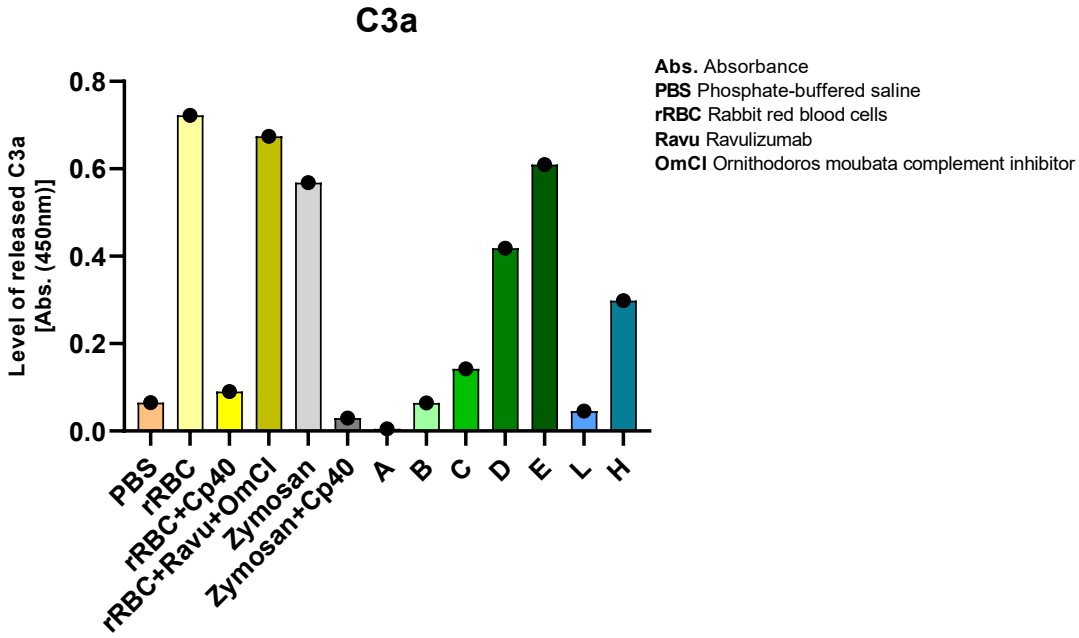

Figure 25: C3a ELISA. *After the addition of PBS, rRBC, rRBC+Cp40, rRBC+Ravu+OmCI, zymosan and zymosan+Cp40 to the samples, the absorbance of the supernatant was measured at 450nm showing the amount of C3a. A-E are buffers from the manufacturer for a standard row, L means low control, H high control.*

3.1.10 PAF

PAF was added to the whole blood either alone or together with Ravuliuzumab + OmCI (as C5 inhibitors) on the one hand or Cangrelor and MRS2170 (as ADP inhibitors) on the other. Additionally, PBS, ADP, rRBC and rRBC with Ravulizumab + OmCI were used as conditions. With regard to hemolysis the results from before could be reproduced. PAF has no effect on the complement system and no hemolysis is measured.

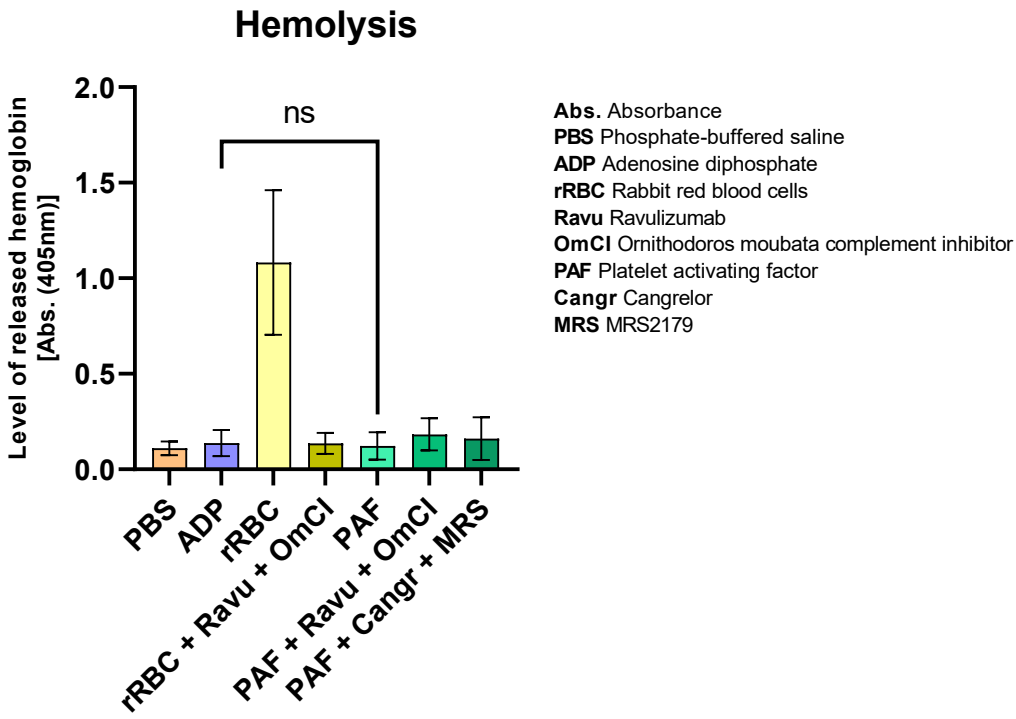

Figure 26: Hemolysis after the addition of PAF. *After treatment of the blood samples with PBS, ADP, rRBC and PAF combined with complement and ADP inhibitors, the absorbance of the supernatant was measured at 405nm. The average of four independent experiments with standard deviation is shown. PAF+Ravu+OmCI and PAF+Cangr+MRS was only used in three experiments. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. ns: non-significant*

The activation assay showed that PAF is able to induce platelet activation, as expected. The signal is not as high as with rRBC or ADP, but definitely higher than with PBS. The C5 inhibitors Ravulizumab and OmCI are unable to inhibit activation, whereas Cangrelor and MRS2179 inhibit complete activation of platelets, although PAF binds to a different receptor. The ADP inhibitors inhibit total activation of platelets also when combined activation signals were present in the assay like rRBC as a complement activator, ADP as direct activator, and PAF as another direct activator.

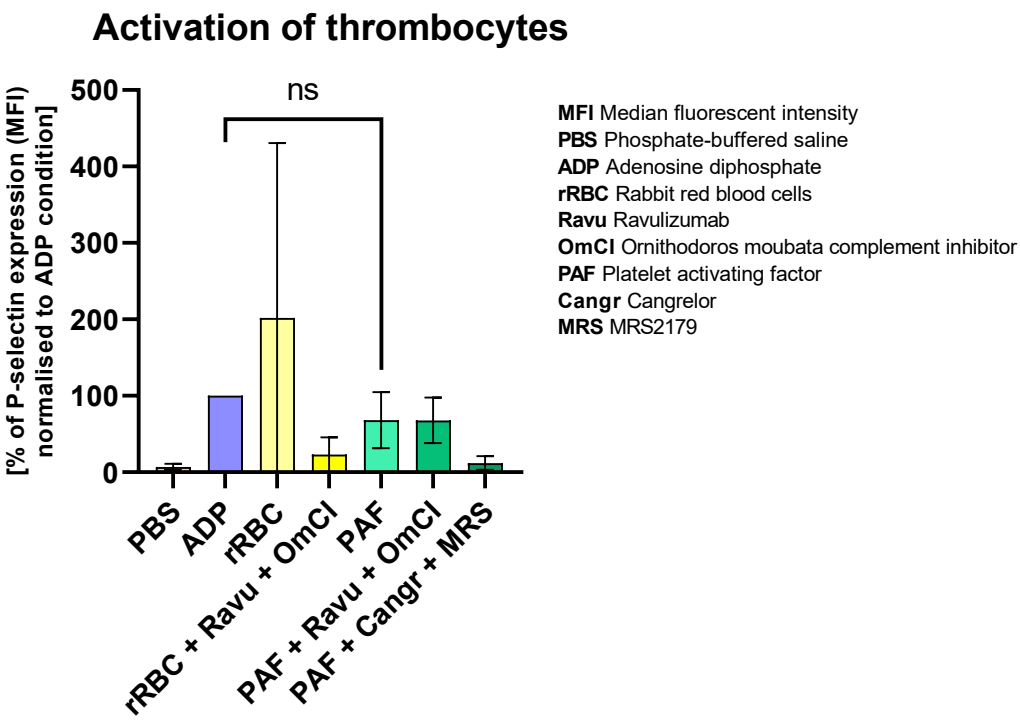

Figure 27: Activation of thrombocytes after the addition of PAF. *After the addition of PBS, ADP, rRBC and PAF combined with complement and ADP inhibitors, the MFI of P-selectin was determined and shows the platelet activation. ADP was defined as 100% activation and used as a reference. The average of four independent experiments with standard deviation is shown. PAF+Ravu+OmCI and PAF+Cangr+MRS was only used in three experiments. Data sets were tested for outliers using ROUT outlier test (Q=5%). No outliers were removed. The data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. ns: non-significant*

3.1.11 Serotonin

To substantiate the previous results, another platelet activator reported in the literature was tested. Serotonin is known enhance platelet responses to ADP. Therefore, the platelets are treated with serotonin alone or combined with either ADP or rRBC to see if the P-selectin response is higher. The agents are also combined with complement inhibitors; the conditions used are shown in Figures 28 and 29. Only condition with rRBC led to hemoglobin release, as expected.

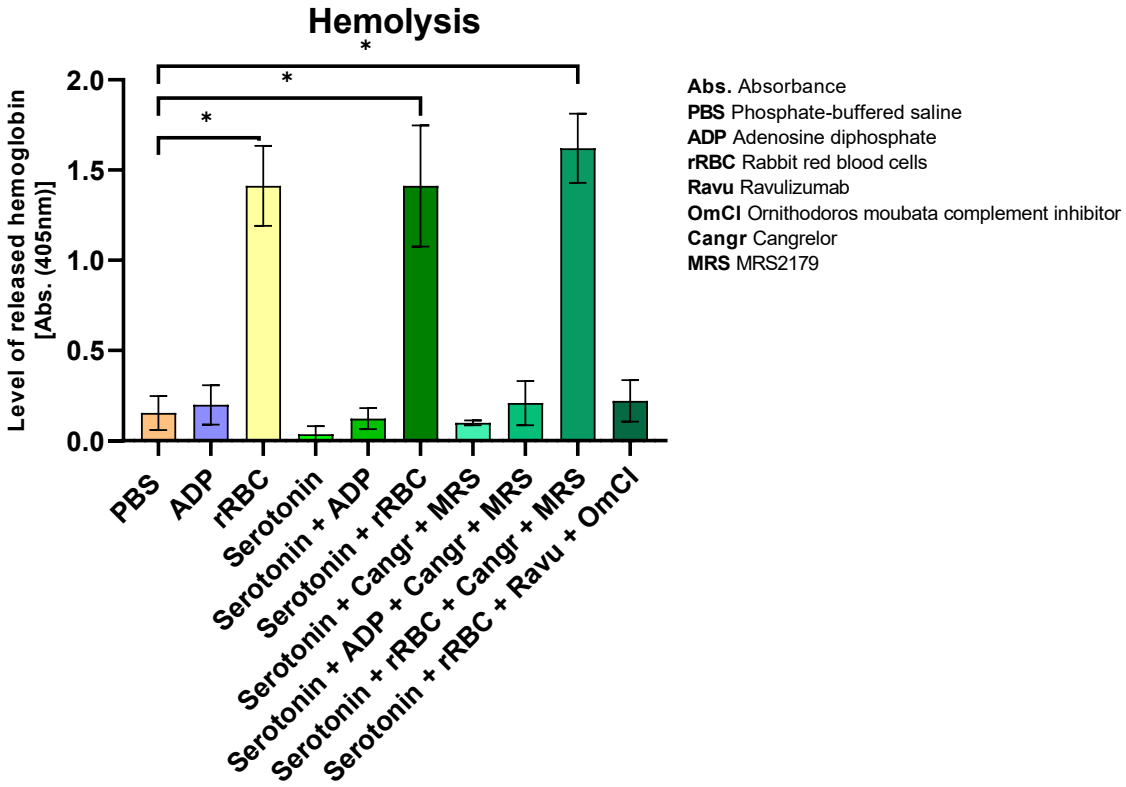

Figure 28: Hemolysis after the addition of Serotonin. *After treatment of the blood samples with PBS, ADP, rRBC and Serotonin combined with complement and ADP inhibitors, the absorbance of the supernatant was measured at 405nm. The average of four independent experiments with standard deviation is shown. Serotonin+ADP+Cangr+MRS, Serotonin+rRBC+Cangr+MRS and Serotonin+rRBC+Ravu+OmCI was only used in three experiments. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$*

Serotonin by itself was not able to induce activation of the thrombocytes. In combination with either ADP or rRBC, the P-selectin signal was higher than without Serotonin. In combination with the different inhibitors the results were basically similar as observed before. ADP inhibitors prevented platelet activation in every combination, Ravulizumab and OmCI only in combination with complement activators. Serotonin does not interfere in any way as measured in this assay. This means that Serotonin is not able to trigger platelet activation by itself, it can only enhance the P-selectin response when platelets are preactivated, whether with rRBC or ADP. So in essence, serotonin has an intensifying effect on platelet activation.

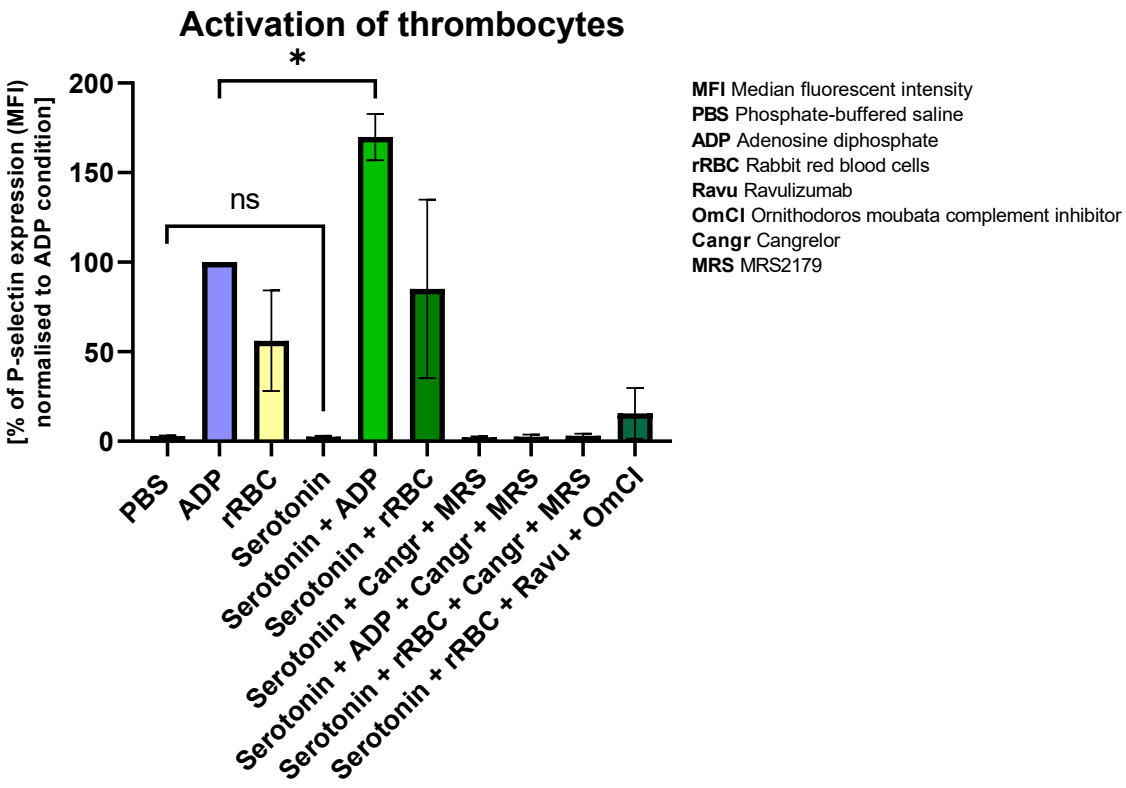

Figure 29: Activation of thrombocytes with Serotonin. *After the addition of PBS, ADP, rRBC and Serotonin combined with complement and ADP inhibitors, the MFI of P-selectin was determined and shows the platelet activation. ADP was defined as 100% activation and used as a reference. The average of four independent experiments with standard deviation is shown. Serotonin+ADP+Cangr+MRS, Serotonin+rRBC+Cangr+MRS and Serotonin+rRBC+Ravu+OmCI was only used in three experiments. Data sets were tested for outliers using ROUT outlier test (Q=5%). No outliers were removed. The data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant*

3.1.12 Platelet activation measured with the marker: CD41

In all the above experiments, the activation of the thrombocytes was defined by the presence of P-selectin. To verify that all substances used in the experiments did not act only on P-selectin, another activation marker, called CD41, was used. The corresponding antibody used in the experiments is called PAC-1. The experiment was performed with both antibodies together (CD41 and P-selectin) to check for congruent results. PBS, ADP, rRBC and rRBC + Ravulizumab + OmCI were added to the samples.

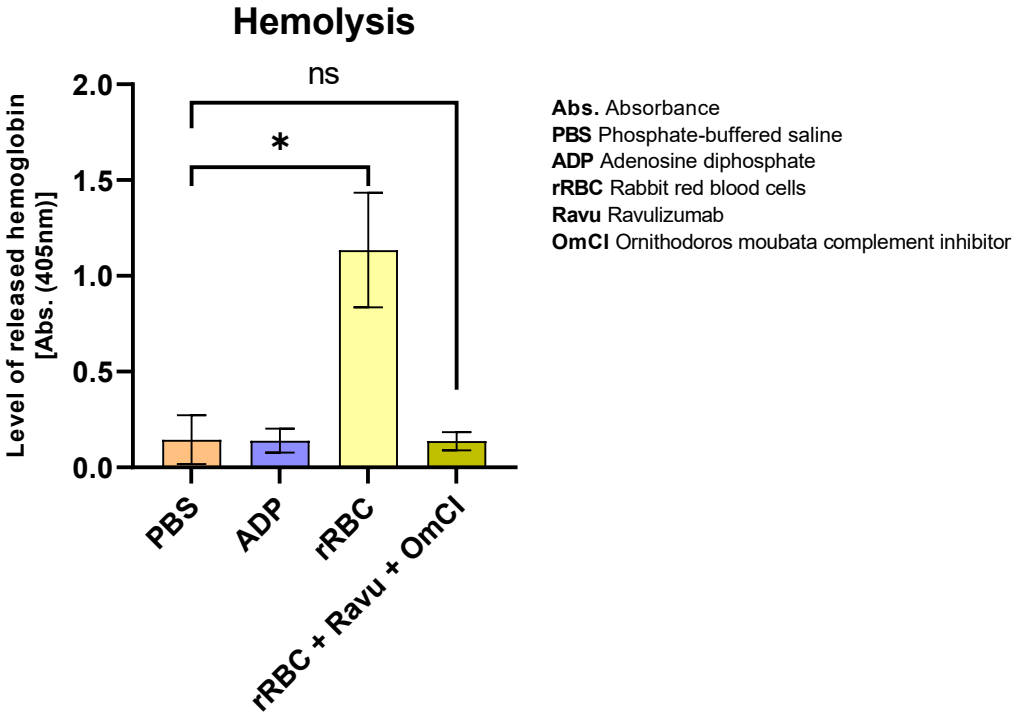

Figure 30: Hemolysis with P-selectin and CD41 as activation markers. *After treatment of the blood samples with PBS, ADP, rRBC and rRBC+Ravu+OmCI, the absorbance of the supernatant was measured at 405nm. The average of seven independent experiments with standard deviation is shown. RRBC+Ravu+OmCI was only used in five experiments. Data sets were analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: $p < 0.05$; ns: non-significant*

The two orthogonal activation markers appear to act in the same direction. PBS shows no activation, ADP and rRBC show activation and rRBC with complement inhibitors also show no activation. The results are congruent.

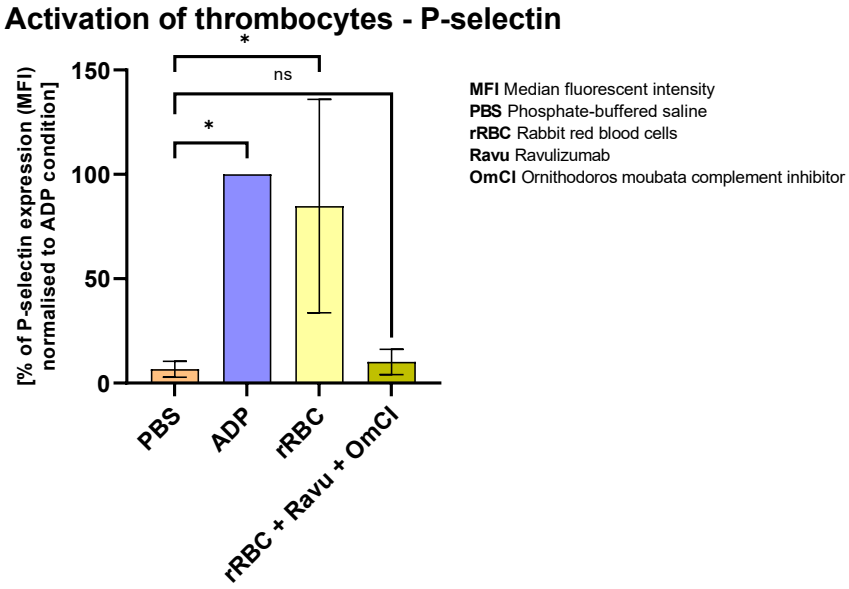

Figure 31: Activation of thrombocytes, P-selectin. *After the addition of PBS, ADP, rRBC and rRBC+Ravu+OmCI, the MFI of P-selectin was determined and shows the platelet activation. ADP was defined 100% activation and used as a reference. The average of seven independent experiments with standard deviation is shown. RRBC+Ravu+OmCI was only used in five experiments. 1 outlier each for rRBC and rRBC+Ravu+OmCI was removed before statistical testing. The cleaned data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: p < 0.05; ns: non-significant*

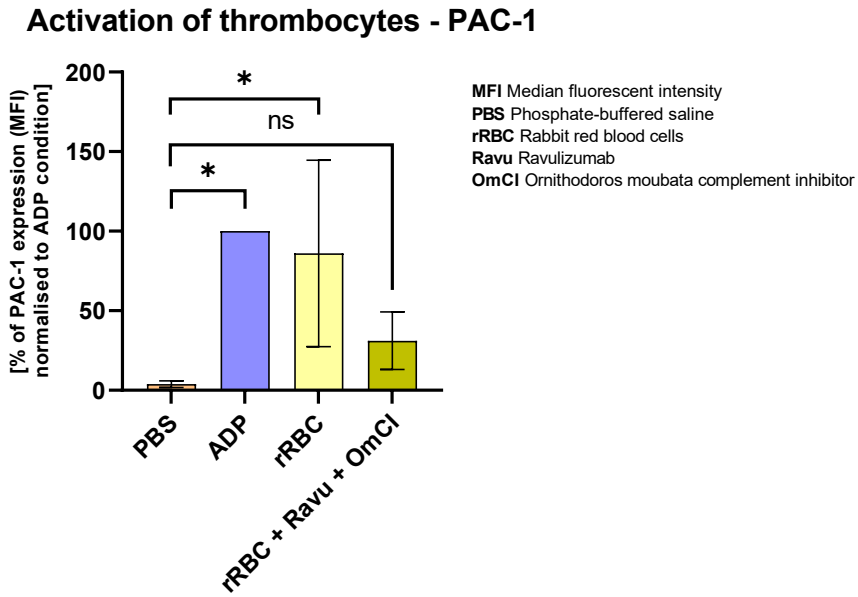

Figure 32: Activation of thrombocytes, PAC-1. *After the addition of PBS, ADP, rRBC and rRBC+Ravu+OmCI, the MFI of P-selectin was determined and shows the platelet activation. ADP was defined as 100% activation and used as a reference. The average of seven independent experiments with standard deviation is shown. RRBC+Ravu+OmCI was only used in five experiments. 1 outlier each for PBS and rRBC was removed before statistical testing. The cleaned data were then analyzed using repeated measures one-way analysis of variance (ANOVA) and post hoc tested for statistical significance with Tukey's multiple comparisons test. For the sake of visibility, only significance levels of selected comparisons that are relevant to the experimental hypothesis are shown. *: p < 0.05; ns: non-significant*

3.2 In vitro whole blood model using PNH thrombocytes shows that C5 inhibition protects both CD59+ and CD59- cells from complement attack

3.2.1 Experimental setup

Blood was drawn from 14 different patients with the disease PNH and the experiments on the whole blood were performed as before. Various clinical parameters of the patients were collected, which are listed in the corresponding tables for each patient in the Appendix. The parameters were measured in the institute, where the patients are treated. Since the PNH patients lack the GPI anchor in all or a part of the hematopoietic cells, thrombocytes are also affected. This means that the patients have no CD59 protein on the membrane of a proportion of their cells. Consequently, the patients have two different CD59 phenotypes: *i.e.* CD59+ (there is CD59 protein on the membrane) and CD59- (no CD59 proteins). The aim was to find out any functional difference between these two platelet populations in the whole blood assay with complement activation. Flow cytometry was used to see if the GPI anchor and subsequent expression of CD59 on the outer membrane, which normally blocks the formation of the membrane attack complex to protect the cells from a complement attack, has an influence on the activation of thrombocytes.

Again, PBS and ADP served as negative and positive controls, respectively. RRBC were used to activate the complement system and to determine whether the absence of the CD59 protein results in lower or higher activation of thrombocytes. Because most patients are treated with a C5 inhibitor, the complement system in the collected blood is automatically inhibited. To circumvent the inhibition, the samples with rRBC were also supplemented with purified C5 at high concentration to allow the membrane attack complex to be formed. With regard to the previous results with ADP inhibitors, Cangrelor and MRS2179 were added to either the ADP or rRBC + C5 conditions.

3.2.2 The patients

3.2.2.1 Patient 1

This patient has cells with and without a GPI anchor, *i.e.*, there are two different thrombocyte clones regarding the CD59 expression. This can be seen in Figure 33. The clone size in table 7 (based on red blood cells) in the Appendix was measured during the clinical routine workup of the patient's sample. The figure shows the data measured with the flow cytometer in our experiments. Only PBS, ADP and ADP + ADP inhibitors were used as experimental conditions. The first patient received Ravulizumab as treatment. No hemolysis was detected in the blood because of the absence of an active complement system. The healthy cells (CD59+) respond to the ingredients like the platelets of the healthy patients. PBS does not lead to platelet activation, whereas ADP does. As expected, the ADP inhibitors inhibit activation via ADP. The thrombocytes without CD59 respond to ADP and the inhibitors in relatively the same way, but in direct comparison the activation is much weaker than for the CD59+ patients.

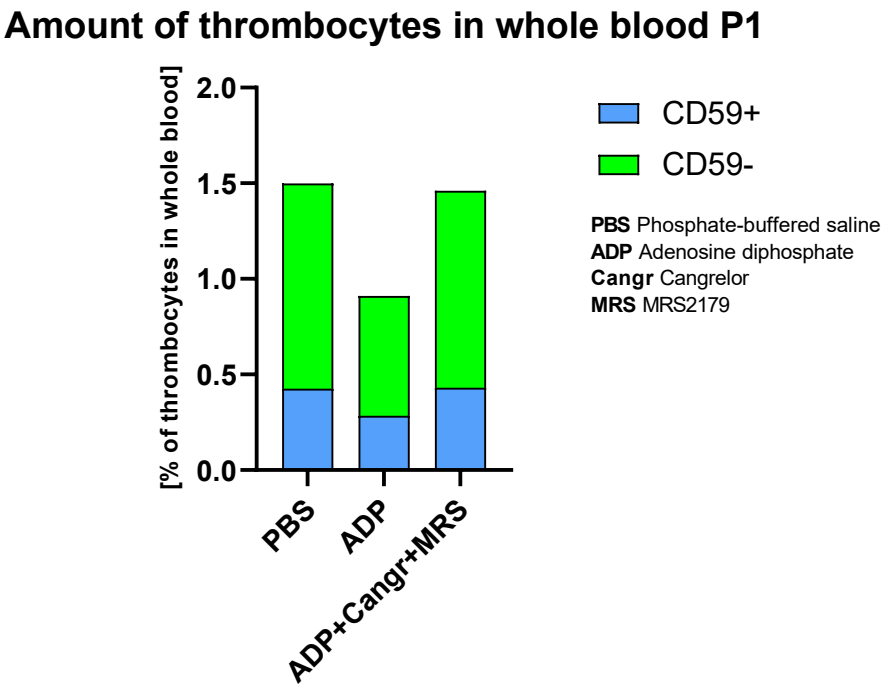

Figure 33: Amount of thrombocytes in whole blood of patient 1. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed.*

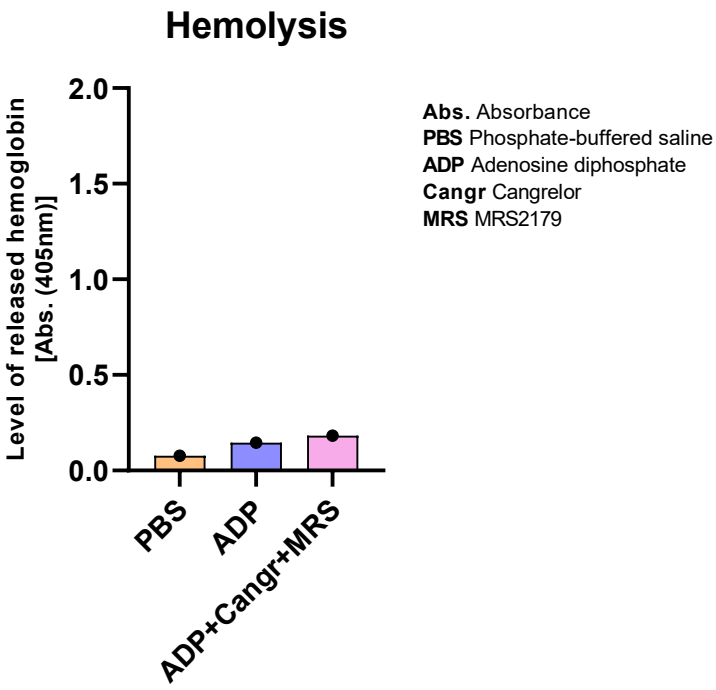

Figure 34: Hemolysis of patient 1. *After treatment of the blood samples with PBS, ADP and ADP+ADP inhibitors, the absorbance of the supernatant was measured at 405nm. None of the samples show hemolysis*

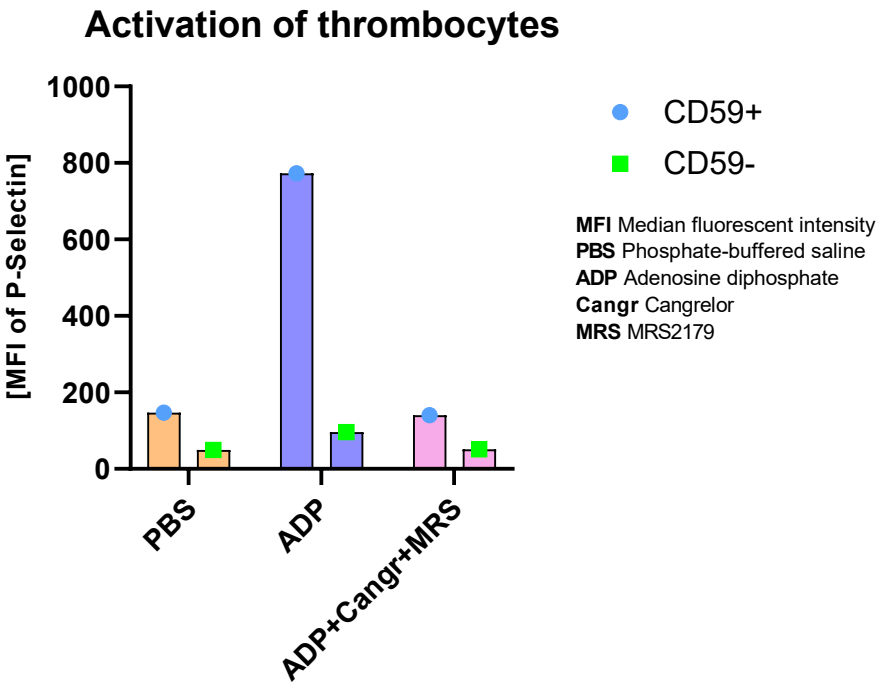

Figure 35: Activation of thrombocytes of patient 1. *After the addition of either PBS, ADP or ADP+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (either CD59+ or CD59-).*

3.2.2.2 Patient 2

Patient 2 is likewise treated with a C5 inhibitor (Eculizumab). Blood samples were treated with the previously described products and incubated. The samples with PBS, ADP (+ ADP inhibitors) and rRBC show no lysed red cells. RRBC + C5 (+ ADP inhibitors) show lysis. Eculizumab seems to do its job and inhibit C5 properly, because only if enough C5 is supplied, the complement system can finish the cascade and destroy the erythrocytes. ADP activation of thrombocytes works, but the CD59- cells are less activated. The other samples show no activation, not even the sample with rRBC + C5 despite expired lysis. This is a difference to the experiments with healthy platelets.

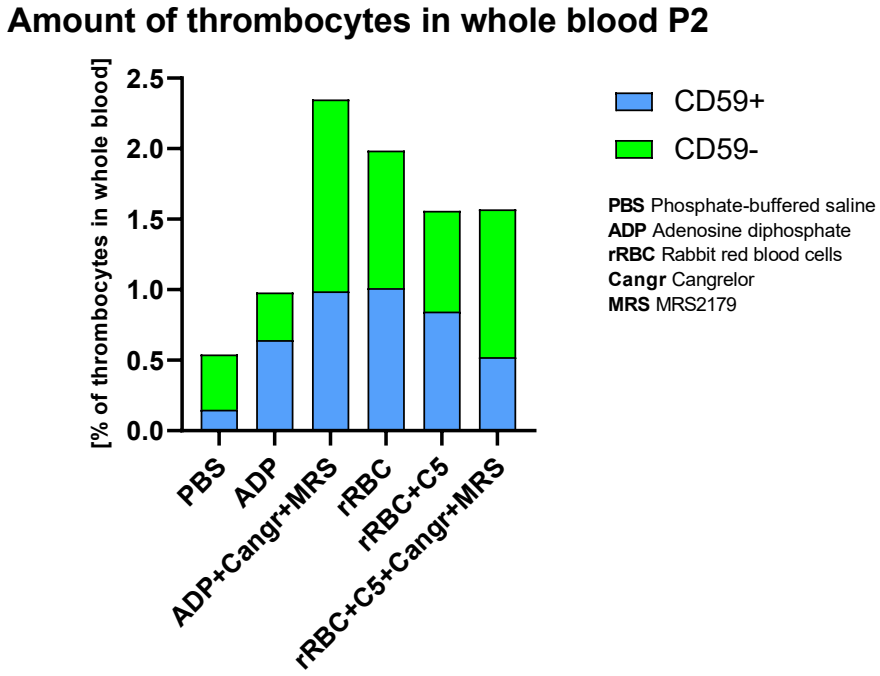

Figure 36: Amount of thrombocytes in whole blood of patient 2. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed.*

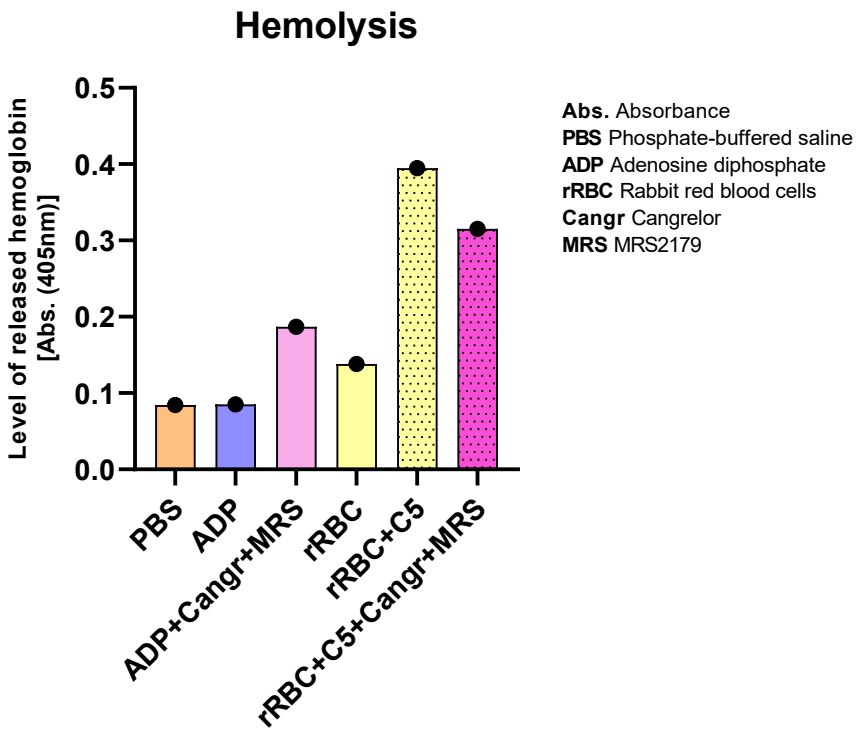

Figure 37: Hemolysis of patient 2. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

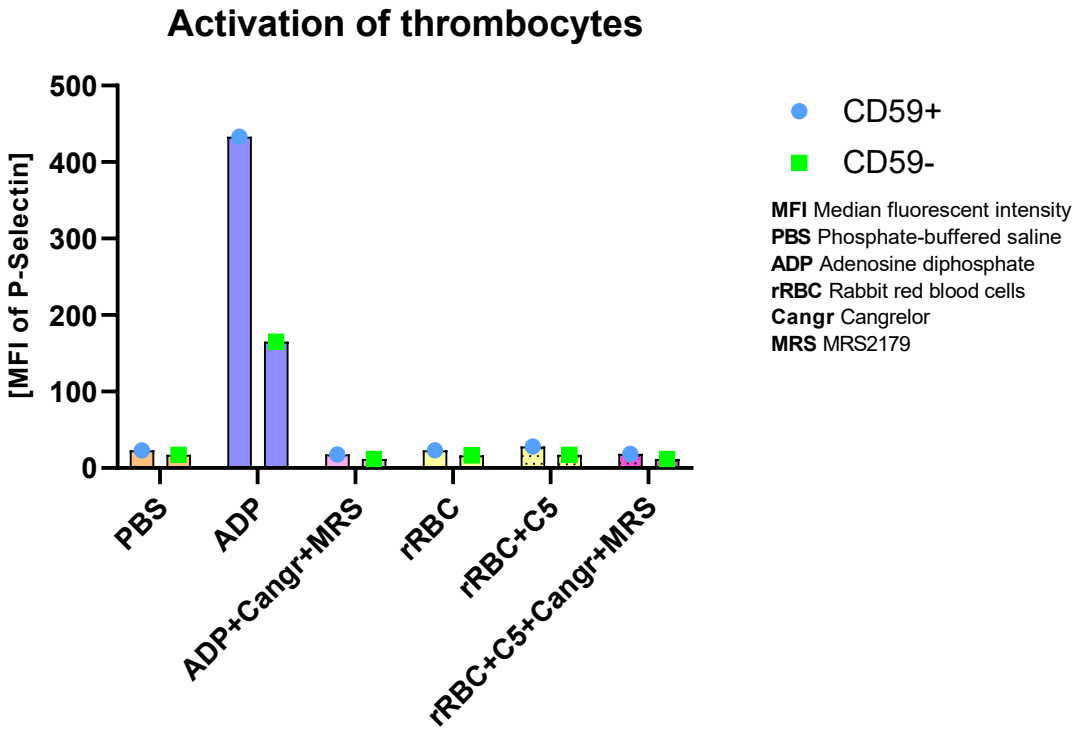

Figure 38: Activation of thrombocytes of patient 2. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (either CD59+ or CD59-).*

3.2.2.3 Patient 3

Patient 3 also received Eculizumab. In addition, this patient has almost no CD59+ cells. Hemolysis shows that rRBC leads to little lysis, but with the addition of C5, lysis is stronger. Compared the positive and negative control, the sample with added C5 activated platelets more strongly than with rRBC alone, but neither particularly strongly, especially rRBC is close to the PBS level. Cangrelor and MRS2179 are also able to inhibit the activation to the level of PBS, even when C5 is added. In this patient, the amount of CD59+ cells was too low to detect by flow cytometer, so comparison between the two clones is not possible.

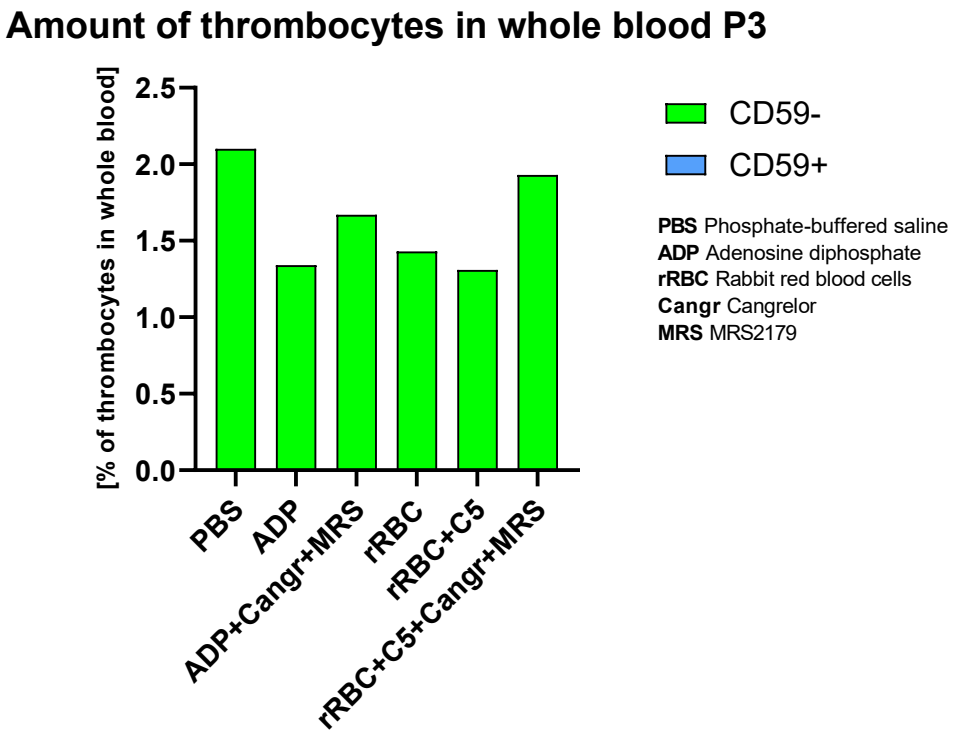

Figure 39: Amount of thrombocytes in whole blood of patient 3. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed. No CD59+ cells could be detected.*

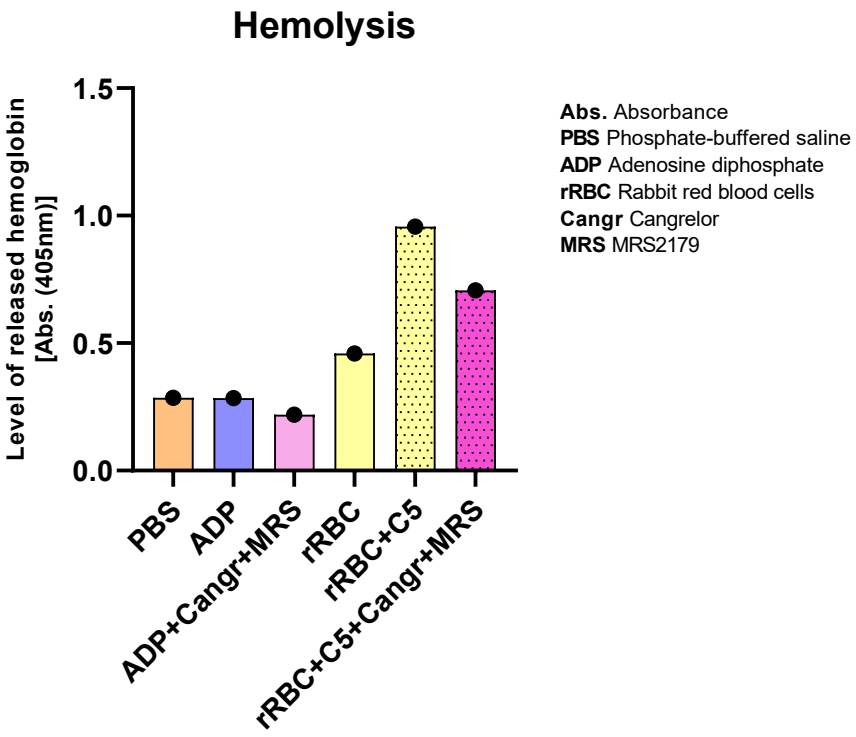

Figure 40: Hemolysis of patient 3. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

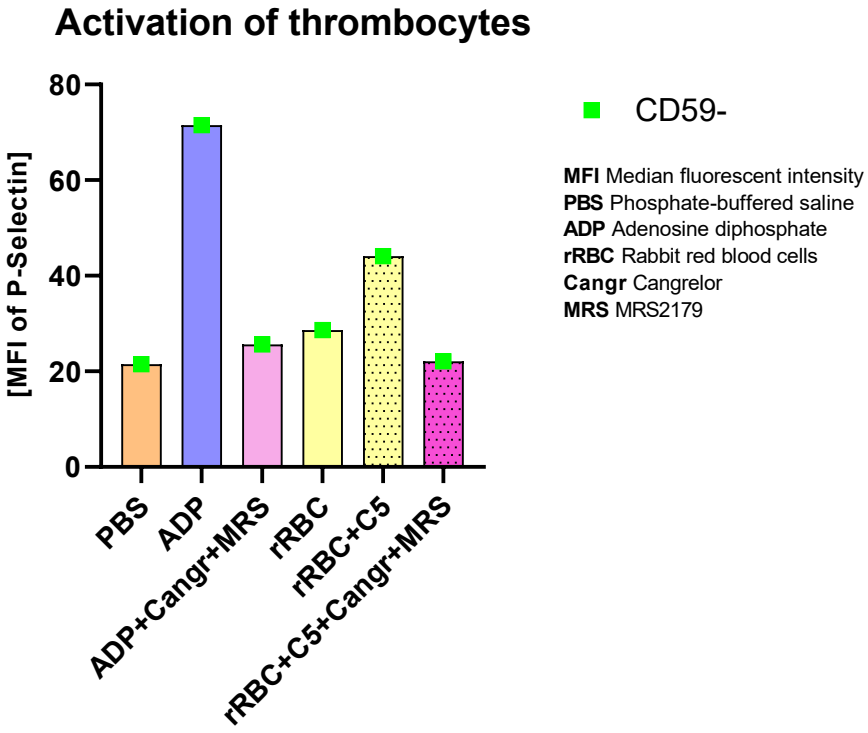

Figure 41: Activation of thrombocytes of patient 3. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (only CD59- cells could be detected).*

3.2.2.4 Patient 4

This patient is treated with Ravulizumab. The patient has two phenotypes, but the CD59- phenotype is quite large. All described products are added to the samples. With the complement system active (after addition of C5), lysis could be measured. RRBC alone could not lead to the same lysis as with C5. Only ADP was able to activate the thrombocytes properly, even after addition of C5 and associated activation of the complement cascade, the platelets were again not activated to the same extent as with ADP. CD59- cells were even more difficult to stimulate, but relatively speaking, the results are the same.

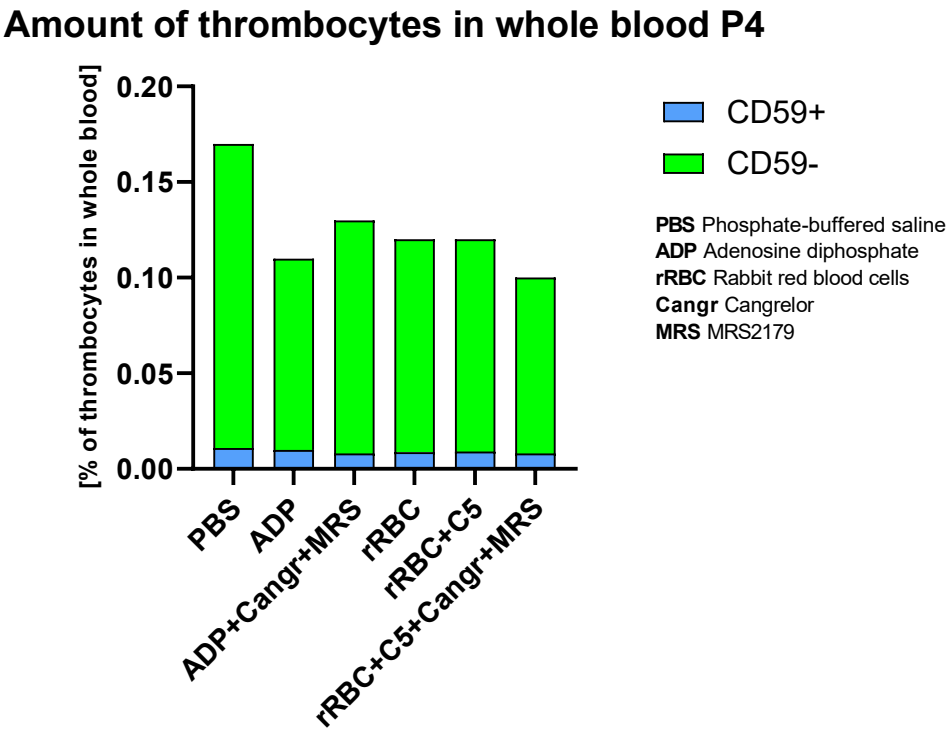

Figure 42: Amount of thrombocytes in whole blood of patient 4. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed.*

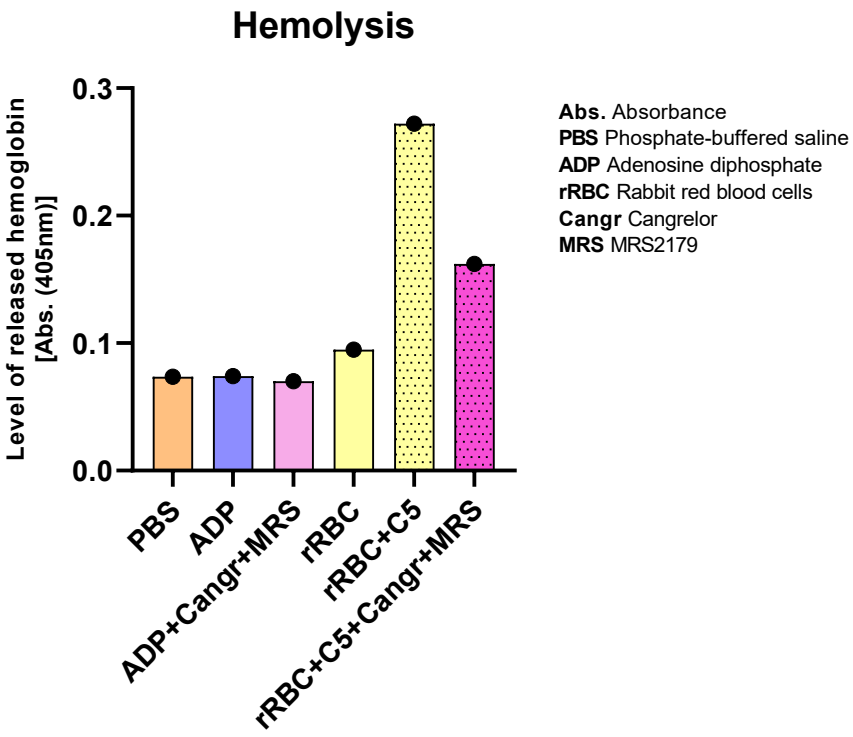

Figure 43: Hemolysis of patient 4. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

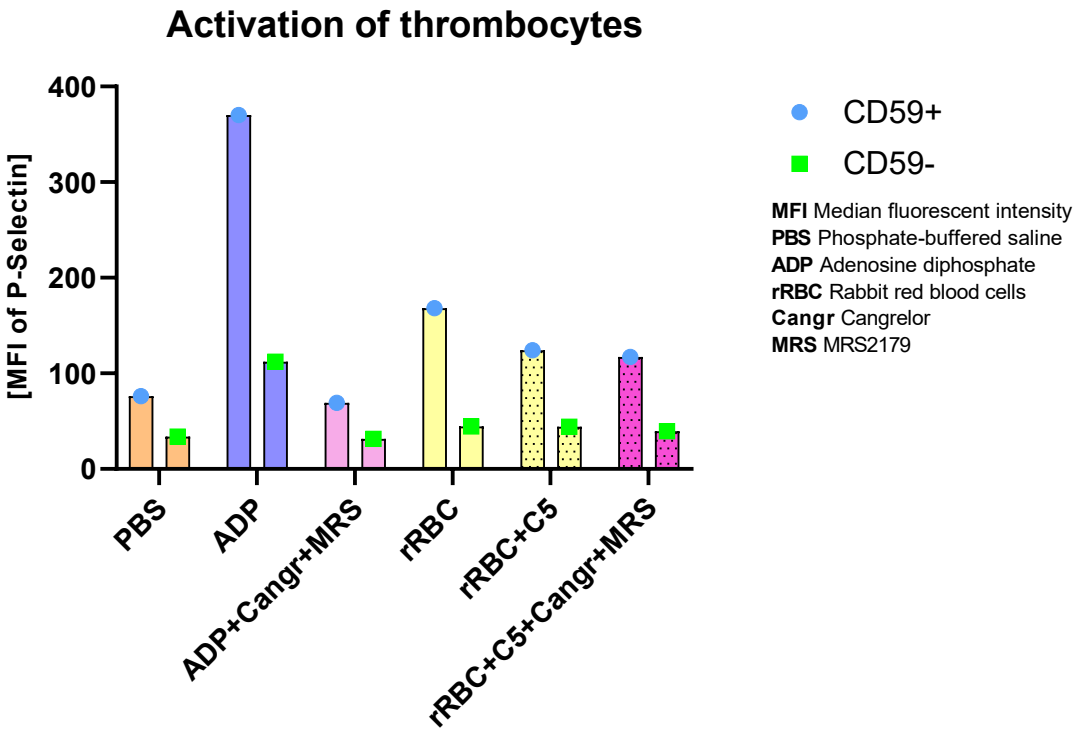

Figure 44: Activation of thrombocytes of patient 4. After *the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-Selectin was determined and shows the platelet activation (either CD59+ or CD59-).*

3.2.2.5 Patient 5

The flow cytometer could only detect CD59- cells. This patient receives Ravulizumab as C5 inhibitor. Hemolysis works properly, rRBC shows slightly more lysis than PBS, more erythrocytes are destroyed after the addition of C5. In this patient only ADP leads the platelets to activation, lysed rRBC (rRBC + C5) are not able to activate the platelets. This seems to be a peculiarity of anti-C5 treated patients.

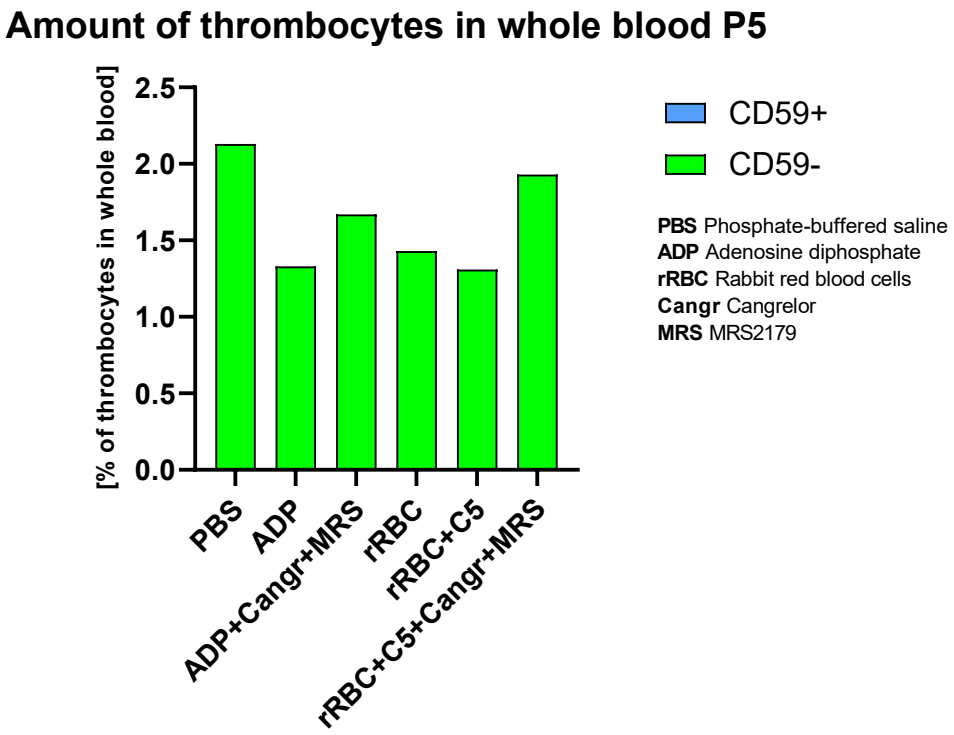

Figure 45: Amount of thrombocytes in whole blood of patient 5. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed. No CD59+ cells could be detected.*

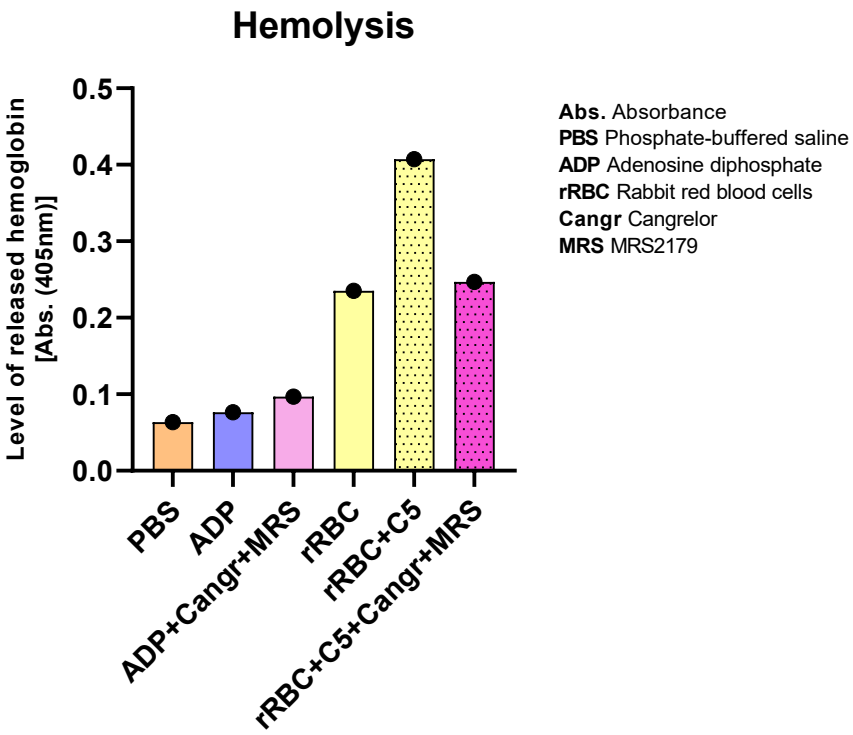

Figure 46: Hemolysis of patient 5. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

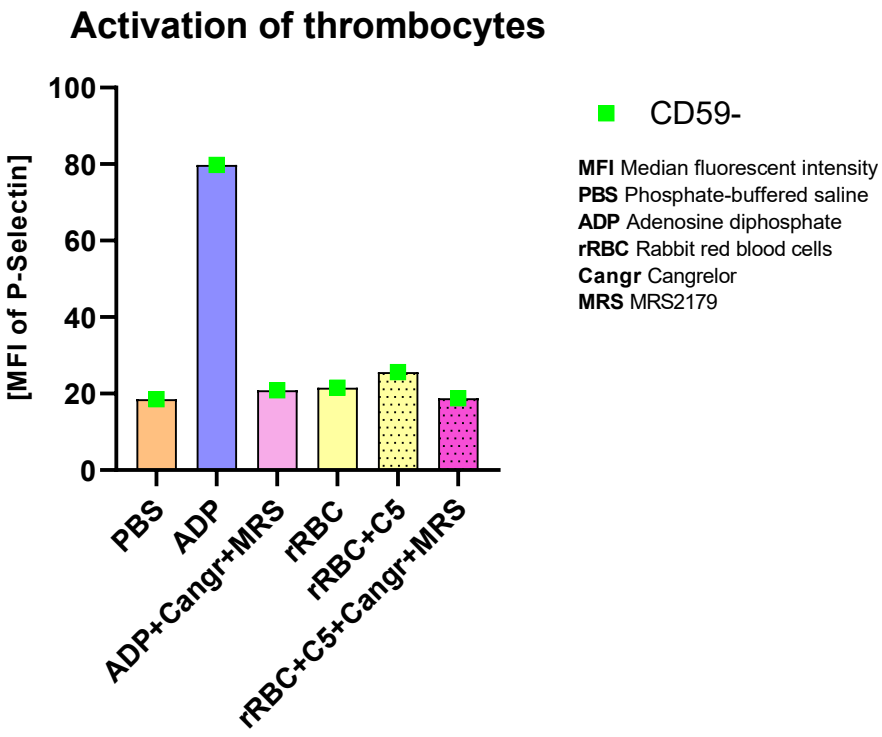

Figure 47: Activation of thrombocytes of patient 5. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (only CD59- cells could be detected).*

3.2.2.6 Patient 6

This patient is the only one treated with a C3 inhibitor (APL). This was not known at the time the experiments were performed. The same products were used to stimulate the thrombocytes. Furthermore, no CD59+ platelets could be detected with the flow cytometer. The erythrocytes lyse upon addition of rRBC, and even greater lysis occurs after addition of C5. The patient may have antibodies against rRBC, the CP is activated and the rRBC are therefore lysed. ADP activates the platelets, rRBC shows low activation and addition of C5 causes higher activation (both fit the hemolysis results). Cangrelor and MRS2179 were able to inhibit the activation.

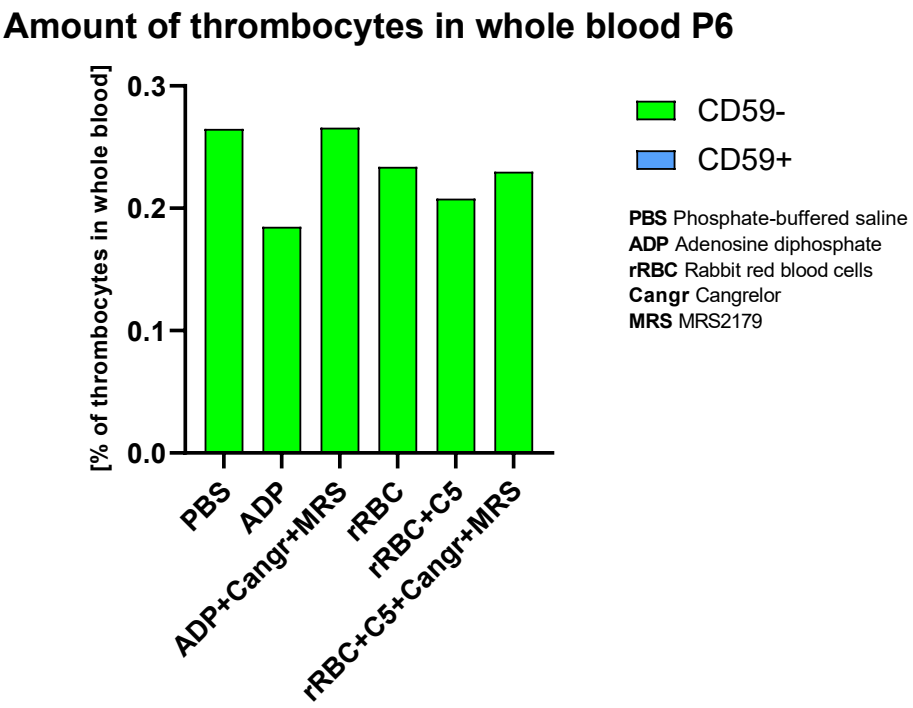

Figure 48: Amount of thrombocytes in whole blood of patient 6. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed. No CD59+ cells could be detected.*

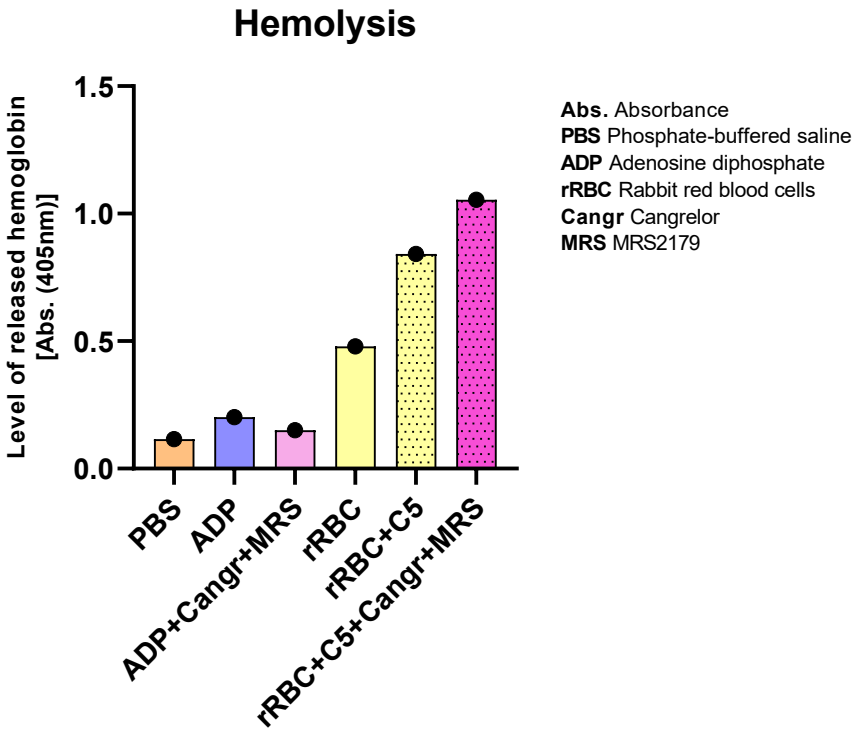

Figure 49: Hemolysis of patient 6. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

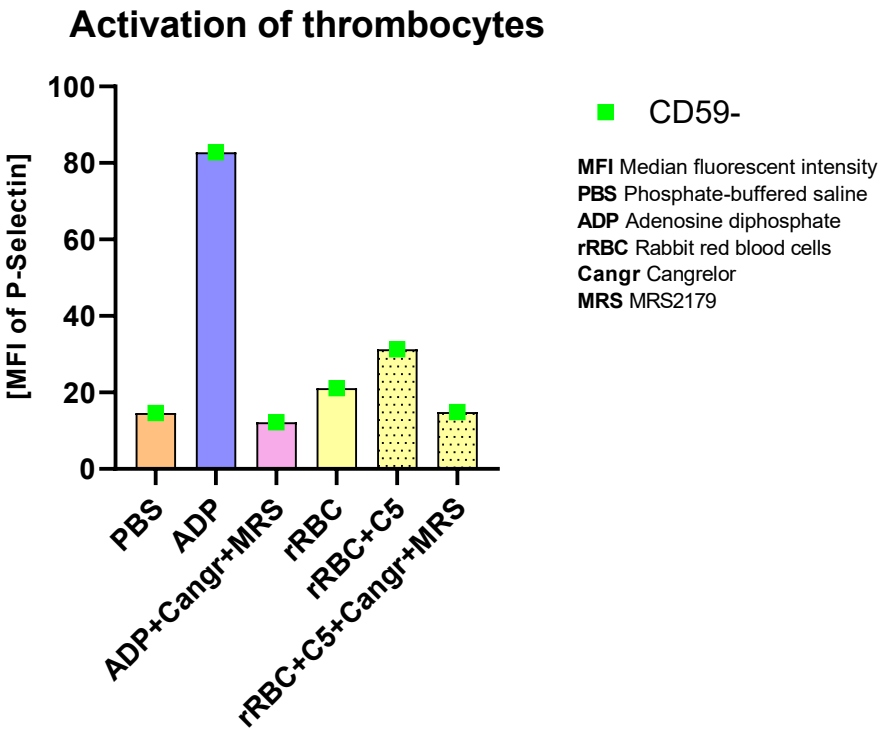

Figure 50: Activation of thrombocytes of patient 6. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (only CD59- cells could be detected).*

3.2.2.7 Patient 7

This patient also has almost no CD59+ thrombocytes and C5 is inhibited with Ravulizumab. The addition of rRBC results in low lysis, combined with C5 the hemolysis is higher. The sample with ADP+Cangr+MRS also shows lower lysis than PBS, but this could also have been an implementation problem. Only the sample with ADP shows a clear activation, and as in some patients before, the sample with rRBC + C5 does not.

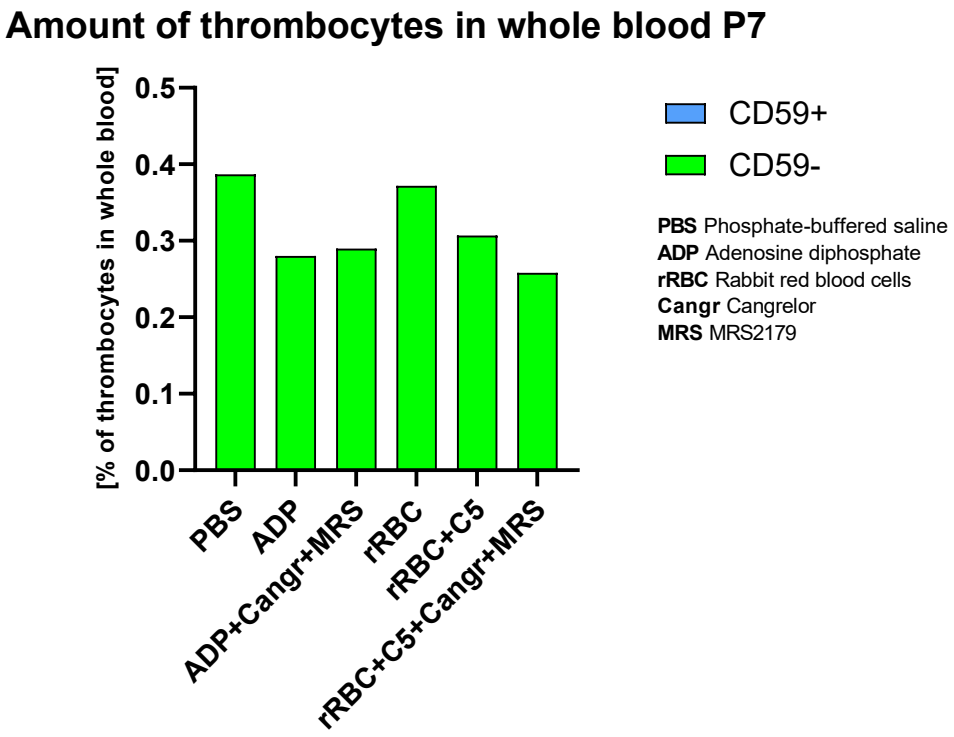

Figure 51: Amount of thrombocytes in whole blood of patient 7. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed. No CD59+ cells could be detected.*

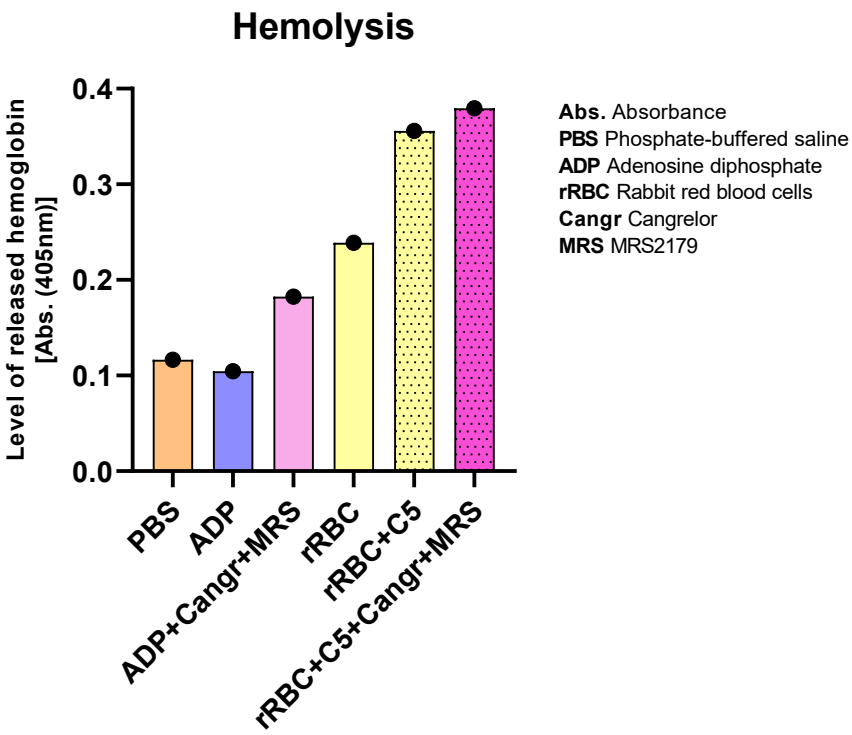

Figure 52: Hemolysis of patient 7. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

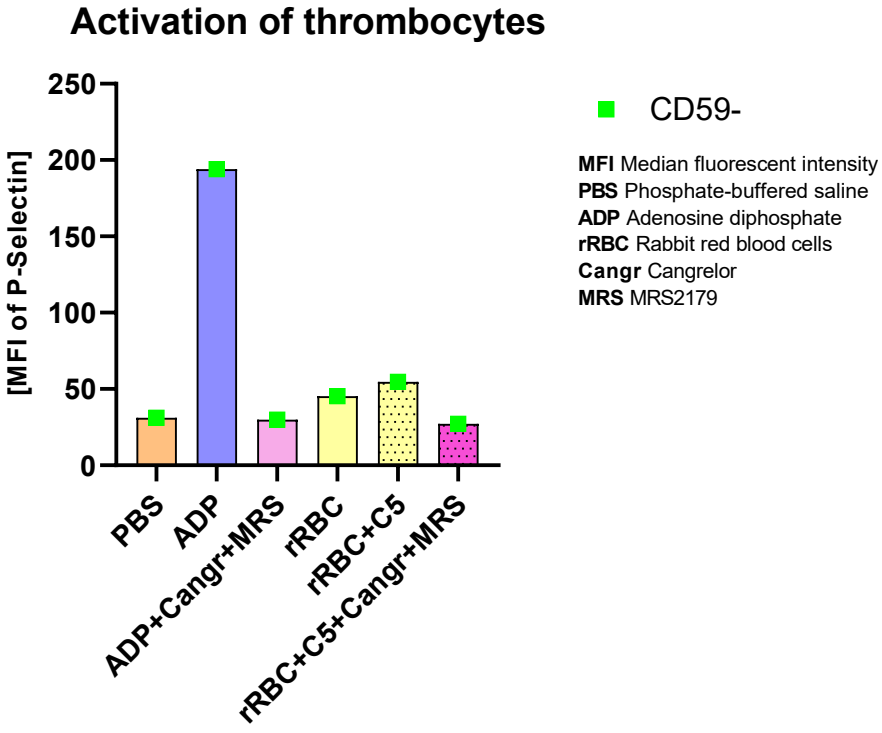

Figure 53: Activation of thrombocytes of patient 7. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (only CD59- cells could be detected).*

3.2.2.8 Patient 8

Patient 8 has a higher healthy CD59+ clone, but CD59- thrombocytes can also be detected. Besides Ravulizumab to inhibit the complement system, the patient receives Eltrombopag, a thrombopoietin receptor agonist to stimulate platelets neogenesis in the bone marrow. The patient develops hemolysis only with addition of rRBC, added C5 doesn't have more influence. Only ADP leads to activation of the thrombocytes – CD59+ cells more than CD59- cells. The other samples are at PBS level, also rRBC + C5 despite expired hemolysis. Addition of ADP+Cangr+MRS also leads to low lysis, which could also be a performance error.

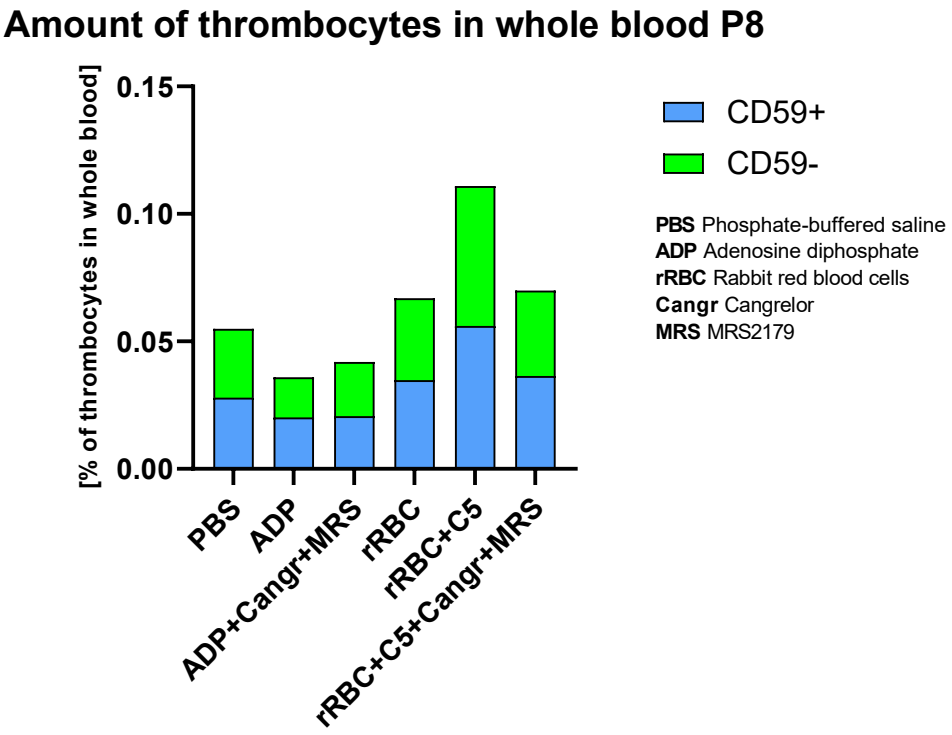

Figure 54: Amount of thrombocytes in whole blood of patient 8. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed.*

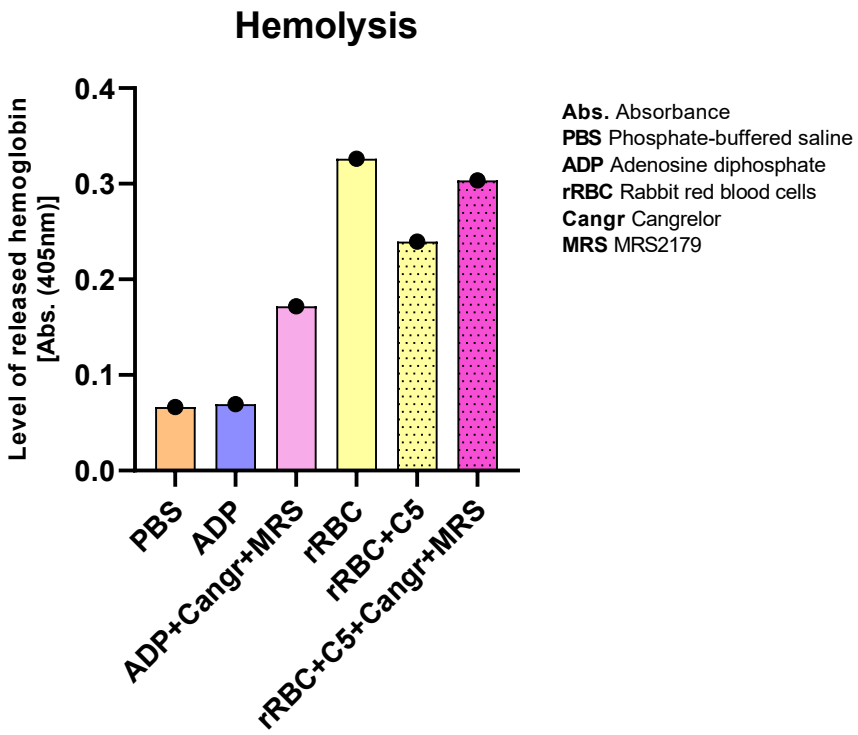

Figure 55: Hemolysis of patient 8. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

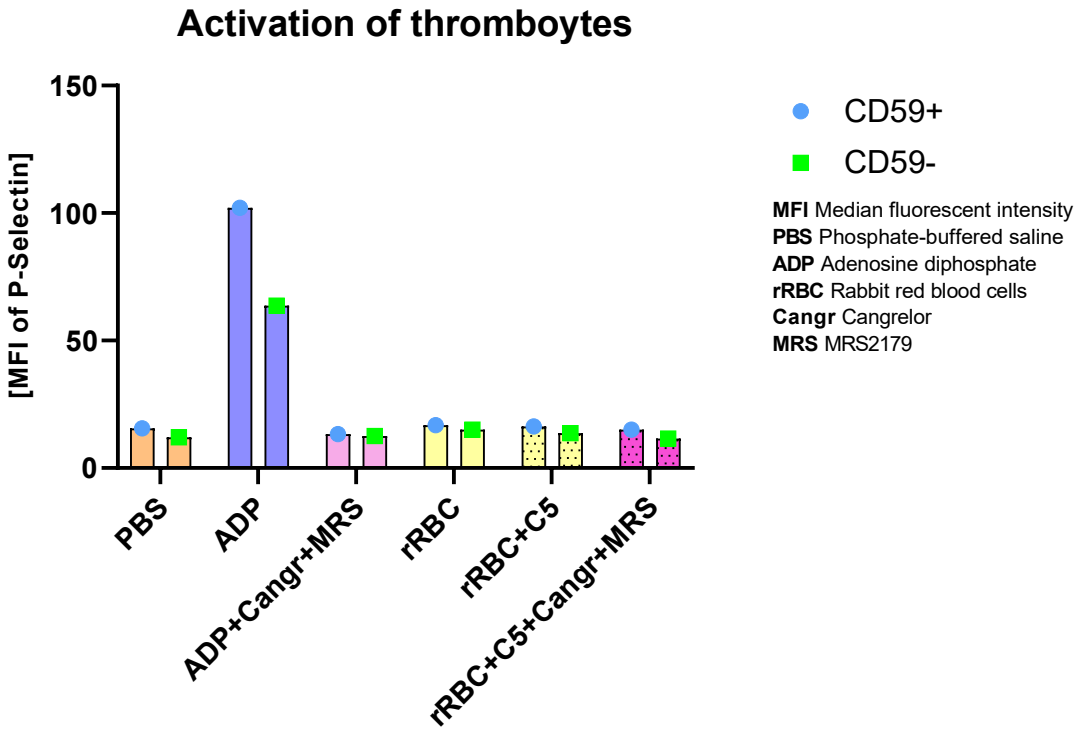

Figure 56: Activation of thrombocytes of patient 8. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (either CD59+ or CD59-).*

3.2.2.9 Patient 9

Patient 9 was not treated with a complement inhibitor. But he is receiving Eltrombopag, the thrombopoietin receptor agonist, and Cyclosporin A (CSA), an immunosuppressant. The platelet count is really low at $17*10^3$/µl. Hemolysis occurs with the addition of rRBC alone, as there is no C5 inhibitor in the blood. C5 does not lead to a higher lysis. However, ADP in combination with ADP inhibitors also leads to low lysis. The platelets seem to be preactivated, because the MFI is quite high compared with the other experiments. ADP activates the thrombocytes, Cangrelor and MRS2179 inhibit signalling. RRBC alone are sufficient to activate platelets in the absence of complement inhibitor in the blood. C5 seems to intensify the effect, and the ADP inhibitors prevent platelets from being activated, even when stimulated with rRBC and C5. Furthermore, there does not appear to be much difference between the two clones.

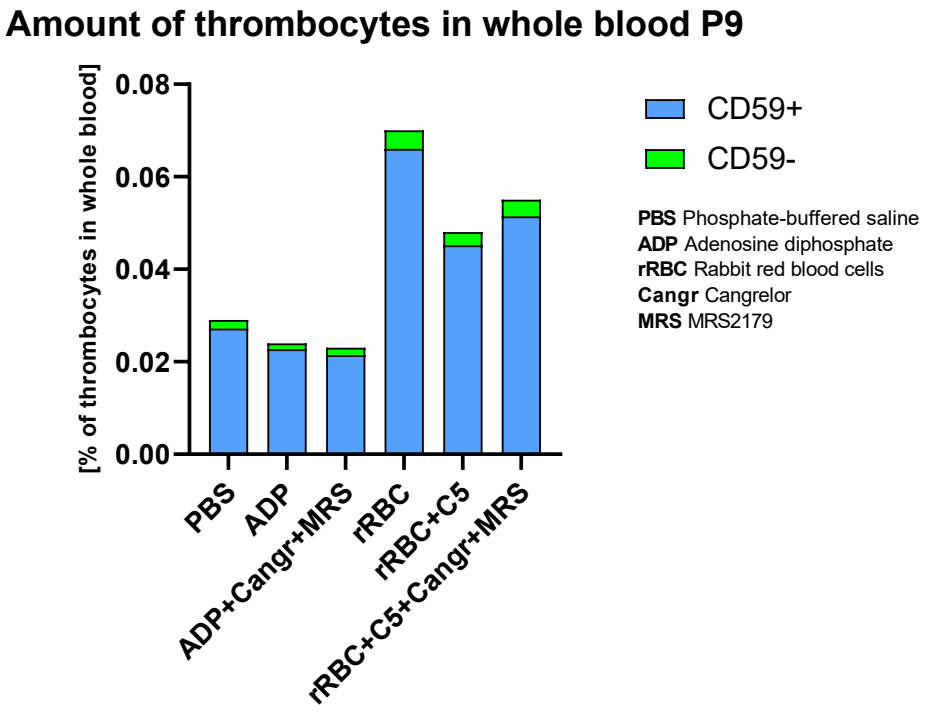

Figure 57: Amount of thrombocytes in whole blood of patient 9. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed.*

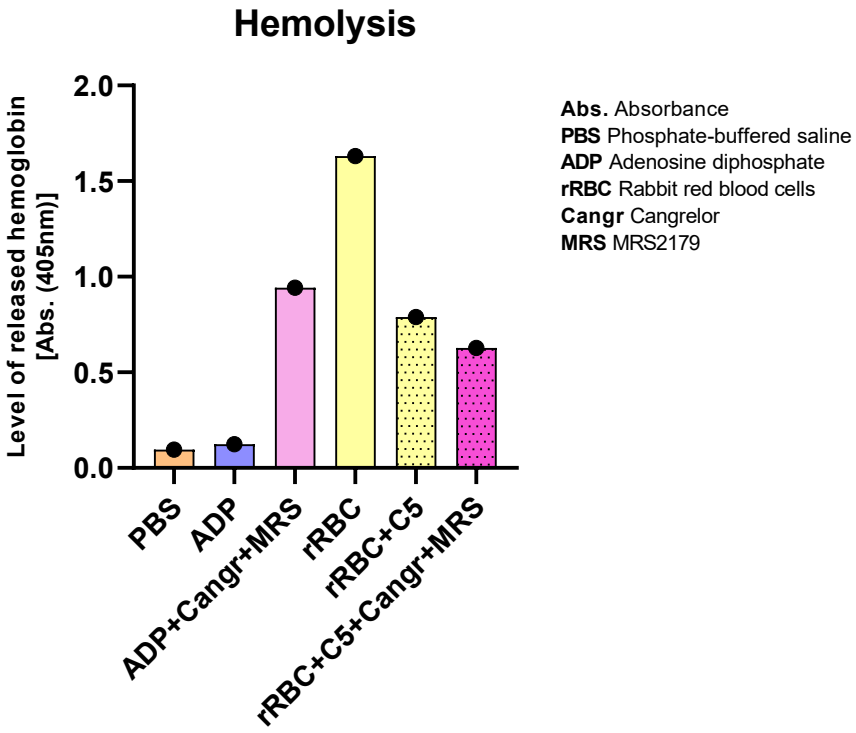

Figure 58: Hemolysis of patient 9. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

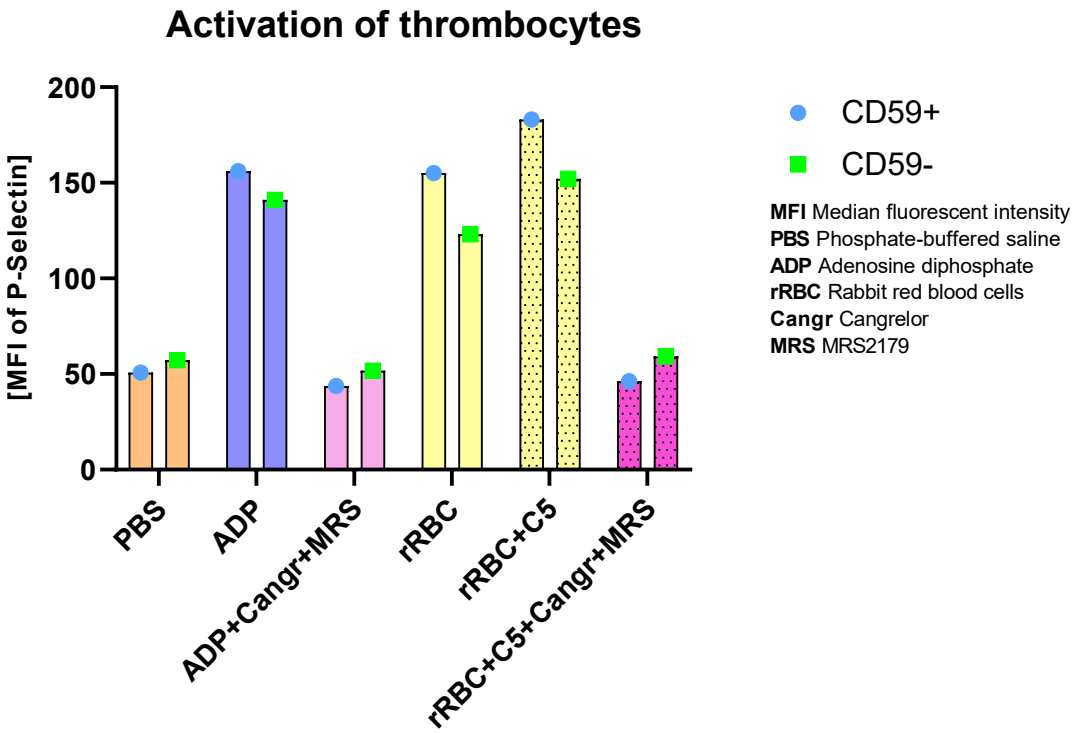

Figure 59: Activation of thrombocytes of patient 9. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (either CD59+ or CD59-).*

3.2.2.10 Patient 10

The complement system of this patient is inhibited with Ravulizumab. In addition, the patient is given CSA. The erythrocytes are lysed by the only addition of rRBC, even if a C5 inhibitor is present in the blood. Combination with C5 also results in lysis. Activation of platelets only occurs with ADP, not with rRBC or rRBC + C5, although lysis was measured, as previously observed. The CD59- thrombocytes are again more difficult to stimulate than the CD59+ clone.

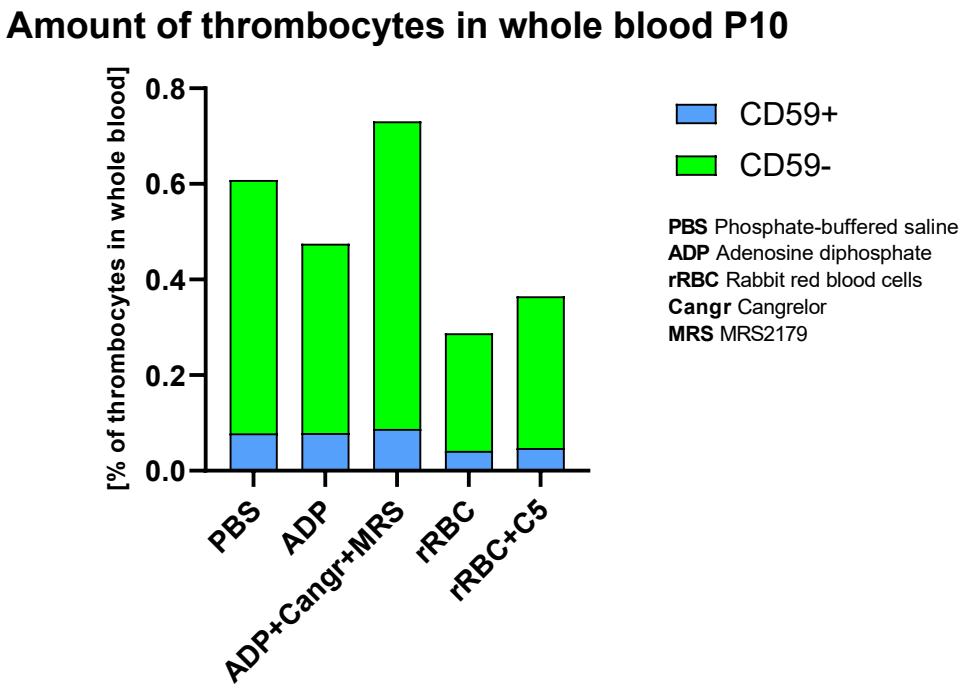

Figure 60: Amount of thrombocytes in whole blood of patient 10. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed.*

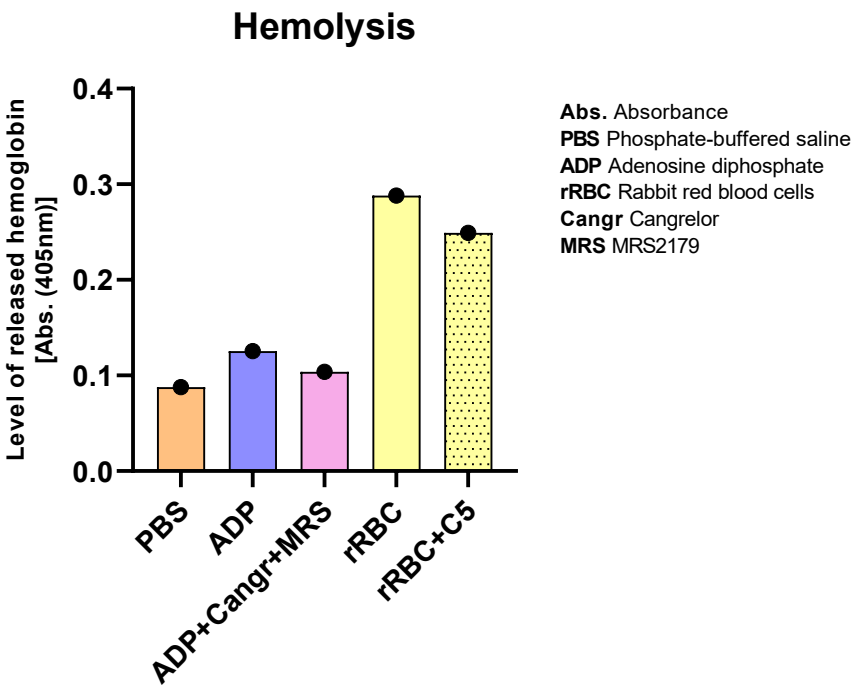

Figure 61: Hemolysis of patient 10. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC and rRBC+C5 the absorbance of the supernatant was measured at 405nm.*

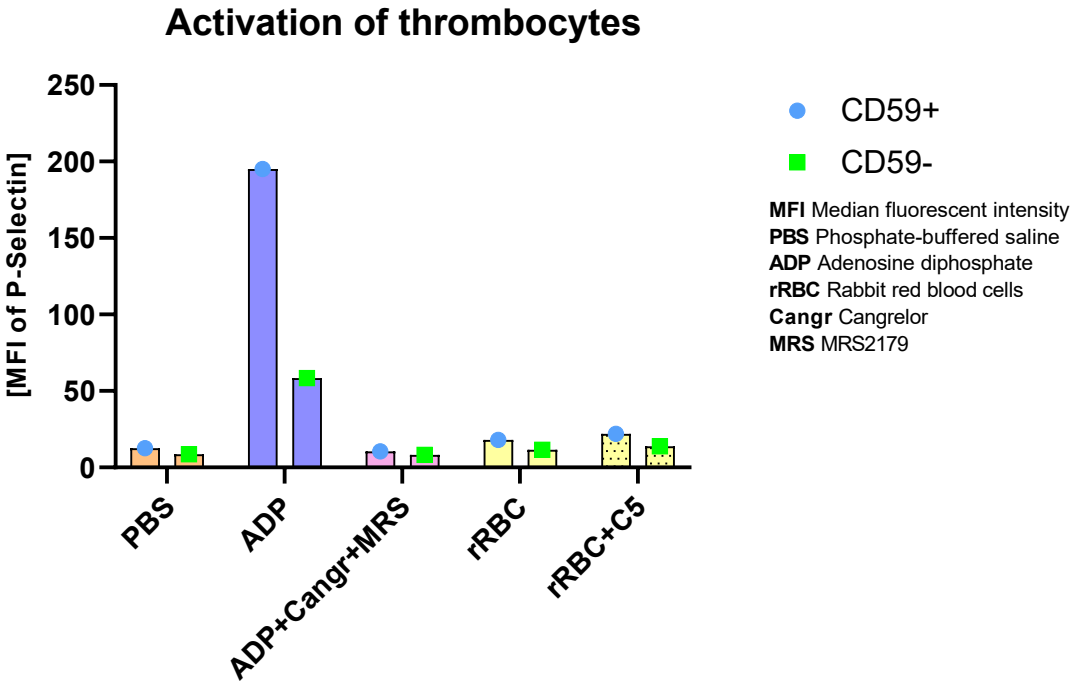

Figure 62: Activation of thrombocytes of patient 10. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC and rRBC+C5 to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (either CD59+ or CD59-).*

3.2.2.11 Patient 11

This patient has a very small CD59- clone; in fact, no CD59- thrombocytes could be detected in my experiments. The patient is not treated with a C5 inhibitor, but with CSA and Eltrombopag. Hemolysis occurs with rRBC and rRBC + C5, matching the lack of complement treatment. The platelets, which are nearly all CD59+, are activated with ADP and also, but to a lesser extent, with rRBC and rRBC + C5.

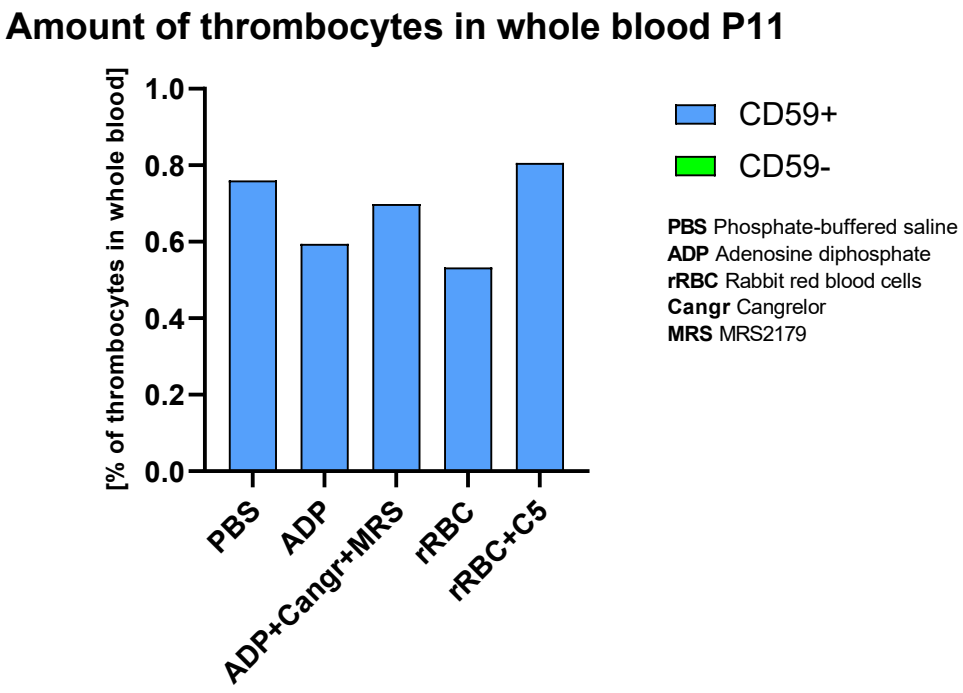

Figure 63: Amount of thrombocytes in whole blood of patient 11. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed. No CD59- cells could be detected.*

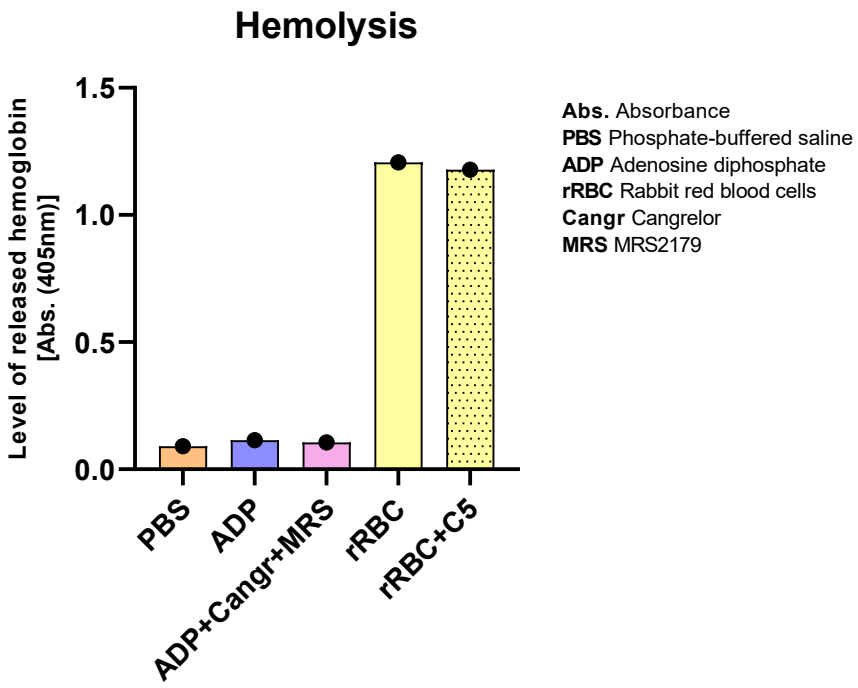

Figure 64: Hemolysis of patient 11. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC and rRBC+C5 the absorbance of the supernatant was measured at 405nm.*

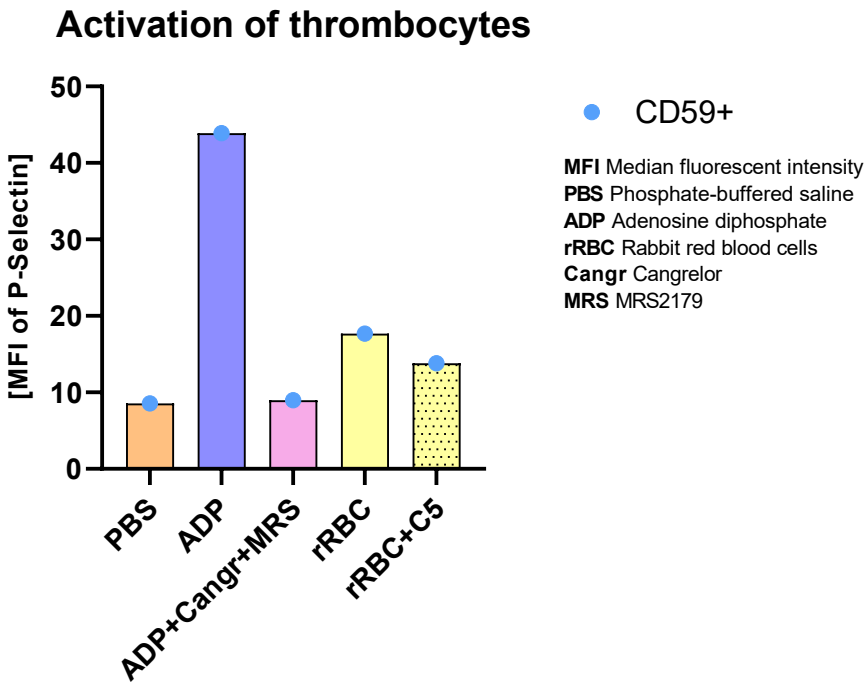

Figure 65: Activation of thrombocytes of patient 11. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC and rRBC+C5 to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (only CD59+ cells could be detected).*

3.2.2.12 Patient 12

The complement system is not inhibited here. No CD59- platelets could be found. No C5 was used in this experiment. Hemolysis after addition of rRBC was slightly higher than with PBS and ADP, but even without prior C5 inhibition, platelets are activated only with ADP, not with rRBC, reflecting the scarcity of hemolysis.

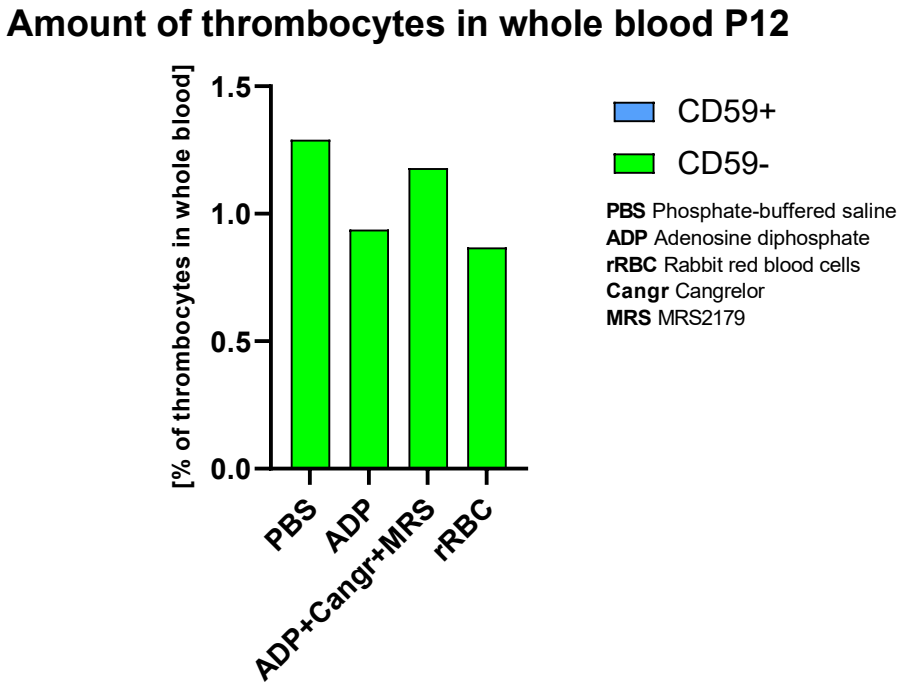

Figure 66: Amount of thrombocytes in whole blood of patient 12. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed. No CD59+ cells could be detected.*

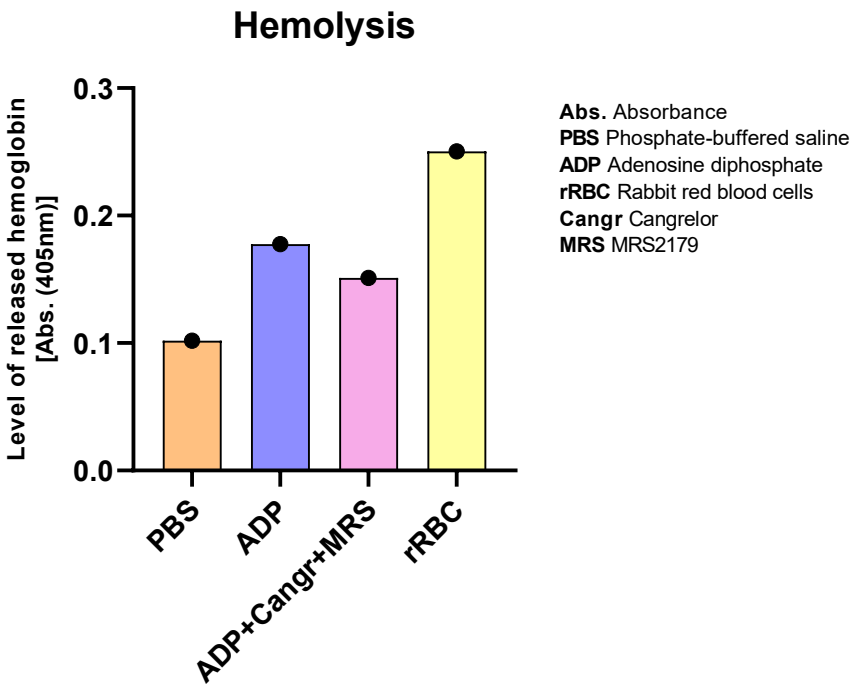

Figure 67: Hemolysis of patient 12. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS and rRBC the absorbance of the supernatant was measured at 405nm.*

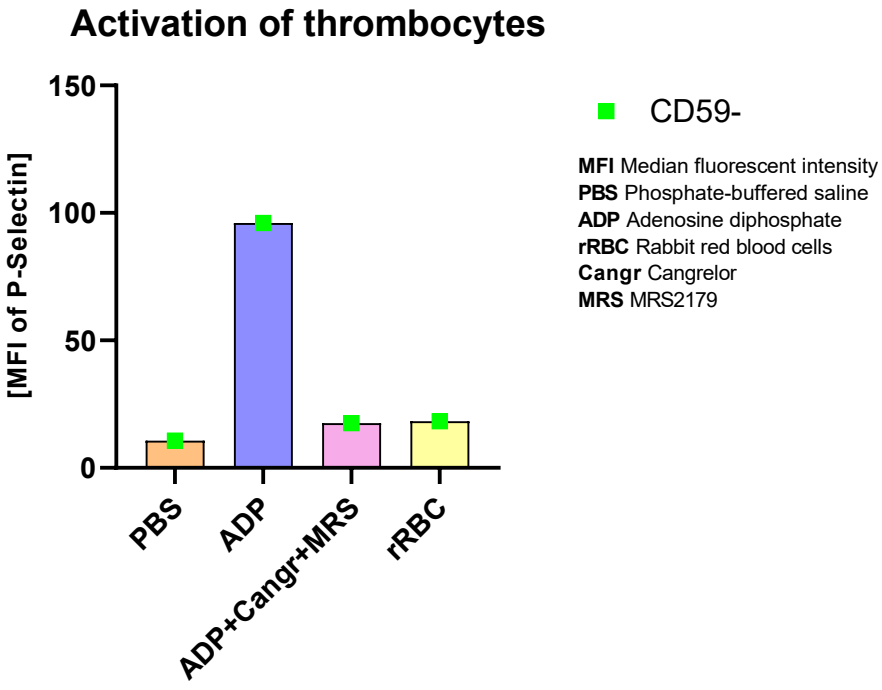

Figure 68: Activation of thrombocytes of patient 12. *After the addition of either PBS, ADP, ADP+Cangr+MRS or rRBC to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (only CD59- cells could be detected).*

3.2.2.13 Patient 13

The patient`s platelet count is very low at $20*10^3/\mu l$. Treatment consists of Eltrombopag and CSA, but no C5 inhibitor. This explains why lysis already occurs with the addition of rRBC, since the complement system is not inhibited at C5 level. Platelets are activated with ADP, rRBC and rRBC+C5, Cangrelor and MRS2179 could prevent platelet activation. There is not much difference between CD59+ and CD59- thrombocytes. The results of this patient are similar to those of the experiments with healthy blood.

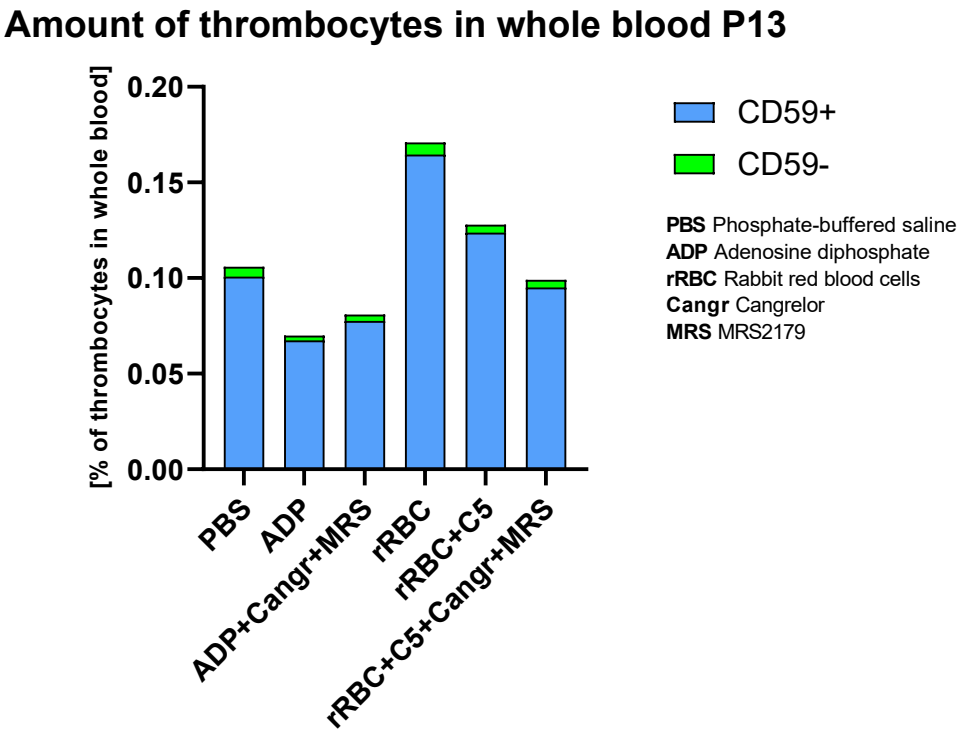

Figure 69: Amount of thrombocytes in whole blood of patient 13. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed.*

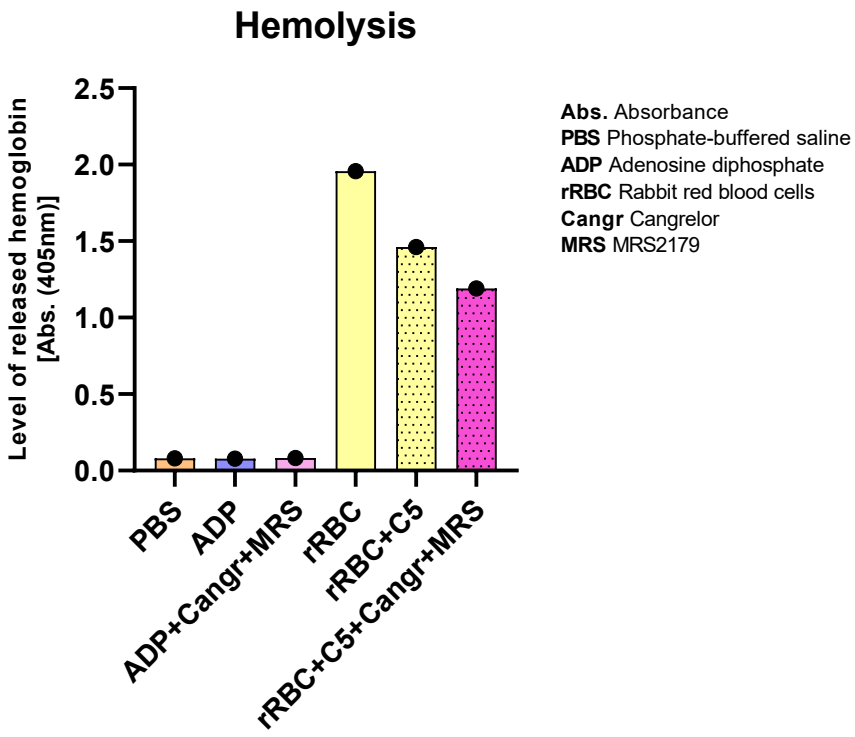

Figure 70: Hemolysis of patient 13. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

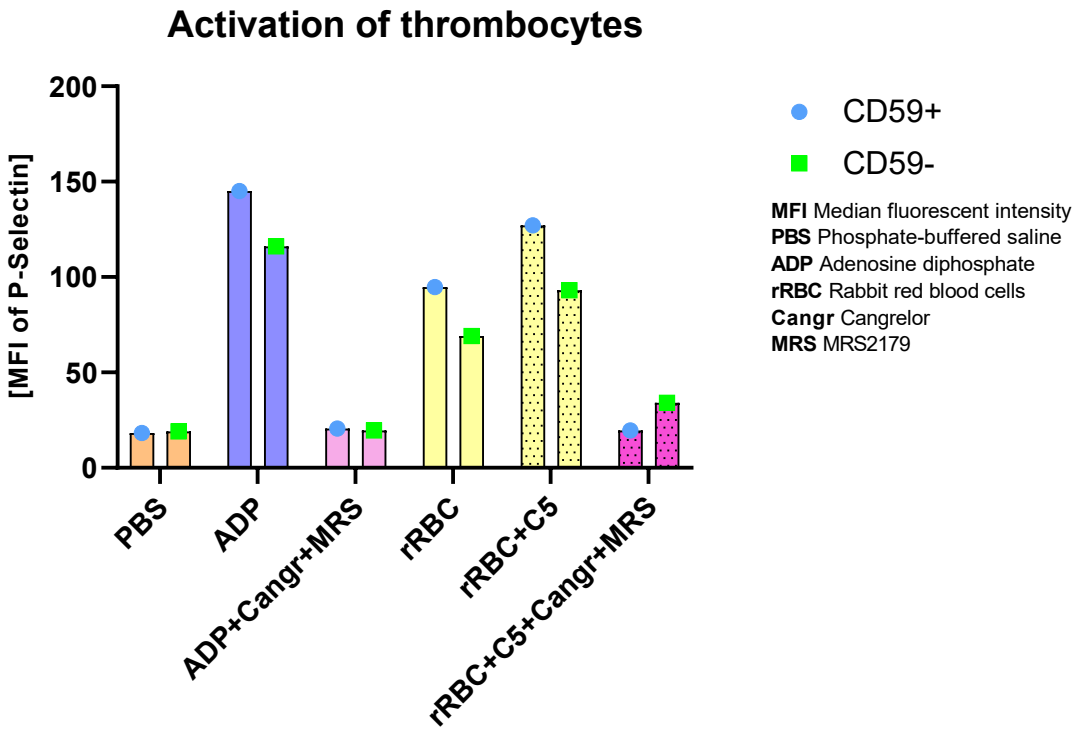

Figure 71: Activation of thrombocytes of patient 13. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (either CD59+ or CD59-).*

3.2.2.14 Patient 14

Patient 14 gets Ravulizumab, the CD59+ clone is very small, so the patient has almost only platelets without GPI anchor. RRBC leads to low lysis, after addition of C5 more erythrocytes are destroyed. Thrombocytes are not activated with rRBC alone, but with the addition of C5 and subsequent activation of the complement system, some of the platelets are activated (but only CD59+ platelets). Cangrelor and MRS2179 inhibited the activation. Again, CD59+ and CD59- thrombocytes show relatively the same results, but CD59- cells can be stimulated much less.

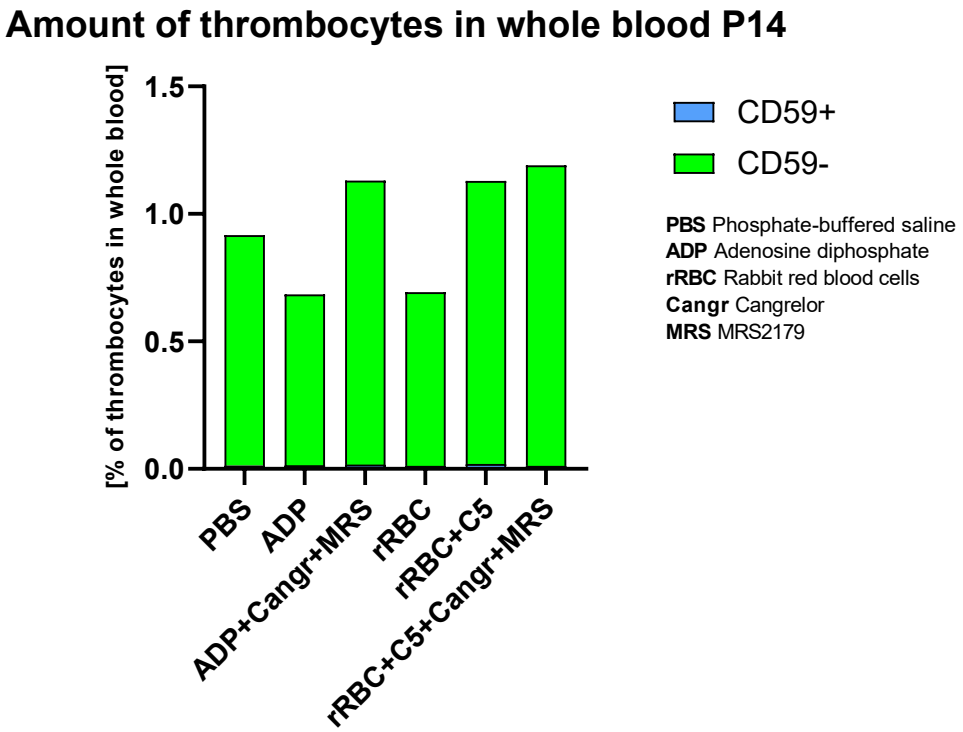

Figure 72: Amount of thrombocytes in whole blood of patient 14. *After performing the experiment, the percentage of thrombocytes in the whole blood of each sample was measured by flow cytometry. The proportion of CD59+ and CD59- cells is displayed. Nearly no CD59+ cells could be found.*

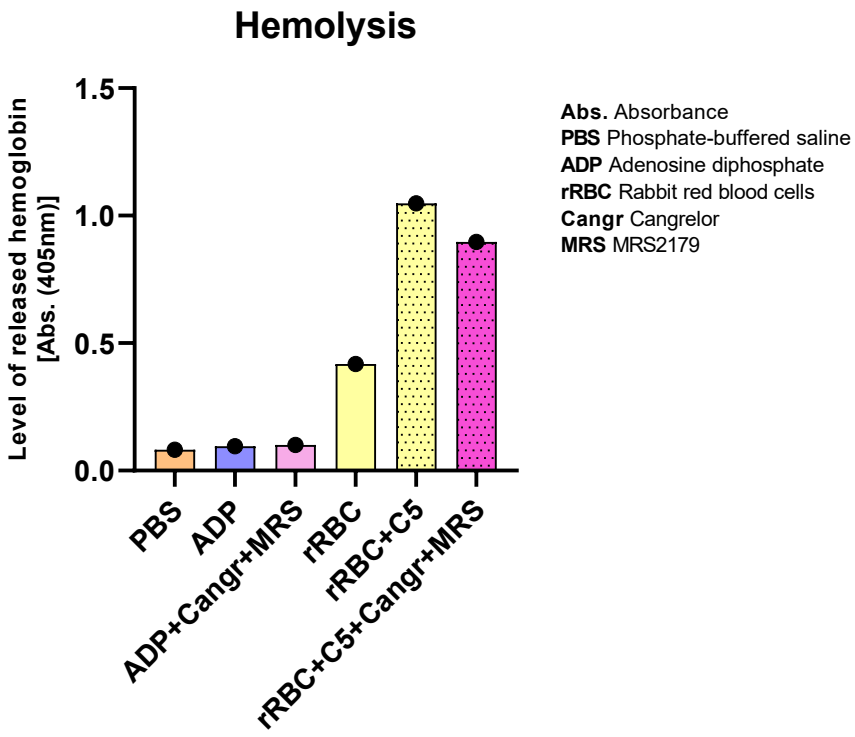

Figure 73: Hemolysis of patient 14. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS the absorbance of the supernatant was measured at 405nm.*

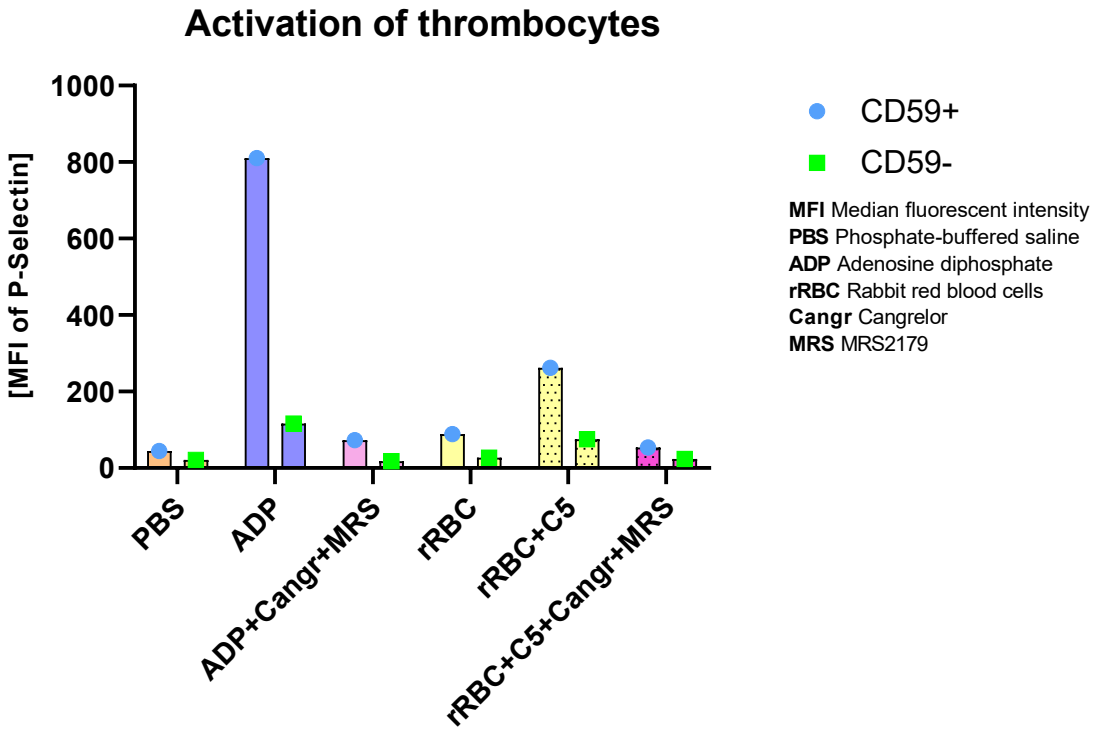

Figure 74: Activation of thrombocytes of patient 14. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation (either CD59+ or CD59-).*

3.2.3 Summary of thrombocyte activation response of patients with C5 inhibition

The results of all patients treated with a C5 inhibitor (*I.e.*, patients 1,2, 3, 4, 5, 7, 8, 10 and 14), were pooled. Hemolysis was measured as previously described. When PBS, ADP and ADP+ADP inhibitors were added to the samples, no erythrocytes were lysed. After addition of rRBC, complement should be active, but only up to the C5 level, because patients were treated with Ravulizumab/Eculizumab. A small fraction of erythrocytes was still lysed, but probably not due to complement activation. Potentially and hypothetically small changes in isotonic settings could be responsible for lysis of PNH RBCs in conditions with other chemicals present leading to some background hemolysis. Also it is known that single C5 inhibitors lead to residual levels of lysis of strongly complement activating cells like rRBCs. Hence, hemolysis of rRBCs in absence of supplementation of C5 also occurred to some extent, but to a lower level when compared to the addition of C5. After the supplementary addition of C5, more erythrocytes are lysed, as expected.

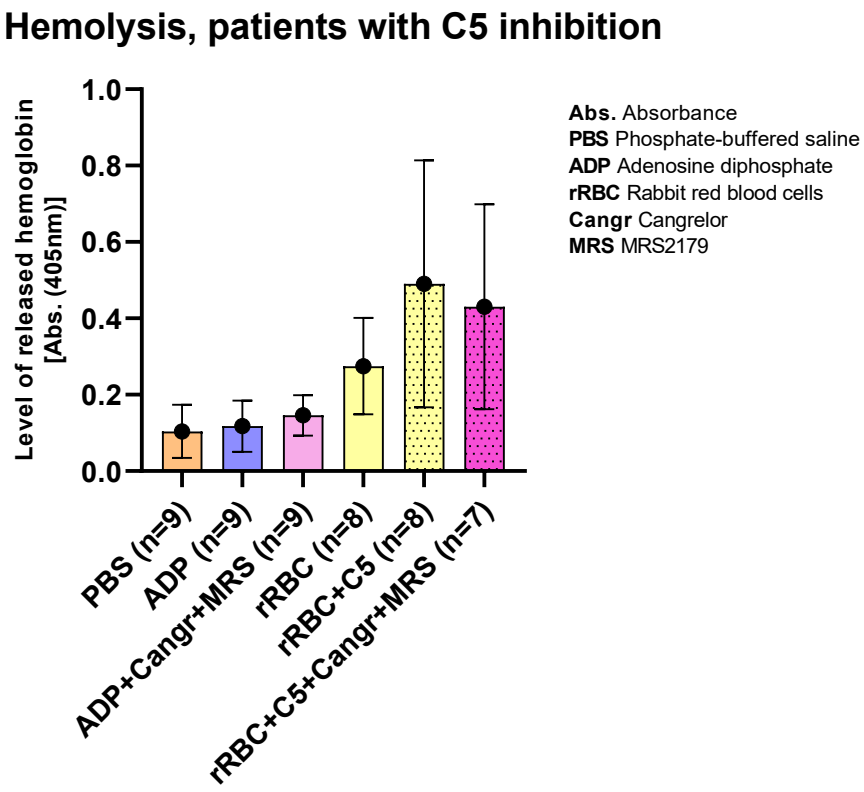

Figure 75: Hemolysis of the blood of PNH patients with C5 inhibition. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS, the absorbance of the supernatant was measured at 405nm. The average of nine independent experiments with standard deviation is shown.*

When comparing thrombocyte activation, ADP is the only agent that really has a consistent increasing impact on platelet activation. Cangrelor and MRS2179 manage to successfully inhibit ADP signalling. RRBC do not activate PNH thrombocytes, even when hemolysis is present. Thus, complement inhibition - in the sense of thrombocyte activation - works. The samples with rRBC and C5 lead to a small activation of thrombocytes, but surprisingly not in all cases. Some activation above the levels of the negative control PBS are observable, but do not reach the same level as observed for the positive control ADP. Even if the complement system was active (occurrence of hemolysis), platelets are hardly stimulated. The occurrence of hemolysis proves the activation of the complement system, but the thrombocytes appear to be somehow protected. Possible reasons for this are discussed in 4.3.1. When ADP receptors are blocked with Cangrelor and MRS2179, the thrombocytes can be completely inhibited even if the complement system was activated with rRBC and C5.

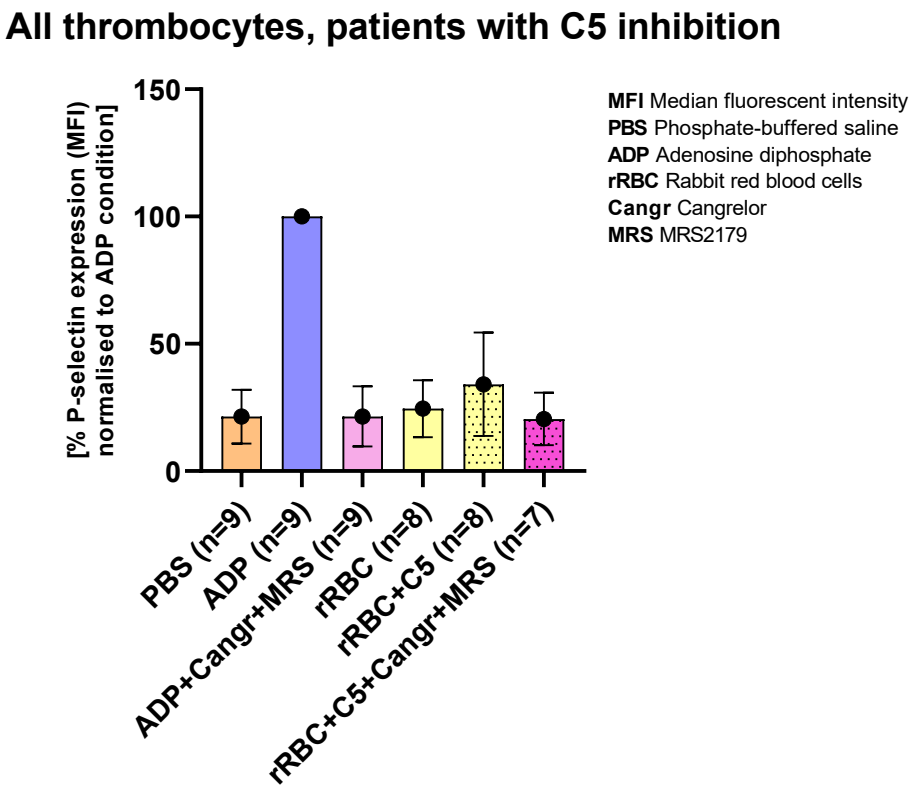

Figure 76: Activation of thrombocytes of PNH patients with C5 inhibition. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 or rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation. ADP was defined as 100% activation and used as a reference. The average of nine independent experiments with standard deviation is shown.*

All cells, or in the most cases a part of the cells of PNH patients, lack the GPI anchor, which is why only some of them have CD59 on their membranes. Platelets can also be divided into two groups (CD59+ and CD59-). To find out whether the CD59- cells are more susceptible to complement attack or complement-mediated platelet activation, the two phenotypes are compared separately. ADP of all platelets was set as 100% activation level (within one experiment) to obtain normalized results to facilitate the comparisons of the groups. If there was only one CD59 phenotype present, then the ADP activation level of this clone was taken as 100% activation and the cells were assigned to the appropriate group. Overall the CD59+ and CD59- platelets seem to react similarly to normal platelets. ADP activates the cells, ADP inhibitors prevent activation. However, rRBC are (almost) not able to stimulate the thrombocytes due to the failure of lysis in C5 inhibited whole blood. But even with added C5 and occurring hemolysis, platelet activation levels were only slightly higher. What is remarkable is, that for some patient samples hemolysis of rRBCs did not lead to a substantial platelet activation phenotype of the PNH platelets, while addition of ADP did so. CD59- cells, even if they cannot fight off the complement system, are not more activated than the CD59+ cells. On the contrary, it seems that CD59- are much more difficult to stimulate than the CD59+ thrombocytes. Overall, the variation of activation levels is rather high among these patients as judged by the standard deviation. Reasons for this could be the different clone sizes, different polymorphisms and also the different supportive treatments of the different PNH patients leading to a substantial biological variability of the cell responses in the whole blood assay. Especially the platelet characteristics were individually rather diverse. Most PNH patients had only or mostly CD59- platelets. Rarely a patient would have mostly CD59+ platelets and they were those who did not receive anti-complement therapy.

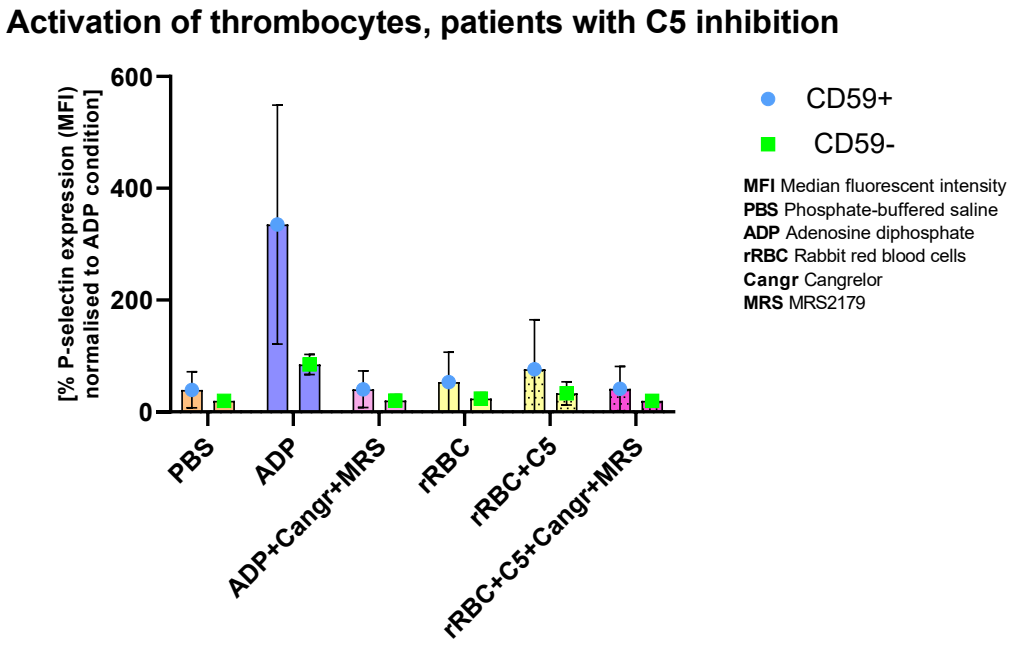

Figure 77: Activation of thrombocytes of patients with C5 inhibition, comparison of CD59+ and CD59- cells. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 or rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation. ADP of all platelets within one experiment was defined as 100% activation and used as a reference. The average of nine independent experiments with standard deviation is shown. If there was only one clone (only CD59+ or -) the results were nevertheless added to the corresponding group.*

3.2.4 Summary of thrombocyte activation response of patients without C5 inhibition

Four of the PNH patients are not treated with any inhibitor of the complement system. The results of these four patients are pooled extra. Addition of PBS, ADP and ADP+ADP inhibitors do not show hemolysis. In the absence of C5 inhibitor in the blood of the patients, rRBC alone are sufficient to cause lysis. Addition of C5 shows the same result, and rRBC + C5 + ADP inhibitors also induce lysis, as expected.

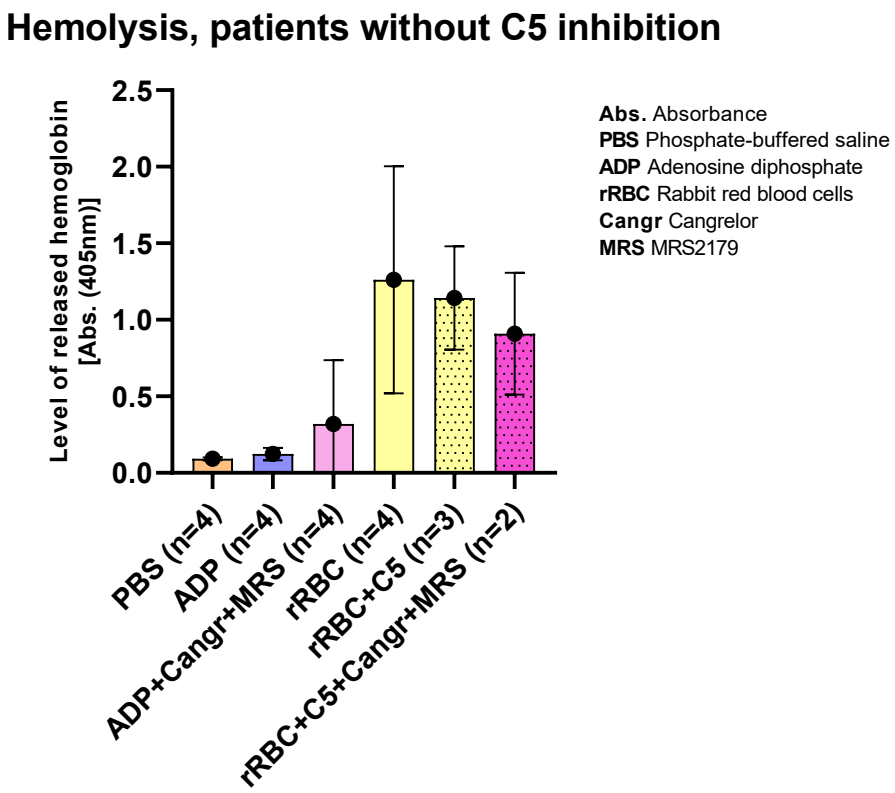

Figure 78: Hemolysis of the blood of PNH patients without C5 inhibition. *After treatment of the blood samples with PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 and rRBC+C5+Cangr+MRS, the absorbance of the supernatant was measured at 405nm. The average of four independent experiments with standard deviation is shown.*

The results of the activation of the thrombocytes are similar to those of healthy patients (since there is no C5 inhibitor already in the patients` blood). PBS was the negative control, ADP the positive control. Cangrelor and MRS2179 prevented platelets from being stimulated via the ADP receptors. All conditions with rRBC activated the platelets, unless Cangrelor and MRS2179 were added.

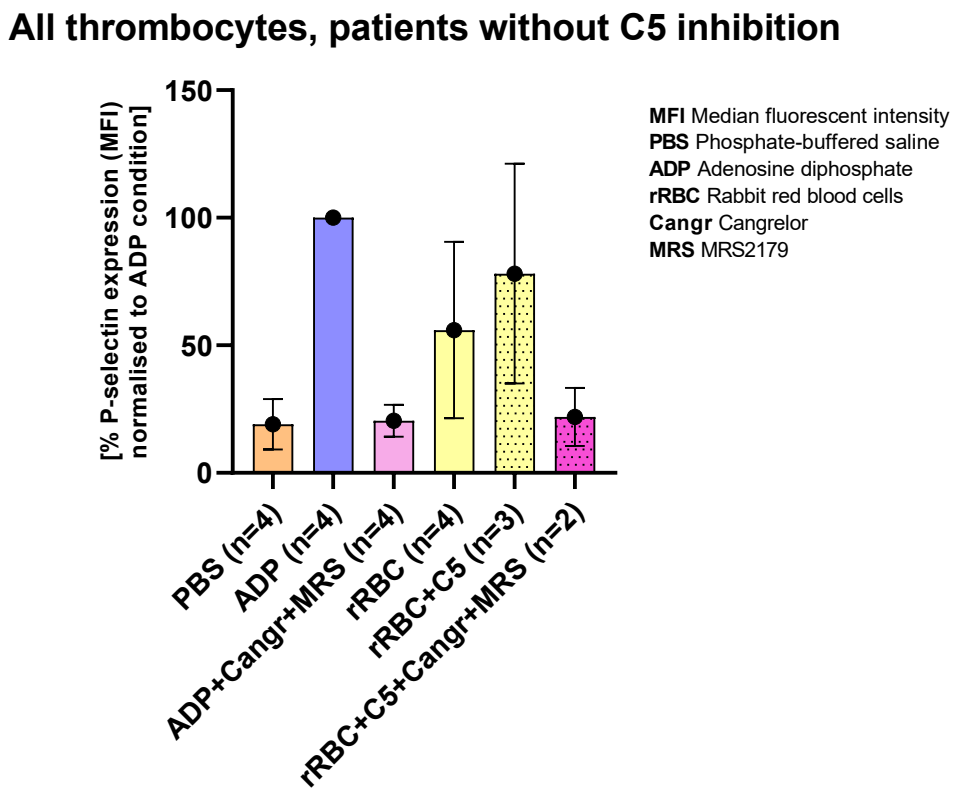

Figure 79: Activation of thrombocytes of PNH patients without C5 inhibition. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 or rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation. ADP was defined as 100% activation and used as a reference. The average of four independent experiments with standard deviation is shown.*

Since these PNH patients had a mixture of CD59+ and CD59- platelets, the activation propensity of these two populations can be compared. Within one experiment, corresponding to a sample from one individual PNH patient, the result of all platelets measured in the ADP conditions was normalized to 100%. If there was only one clone, the ADP-value for that clone was normalized to 100%. Compared with each other, CD59 + and CD59 - platelets from patients not treated with a complement inhibitor showed almost equal levels of cell activation upon stimulation. This is in remarkable contrast to the platelets derived from patients on C5 inhibitors (see above; for which the CD59- platelets showed much less propensity for prothrombotic cell activation by ADP). For the platelets from non-complement inhibited patients, as expected, ADP leads to activation, Cangrelor and MRS2179 inhibit it. RRBC alone also stimulate the platelets of patients on C5 inhibitors to some extent, after supplementation of C5 platelets were even more active. However, the ADP inhibitors can prevent this stronger activation completely. In this regard the

responses of the platelets derived from non-complement inhibited patients are more comparable in their activation behavior to the platelets from healthy volunteers rather than to cells derived from C5-inhibited patients.

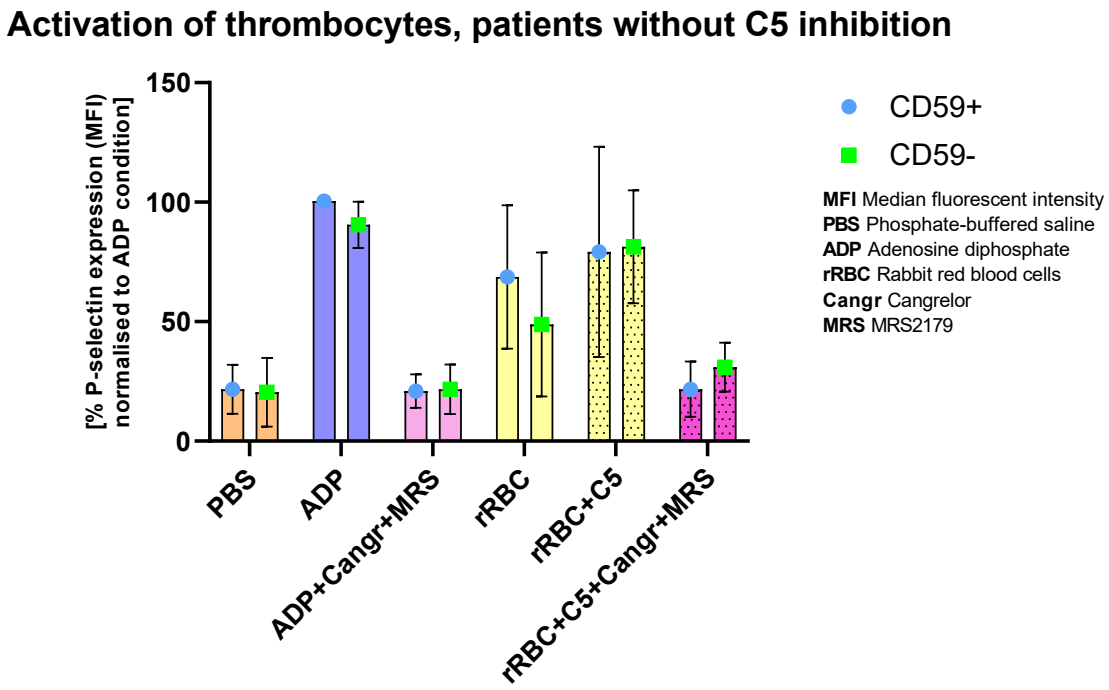

Figure 80: Activation of thrombocytes of PNH patients without C5 inhibition, comparison of CD59+ and CD59- cells. *After the addition of either PBS, ADP, ADP+Cangr+MRS, rRBC, rRBC+C5 or rRBC+C5+Cangr+MRS to the samples, the MFI of the activation marker P-selectin was determined and shows the platelet activation. ADP of all platelets within one experiment was defined as 100% activation and used as a reference. The average of four independent experiments with standard deviation is shown. If there was only one clone (only CD59+ or -) the results were nevertheless added to the corresponding group.*

3.3 Conclusion

In summary, my experiments have shown that the complement system has no direct influence on prothrombotic cell activation of platelets. Platelet activation occurs indirectly through the formation of the membrane attack complex and, as is the case in PNH or rabbit erythrocytes, by subsequent hemolysis with release of ADP. Based on experiments with both cells from healthy volunteers and PNH patients, it can be said that the ADP inhibitors reliably inhibited platelets, regardless of whether the ADP is added directly to the samples or released through lysis. Consequently, ADP must be the responsible danger signal leading to prothrombotic cell activation when complement-mediated hemolysis occurs.

4 Discussion

The complement system plays an important role in both immune and coagulation system. Already small defects or imbalances are leading to severe diseases and the patients suffer from thromboembolic events which cannot be controlled by anticoagulant or antiplatelet therapies. [64, 65] Also the cleavage products and anaphylatoxins C3a and C5a are described in the literature to have an impact on different cells and finally leading to a prothrombotic state. [1, 23, 58, 63, 75] Thus, complement activation is thought to be responsible for the prothrombotic cell activation that appears in PNH and which is largely responsible for the increased mortality of this disease (prior the onset of complement inhibitor). The absence of CD59 and CD55 on the membranes of different hematopoietic cells, which normally protects the cells from a complement attack, is leading to intravascular hemolysis and the related complications like thrombosis, anemia and different others. [65, 66] Patients with thromboembolic complications are bearing the risk of a poor survival and the survival rate at 4 years of patients presenting themselves with thrombosis was only 40%. [28] This poor outlook was abruptly improved with the introduction of the C5 inhibitor Eculizumab. In the study by Hillmen et al. the thromboembolism rate after treatment with Eculizumab was reduced from 10.61 to 0.62 events per 100 patient-years. [29] Many other studies trials were performed not only concerning the thromboembolism rate but also overall survival, number of transfusions, intravascular hemolysis and a corresponding LDH rate, hemoglobin levels and several others. [28–30, 35, 45] Eculizumab is inhibiting the cascade at C5 level. Inhibited C5 is not cleaved and neither the anaphylatoxin C5a, nor C5b is produced. Consequently, the membrane attack complex cannot be formed. Since Eculizumab improves the prognosis of patients in many ways, it is reasonable to assume that the key factor of the link between complement and prothrombotic state lies in C5a/C5b including the membrane attack complex or anything that happens after C5 cleavage. Everything that happens up to the cleavage of C5 is still generated under C5 inhibition, like the anaphylatoxin C3a and C3b, which opsonizes cells. Like this, invaders can still be recognized and repelled, but the lytic terminal pathway is inhibited. [51] In vitro findings from different studies suggested, that even C3a and C3b have an impact on coagulation and bleeding time and also on activating platelets. [1, 23, 58, 63] However, these in vitro findings cannot be confirmed by the clinical in vivo evidence, as the use of Eculizumab tremendously improves the clinical situation of

PNH patients including the occurrence of thrombotic events. There is a discrepancy between the in vitro findings and the in vivo observations. The mechanistic clarification of this discrepancy or at least the finding of an important link between the prothrombotic state and the complement system was the aim of this thesis.

4.1 Experimental setup

When establishing the experimental setup several things had to be considered. In order to be as close to the reality as possible a full blood model was chosen instead of *e.g.* platelet rich plasma as used by others before. The setup is described in 2.2.1. The only component missing in the blood is functional thrombin, because a hirudin tube was chosen to draw blood from the donors. Hirudin is inhibiting thrombin directly but does not have an influence on thrombocytes or the complement system. [39, 40, 77] Although thrombin could cleave C5 independently of C3, it does not appear to be very effective at physiological concentrations. [13, 32, 36, 37]. To prevent platelets form activating on their own by mechanical shear force and to obtain comparable results, blood was drawn slowly from an antecubital vein and the cells in whole blood were fixed as early as possible prior to the analysis by flow cytometry.

4.2 Whole blood experiments with blood from healthy volunteers demonstrate the importance of the MAC for platelet activation

When adding rRBC to the complement active whole blood, red cells are opsonized and subsequently lysed, which was measured by quantifying the release of hemoglobin. Whether the active complement system also lysed human red cells could not be found out but it is assumed that this did not happen (as no lysis occurred under the same conditions in absence of rRBCs). Complement mediated rRBC lysis correlated with platelet activation.

Platelet activation was measured by determining the increase in surface expression of the well-known activation marker P-selectin. Moreover, an additional platelet activation marker was investigated in exemplary experimental settings and shown to correlate with the expression of P-selectin. Glycoprotein IIb/-IIIa (CD41) showed congruent results, what is described in 3.1.12.

Next, it was clarified, which level of complement activation is required to activate the platelets. To do so, the complement cascade was intercepted at specific levels using specific and well-known pharmacological complement inhibitors. Addition of rRBC to complement

active whole blood led to hemolysis and thrombocyte activation. Supplementing these reactions with the C3-inhibitor Cp40 or the C5-inhibitor eculizumab stopped hemolysis and platelet activation. Interestingly, C5-inhibition inhibits the MAC, but allows complement activation at the level of C3. Hence the anaphylatoxins C3a and the opsonin C3b still get produced under C5-inhibition. As samples with C5 inhibition failed to show hemolysis and thrombocyte activation, it can be ruled out that C3a and C3b act as platelet activators. This is in line with the clinical evidence as Eculizumab largely prevents thrombosis in PNH [29, 30, 35] and is in contrast to several mechanistic reports that had looked at this question using isolated cells rather whole blood. [55, 58]

In aggregate, it appears that the proteolytic activation of C5 is the key step in the whole blood assay that leads to platelet activation. Therefore, two complement effector functions could be responsible for C5-mediated platelet activation; the anaphylatoxin C5a and C5b, which leads to the formation of the MAC. MAC may induce cell signalling like C5a, but it also lyses cells and hence induces the release of intracellular danger signals.

Thus, I hypothesized that not the complement system but intracellular danger signals released during complement-mediated cell lysis are responsible for the activation of the thrombocytes.

To test this, the erythrocytes were lysed mechanically (not via the MAC) before being added to the whole blood samples. Indeed, even without complement activation the addition of shattered RBCs led to a strong and concentration dependent activation of the thrombocytes.

One known activator of thrombocytes is ADP (also used as our positive control), which is also released upon RBC lysis. To find out whether this is the danger signal responsible for the platelet activation, two known ADP-receptor antagonists were probed in my setup. ADP interacts with the platelets via two receptors (P2Y1 and P2Y12). Cangrelor is inhibiting P2Y12 and MRS2179 the P2Y1 receptor. After titrating the correct dose of the inhibitors, the samples were supplemented with both inhibitors to rule out any ADP-signalling. Although Cangrelor and MRS2179 did not interfere with the complement cascade, as complement mediated-hemolysis also developed in presence of the two ADP inhibitors, the ADP receptor antagonists completely prevented thrombocyte activation. These data support the hypothesis that the platelet activation is a consequence of lysis and not of

complement activation. In one experimental setup a completely human model was established where only cells from one donor were mixed within the whole blood model utilizing shattered (mechanically lysed human RBCs). These results were consistent with the results of experiments using the rRBCs ruling out any effects that could have been introduced via having cells from two different species in one assay (*i.e.* human and rabbit).

4.2.1 Purified complement components C3a and C5a show no effect on platelet activation

There are sources reporting that the anaphylatoxins C3a and C5a are able to induce platelet activation and aggregation [20, 55], but my mechanistic experiments in whole blood using different inhibitors did not confirm this. Therefore, I cross-validated my observations by investigating the effect of the purified components C3a and C5a. Addition of C3a or C5a alone was not able to induce thrombocyte activation. Also, addition of a combination thereof was not able to influence platelet activation even when the platelets had been preactivated with ADP (Figure 20). These experiments as well support the hypothesis, that not the complement system by itself but the signals, which are released upon lysis, are responsible for thrombocyte activation.

4.2.2 Activation of complement with zymosan induces complement activation but fails to induce platelet activation

Next to rRBC as complement activator also zymosan was used. Zymosan activated the complement system, but did not lead to any hemolysis, as expected. So, the complement cascade produced C3a and probably C5a (not measured), but the platelets stayed quiescent. This once more confirmed my hypothesis. In order to see, whether or not the ADP-inhibitors and/or complement inhibitors have an overall meta-level inhibitory effect on platelets, platelet activating factor (PAF) was used as an additional platelet activating factor. PAF activates the platelets independently from ADP or the complement system. As already assumed before, the ADP receptors seem to be really important for the thrombocytes, because even if PAF is used as activation marker, platelet activation can be prevented with ADP inhibitors (but importantly not with complement inhibitors). Electrodense granules of platelets hold stores of ADP among other things and hence platelets, upon activation, could enhance any initial activation signal by releasing the ADP from the ADP from the electrodense granules. Thus, it could be that PAF signalling works

via release of ADP from platelet intracellular stores and therefore, ADP-receptor antagonists inhibit also PAF-mediated platelet activation.

4.3 Platelets in whole blood derived from PNH patients behave differently than platelets from healthy volunteers depending on the anti-complement agent used for PNH therapy

All the previous results were performed with blood from healthy volunteers. Next it was investigated whether or not complement vulnerable cells like RBCs and platelets from PNH patients, which are sensitive to complement attack, react differently to platelet activating stimuli.

The design of the experiment was the same than before. The blood was drawn from patients who came for a regular check-up to the institute. Therefore, one difference compared to before was that other people drew the blood. The blood samples were taken immediately after collection to achieve a high degree of comparability. Nevertheless, it cannot be excluded, that the platelets have been partially pre-activated by the short storage and transport. Another source of variance came from the different patients. All suffer from PNH, but to varying degrees. The clones are different, some have nearly no CD59+ cells, some nearly no CD59- cells. Accordingly, it was difficult to bring the different results together, meaning that the pooled data show a rather huge spread of variability (high standard deviations over averaged data from different patients). Furthermore, some patients had very low to almost no thrombocytes. To perform the results with just a few platelets was not easy, because it was hard for the flow cytometry analysis to detect enough platelets for comparable results. The reason, why some patients have a low platelet count is unclear. Theoretically platelet numbers could be reduced because they may also lyse in a complement dependent manner, or because they are consumed due to in vivo activation of platelets. To treat the thrombocytopenia of some patients, Eltrombopag, an agent for stimulating platelet formation in the bone marrow, had been administered to some patients, which further increased the variance in the experimental conditions when working with PNH patient blood. It is unclear, what effect this drug has on the behavior of platelets and other cells.

Nine patients were treated with a C5 inhibitor, one patient with a C3 inhibitor and the remaining four patients received no complement-inhibiting therapy. The results of the respective groups were combined.

Overall, the addition of rRBCs to PNH whole blood resulted in hemolysis as long as the complement system was active. This was the case, when PNH patients were not treated with a complement inhibitor, or when purified C5 was supplemented into PNH whole blood from patients treated with a C5 inhibitor. Remarkably ADP always induced platelet activation of the PNH platelets while rRBC lysis did sometimes but not always. The reasons for this are not clear, but a speculative explanation model is provided below.

Of note, the platelet activation propensities of the whole blood from anti-C5 treated PNH patients was different than the one from PNH patients without an anti-complement treatment. By far and large the thrombocyte activation profiles from patients not treated with complement inhibitors was similar to the ones of healthy donors. In contrast, the activation profiles of patients on C5 inhibitors were substantially reduced even though clear and distinct levels of hemolysis could be achieved in the whole blood samples of these patients upon addition of purified C5. As in healthy patients, the ADP inhibitors Cangrelor and MRS2179 were able to inhibit platelet activation completely regardless of the treatment of the PNH patients.

However, it should be noted that the number of patients without C5 inhibition is not high. In addition, it was difficult to standardize the results of the patients without C5 inhibition, because some of them had nearly no platelets with a CD59+ phenotype, some of them none of a CD59- phenotype. Some of them got additional treatment (*e.g.* with CSA or Eltrombopag). This makes it difficult to make the results comparable to each other or to present average values without a wide spread. In order to achieve comparable and significant results, the number of patients must be much higher in future, extended studies.

4.3.1 CD59+ and CD59- platelets of PNH patients receiving C5 inhibitors show different activation profiles

Since the platelets from patients on anti-C5 therapy did not activate as readily upon occurring hemolysis in the samples, a deeper analysis was started stratifying the PNH platelets in two groups: those being CD59 negative and those being CD59 positive. As described before, some patients just had one PNH platelet phenotype. In eight of the 14

patients, both phenotypes could be detected with the flow cytometer, five had only CD59- cells and one patient only CD59+ cells. One in the CD59- group and the patient with only CD59+ cells did not receive anti-complement treatment. The other patients without anti-complement treatment had both phenotypes, but mostly CD59+ platelets. One of the patients with only CD59- cells received an C3 inhibitor, the patient was not included in the pooled data (section 3.2.2).

In order to be able to compare the different clones, the ADP activation levels of all phenotypes together within one experiment was normalised to 100% activation. When comparing the two phenotypes within one patient, it is noticeable that the CD59- platelets are much more difficult to activate than the CD59+ platelets when derived from C5-inhibited patients. The reason for this is not clear. Is the disease changing the content of the granules, which store the P-selectin? Or are the ill platelets already lysed by the complement attack, so that we can only recognize the cells that defend themselves better from complement mediated lysis? Or does even the C5 inhibitor have an impact on the CD59- cells? There are many possibilities, but the fact that CD59- cells are more difficult to stimulate is a surprising preliminary result. Potential reasons why specifically CD59- platelets of anti-C5 treated platelets show less platelet activation when compared to not complement inhibited patients are not clear at all. Potentially and highly speculatively this could be due to complement deposition on blood cells under anti-C5 therapy. It is also noteworthy that CD59- platelets from patients treated with a C5 inhibitor are sometimes not activated at all despite the addition of rRBC + C5 and subsequent hemolysis. In the same assays, APD always activates the platelets, albeit to a lesser extent than the CD59+ platelets, which means that they can be stimulated per se. One consideration could be that it is precisely those CD59- platelets, that are difficult to stimulate, that survive. The missing GPI-anchor could also be a possibility to consider. However, both ideas are unlikely, because the platelets of patients without complement therapy behave in exactly the same way as in healthy people. I have not made any measurements in this regard, but it is known that anti-C5-therapy leads to a loading of C3 on RBC. [60] Due to C5 inhibition, they are not lysed, but are C3 positive. Perhaps this is exactly what happens with the platelets and loading with C3 on their surfaces (and surface receptors and transporters) interferes with activation. Under the ADP condition, platelets are still activated, but the CD59- platelets are activated to a lesser extent. Under the rRBC + C5 condition, where the complement

system is active and C3 loading can occur within the assay, the platelets are normally no longer activated at all.

Future work could explore this topic in more detail. The number of patients for the experiments must be significantly higher in order to verify the results and rule out errors. However, this may not be easy, as PNH is an ultra-rare disease. Further experiments must also be carried out to answer the question of why C5 inhibitors appear to put CD59-platelets into a resting state in which they can hardly be activated. This is a remarkable result. Given the prothrombotic phenotype of the disease PNH, most experts would probably have speculated that platelets of the PNH phenotype would be more readily be activated. Due to the preliminary status of my results, this question cannot be addressed conclusively but further studies are warranted. One could, for example, investigate in the future, whether C3 loading also exists on platelets and whether this is associated with reduced activation.

4.4 Conclusion and significance of the overall results

4.4.1 Significance for the current complement research

Looking at the results of both the healthy experimental group and the patients suffering from PNH, it becomes clear that my results partially differ from previous other reports. Others attribute complement products, particularly C3a, to an important role in the regulation or activation of thrombocytes. [23, 58, 63, 75] However, these are in vitro experiments and in some cases the concentration of the products does not correspond to that in vivo, but is many times higher. But the clinical presentation of patients suffering from complement diseases tells us otherwise. The entire complement cascade up to the cleavage of C5 still proceeds, but does not lead to thrombosis. And this is exactly what my results confirm. C3a and C5a certainly influence many important processes in our body and they may affect platelet function, but not as the main driver of activation. In line with my findings, a recent study mentioned that C3a does not induce prothrombotic cell activation in whole blood assay using a bacterial challenge. [42] Some reports stating that complement products are important for platelet activation do not mention the MAC and subsequent release of ADP. Since ADP inhibitors can prevent all platelet activation in my experiments, whether healthy or PNH-platelets were used, I believe that lysis of erythrocytes with subsequent release of ADP is the main driver of thrombocyte activation

in complement diseases. Furthermore, the experiments with PNH patients showed interesting insights into the different behavior of platelets from PNH patients, especially when treated with a C5 inhibitor. The finding that C5 inhibitors not only prevent the activation of platelets by inhibiting red cell lysis and the subsequent release of ADP, but also by putting them into a more quiescent state (possibly due to C3 loading), particularly when the complement system is activated, is new and worth further research.

4.4.2 Clinical significance

All these findings could have important clinical implications. In PNH, anticoagulation is used for patients, in whom thrombosis cannot be completely prevented by C5 inhibitors. [24, 65] However, there are many side effects and the effectiveness of these drugs is not really promising, because the drugs target the coagulation cascade and not the complement cascade or interfaces between the two systems, which are responsible for the thrombotic state. Since in my experiments the lysis of erythrocytes and the release of ADP is a main driver of platelet activation, in patients suffering from PNH (or suffering from a disease in which hemolysis and thrombosis occurs simultaneously) and especially in patients in whom thrombosis occurs despite of C5 inhibition, an ADP inhibitor such as Clopidogrel could provide much more benefit. Since the C5 inhibitors bring a great benefit to the patients' treatment outcome and also C5 inhibitors seem to "protect" thrombocytes activation, especially when the complement system is active, it is clear that ADP inhibitors cannot replace the use of complement inhibitors. However, they can be an important addition to effective prevention of thrombosis. Comparing the pathophysiology of PNH with C3G (in C3G the AP is strongly activated, but cytolytic MAC is not built and thrombosis does not occur), confirms our results that it is not the activation of the complement system per se that leads to platelet activation and thrombosis, but the release of ADP (what is the case in PNH and aHUS). [48, 57, 65, 71] One limitation of my results is that we have not investigated how endothelial cells behave to complement activation and complement activation products. Likely endothelial cells will react differently to C3a and C5a or other complement activation products. Yet, all such complement activation does also occur in C3G patients (however without resulting in cytolysis) and C3G patients do not have a prothrombotic phenotype. [65]

4.5 Outlook

Granted, there are certainly more reasons for thrombosis in complement diseases than ADP, as described in the introduction, but ADP seems to play an important role and should not be disregarded. The role of ADP in complement diseases and in thrombosis overall should be examined further. The property of the C5 inhibitors, to somehow protect thrombocytes from activation is also a new finding. What influence the inhibitors have not only on the erythrocytes but also on thrombocytes (like C3 loading) opens up a new field of complement research. In addition, this work mainly examined the effects of the complement system on platelets, while the role of endothelial cells or leucocytes was not highlighted. Certainly, the complement system and the released ADP also have an influence on these cells and the interaction between all these systems is also definitively worth taking a closer look at in future studies.

5 Summary

Thrombosis is a feared condition in the clinic leading to pulmonary embolism, heart attack, phlebothrombosis and many other diseases. Since the cause is suspected to be a dysfunction of the coagulation system, treatment consists of anticoagulant or antiplatelet therapy. The disease paroxysmal nocturnal hemoglobinuria (PNH) differs fundamentally in this regard. Thrombosis also occurs, but the cause lies in the complement system. A genetic defect results in the absence of glycosylphosphatidylinositol (GPI) anchors that bind proteins, including proteins that defend against the formation of the membrane attack complex (MAC; the end of the cascade of the complement system, which destroys cells and bacteria). Thus, these cells fail to protect themselves from complement system, and this leads, among other things, to the formation of the MAC with subsequent hemolysis, thrombosis and end organ failure. The link between the complement system and the prothrombotic state is not yet fully understood. The use of Eculizumab (C5 inhibitor) has brought great success for people suffering from PNH. Inhibition of the terminal pathway of the complement system prevents thrombosis and improves many symptoms of the disease. Since Eculizumab inhibits C5, the cascade continues until its cleaving. Previous in vitro experiments had shown that products of the complement cascade before C5 level, *i.e.* already at C3 level, lead to an activation of thrombocytes. As there is a discrepancy between in vitro experiments and the in vivo experience, the aim of this thesis was to clarify this and to characterise complement signalling pathways that lead to a prothrombotic cell activation, which was mainly tested on thrombocytes. The experiments were carried out using diluted whole blood (drawn in Hirudin tubes) in order to be as close to reality as possible. Different agents were added to the samples, from positive (adenosine diphosphate (ADP)) and negative controls (phosphate-buffered saline (PBS)) to activators and inhibitors of the thrombocytes or the complement system. The activation of the thrombocytes was quantified with P-selectin and Glycoprotein IIb/-IIIa and measured with the flow cytometer. Selected experiments were also performed with the blood from PNH patients to compare the two different CD59 populations. Once the negative and positive control had been established, the different agents were tested. Rabbit red blood cells (rRBC) as an activator of the alternative pathway of the complement system activated the platelets when they were lysed. Hence, platelets activation was blocked by C3 or C5 inhibition, which inhibited rRBC hemolysis, proving that the complement system only leads

to an activation of thrombocytes when lysis of cells (cytolysis) occurs. Since C5 inhibition is sufficient to prevent activation, the responsible element must occur after this step. To test the hypothesis that not the complement system, but in fact the intracellular danger signals released during hemolysis caused by MAC are responsible for the activation of the thrombocytes, the rabbit cells were shattered and ADP inhibitors were used (ADP is known to be released upon hemolysis). They completely prevented thrombocyte activation, no matter if combined with ADP, rRBC or shattered rRBC (which activated the thrombocytes tremendously) or if given to the whole blood samples of PNH patients. Purified C3a and C5a were also administered to the samples at a high concentration, but (unlike suggested in previous studies) had no effect on platelet activation. The experiments with PNH patients produced similar results regarding the ADP inhibitors. The results showed that CD59- cells (which lack the GPI anchors and the GPI-tethered proteins) were much harder to activate than the CD59+ (healthy) cells. Furthermore, C5 inhibition of patients appeared to have induced CD59- platelets to assume a phenotype that hinders thrombocyte activation. Speculatively, it is possible that C3 loading (which has been shown to occur in erythrocytes) also occurs on thrombocytes and interferes with their activation. The results show clearly that the complement fragments have no direct impact on thrombocyte activation. The complement system seems to be a “means to an end” to achieve lysis. The danger signal released by complement-mediated cytolysis responsible for prothrombotic cell activation is ADP. Our data are consistent with the clinical evidence and not with some studies that blame complement agents (rather than subsequent hemolysis) to be responsible for platelet activation. Our findings could be important for the clinical management of complement diseases such as PNH or atypical hemolytic uremic syndrome (aHUS). In diseases in which the complement system is activated or cannot be controlled leading to MAC formation and a following release of danger signals, ADP inhibitors, which are not yet used in these patients, could have much greater benefit than anticoagulants. Of course, treatment with C5 inhibitors is the most promising strategy. However, since some patients experience thrombosis despite its use, antiplatelet therapy could be a valuable adjunct in these patients. Since we have only illuminated the connection between the complement system and a prothrombotic cell activation concerning thrombocytes, a lot more experiments must follow involving further connections and other cells such as endothelial cells or leucocytes before harder conclusions can be drawn.

6 References

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Appendix

The following tables contain various parameters of the PNH patients whose blood was used for the experiments (in 3.2). The parameters were measured in the institute, where the patients are treated.

Table 7: Data from patient 1 received from the institute.

Patient 1	
Therapeutic complement inhibitor	Ravulizumab (C5 inhibitor)
Dose	3300mg / 8w
Other drugs	No
Clone size (date)	1/12/2021
RBC (III+II)	18+5 % of all RBC
Platelet count	165 10^3/µl

Table 8: Data from patient 2 received from the institute.

Patient 2	
Therapeutic complement inhibitor	Eculizumab (C5 inhibition)
Dose	900mg / 14d
Other drugs	Folic acid, iron, Vit D, Vit B12
Clone size (date)	1/2/2021
RBC (III+II)	0.4+1.4 % of all RBC
Platelet count	133 10^3/µl

Table 9: Data from patient 3 received from the institute.

Patient 3	
Therapeutic complement inhibitor	Eculizumab (C5 inhibitor)
Dose	900mg / 14d
Other drugs	Folic acid, Vit D
Clone size (date)	1/19/2021
RBC (III+II)	93+10 % of all RBC
Platelet count	147 10^3/µl

Table 10: Data from patient 4 received from the institute.

Patient 4	
Therapeutic complement inhibitor	Ravulizumab (C5 inhibitor)
Dose	3600mg / 7-8 w
Other drugs	No
Clone size (date)	1/26/2021
RBC (III+II)	55+22 % of all RBC
Platelet count	70 10^3/µl

Table 11: Data from patient 5 received from the institute.

Patient 5	
Therapeutic complement inhibitor	Ravulizumab (C5 inhibitor)
Dose	3600mg / 7 w
Other drugs	No
Clone size (date)	1/26/2021
RBC (III+II)	54+7 % of all RBC
Platelet count	84 10^3/µl

Table 12: Data from patient 6 received from the institute.

Patient 6	
Therapeutic complement inhibitor	APL (study; C3 inhibitor)
Dose	1080 mg
Other drugs	No
Clone size (date)	5/5/2021
RBC (III+II)	91+8 % of all RBC
Platelet count	75 10^3/µl

Table 13: Data from patient 7 received from the institute.

Patient 7	
Therapeutic complement inhibitor	Ravulizumab (C5 inhibitor)
Dose	3300mg / 8w
Other drugs	No
Clone size (date)	12/8/2020
RBC (III+II)	2+67 % of all RBC
Platelet count	197 10^3/µl

Table 14: Data from patient 8 received from the institute.

Patient 8	
Therapeutic complement inhibitor	Ravulizumab (C5 inhibitor)
Dose	3300mg / 8w
Other drugs	Eltrombopag 75mg/d
Clone size (date)	2/9/2021
RBC (III+II)	1+17 % of all RBC
Platelet count	103 10^3/µl

Table 15: Data from patient 9 received from the institute.

Patient 9	
Therapeutic complement inhibitor	No
Dose	No
Other drugs	Eltrombopag 150mg/d Cyclosporin A 175/150mg/d
Clone size (date)	2/9/2021
RBC (III+II)	1.3+0 % of all RBC
Platelet count	17 10^3/µl

Table 16: Data from patient 10 received from the institute.

Patient 10	
Therapeutic complement inhibition	Ravulizumab (C5 inhibitor)
Dose	3500mg / 8w
Other drugs	Cyclosporin A 100mg/d
Clone size (date)	2/16/2021
RBC (III+II)	47+6 % of all RBC
Platelet count	163 10^3/µl

Table 17: Data from patient 11 received from the institute.

Patient 11	
Kind of C5 inhibition	No
Dose	No
Other drugs	Eltrombopag 75mg/d Cyclosporin A 150/175mg/d
Clone size (date)	3/23/2021
RBC (III+II)	0.2+1 % of all RBC
Platelet count	167 10^3/µl

Table 18: Data from patient 12 received from the institute.

Patient 12	
Therapeutic complement inhibition	No
Dose	No
Other drugs	No
Clone size (date)	2/16/2021
RBC (III+II)	56+40 % of all RBC
Platelet count	150 10^3/µl

Table 19: Data from patient 13 received from the institute.

Patient 13	
Kind of C5 inhibition	No
Dose	No
Other drugs	Eltrombopag 150mg/d Cyclosporin A 150/75mg/d
Clone size (date)	12/1/2020
RBC (III+II)	1+0,3 % of all RBC
Platelet count	20 10^3/µl

Table 20: Data from patient 14 received from the institute.

Patient 14	
Therapeutic complement inhibition	Ravulizumab (C5 inhibitor)
Dose	3300mg / 7w
Other drugs	Folic acid, Vit D
Clone size (date)	2/23/2021
RBC (III+II)	55+39 % of all RBC
Platelet count	205 10^3/µl

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Curriculum vitae

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