Aus dem

Institut für Klinische und Experimentelle Transfusionsmedizin der Universität Tübingen

The effect of Syk inhibition in platelets- New therapy outlook for thrombotic and immune thrombocytopenia?

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Toma, Filip

2025

Dekan: Professor Dr. B. Pichler

1. Berichterstatter: Professor Dr. T. Bakchoul
2. Berichterstatter: Professor Dr. D. Rath

Tag der Disputation: 27.02.2025

Table of Contents

List of illustrations

List of figures

Table of abbreviations

αIIbβ3,GPIIb/III, CD41/CD61complex:	Fibrinogen receptor
AAb:	Autoantibody
Abs:	Antibody
ACD:	Acid citrate dextrose
ADP	Adenosine diphosphate
ALK:	Anaplastic lymphoma tyrosine kinase
APC:	Allophycocyanin
ATP:	Adenosine triphosphate
BSA:	Bovine serum albumin
CAM:	Cell adhesion molecule
CD:	Cluster of differentiation
CD32:	FcγRIIA
CD62P:	P-selectin
CLL:	Chronic lymphocytic leukemia
CML:	Chronic myeloid leukemia
COVID-19:	Coronavirus disease 2019
CRP:	Collagen-related peptide
CytC	Cytochrome c
DAG:	Diacylglycerol
DAG:	1,2-diacylglycerol

DMSO:	Dimethyl sulfoxide
ECL:	Erythrina crista Galli lectin
ER:	Endoplasmic reticulum
Fab region:	Fragment antigen-binding region
FACS:	Flow Cytometry
FBG:	Fibrinogen
Fc region:	Fragment crystallizable region
FcR	Fc receptor
FITC:	Fluorescein isothiocyanate
FSC:	Forward scatter
Fv:	Variable region
GMP:	Guanosine monophosphate
GP:	Glycoprotein
GPCRs:	G-protein-coupled receptors
HIT:	Heparin-induced thrombotic thrombocytopenia
Ig	Immunoglobulin
IKET:	Institute of Transfusion Medicine Tübingen
IP3:	Inositol 1,4,5-triphosphate
ITAM:	Immunoreceptor tyrosine-based activation motif
ITP:	Immune thrombocytopenia
LAT:	Linker for activation of T cells
MK:	Megakaryocyte
mRNA:	Messenger ribonucleic acid

Neg.:	Negative
P2Y1:	Purinergic receptor P2Y1
PAC-1:	First procaspase activating compound
PAR:	Protease-activated receptor
PF4:	Platelet factor 4
PI3K:	Phosphoinositide 3-kinase
PI3-K:	phosphoinositide 3-kinases
PIP2:	Phosphatidylinositol bisphosphate
PKC:	Protein kinase C
PLC:	Phospholipase C
PLTs:	Platelets
Positive:	Positive
PRP:	Platelet rich plasma
PS:	Phosphatidylserine
PSGL-1:	P-selectin glycoprotein ligand-1
ROS:	Reactive oxygen species
RT:	Room temperature
RyR:	Ryanodine receptor
SAC:	Surface area covered
SARS-COV2:	Severe acute respiratory syndrome coronavirus 2
SD:	Standard deviation
SEM:	Standard error of the mean
SERCA:	Sarco/endoplasmic reticulum Ca^{2+} ATPase

SH2:	Src Homology 2
TF:	Tissue factor
TMRE:	Tetramethylrhodamine, ethyl ester
TRAP:	Thrombin receptor-activating peptide
TXA2:	Thromboxane A_2
VITT:	Vaccine-induced thrombotic thrombocytopenia
Vs.:	Versus
vWF:	von Willebrand factor
WHO:	World health organization
wPLTs:	Washed platelets
ΔΨm:	Mitochondrial transmembrane potential

1 Introduction

1.1 The role of platelets in thrombosis and haemostasis.

Haemostasis is defined as the product of physiological processes enabling the arrest of bleeding. Coagulation-activating and inhibitory mechanisms maintain a tightly regulated balance between blood circulation and clot formation[1];[2]. Platelets occupy a central and diverse role in the regulation of human haemostasis [3].

Platelets are anucleate cells with a diameter of 2–3 µm that originate from the myeloid lineage and directly derived from megakaryocytes [4]. Platelets have already been identified as “blood particles with roles in thrombosis and blood coagulation" in the late 1800s [5]. Following their formation in the bone marrow or lungs [6], they circulate the vascular system, regulating hemostasis for up to an average of nine days[7].

Platelets, especially their count [8] and function [9] play a role to the physiological functioning of hemostasis as they are effectors in both extrinsic and intrinsic pathway of blood coagulation [3]. A disturbance in the well-adjusted balance of hemostasis inevitably lead to negative patient outcome. The excessive activation of intravascular coagulation can lead to the formation of blood clots and thrombotic events [10] constituting one of the leading causes of death in industrialized nations[11]. Insufficient coagulation on the other hand can result in severe bleeding events [12], impaired wound healing [13] and disturbed inflammatory processes [14]. Platelet disorders are therefore a major concern for both patients and treating physicians.

1.1.1 Mechanisms and significance of platelet activation

Despite their anucleate nature, platelets fulfil a wide range of functions and are metabolically active, disposing of mitochondria, endoplasmic reticulum, Golgi apparatus and mRNA, which

allow them to express surface receptors, adhesion molecules and secrete granules in order to fulfil their function as initiators and amplifiers of the coagulation cascade [15]. Chemical and physical interactions between platelets and their environment can lead to their activation, changing platelets from their resting discoid shape to a star-like shape with spreading filopodia and inducing the exposure of procoagulant surface molecules, secretion of procoagulant granules, and effector molecules. Most prominent platelet activators are the interaction with adhesion proteins of the extracellular matrix, soluble platelet agonists and shear stress via numerous platelet receptors and specific signal transduction [16]. Platelet activation can ultimately lead to the creation of an autocrine and paracrine positive feedback loop, resulting in the recruitment of other activated platelets interconnected with one another through fibrin bridges, capable of thrombus stabilization. Most platelet activators trigger the phosphorylation of Phospholipase C (PLC) [17]. The activation of PLC, further discussed below in "The role of the Fcγ receptor IIA in platelets", leads to the elevation of cytoplasmatic calcium levels initiating "inside out signaling" and the integrin signaling of αIIbβ3 also known as fibrinogen receptor, glycoprotein GPIIb/III or CD41/CD61 complex [18], key to platelet aggregation [19]; furthermore, platelet activation entails the release of platelet activators and procoagulant mediators such as thromboxane A2 (TXA2), adenosine triphosphate ATP, platelet factor 4 (PF4) and serotonin, as well as ADP, a central amplifier of autocrine platelet activation via G protein-coupled receptors (GPCRs) [20]. Degranulation of α-granules containing fibrinogen and factor V [21] also leads to the expression of P-selectin on the platelet surface, crucial to cell-cell interactions between platelets and lymphatic cells, discussed in detail below [22].

The following section introduces the most important activators and activation pathways to better understand their roles and effects. Since interactions of platelets with their environment highly diverse, most crucial mechanisms will be introduced without claim to completeness.

1.1.1.1 Platelet activation by collagen and von Willebrand factor (vWF)

A central initiator of platelet activation and primary hemostasis is the platelets' capacity to bind to the extracellular matrix within damaged blood vessels [23]. When the integrity of the

endothelial cell layer is damaged, von Willebrand factor (vWF) present in blood plasma binds to subendothelial collagen [24]. Under shear stress [25], platelets can then bind either to the formed link between vWF and collagen through the platelet glycoprotein GPIb-IX or directly to collagen via various identified collagen receptors, such as the GPVI receptor[26]. Collagen/GPVI-mediated platelet activation, as well as von Willebrand factor/GPIb-IX–mediated platelet activation, occurs after the activation of the immunoreceptor tyrosine-based activation motif (ITAM) and tyrosine kinase Syk, leading to the activation of phospholipase Cγ2 (PLCγ2), resulting in granule secretion and integrin activation [27]. ITAM signaling will be further discussed in "The role of the Fcγ receptor IIA in platelets".

1.1.1.2 Platelet activation through G protein-coupled receptors (GPCRs)

1.1.1.2.1 Thrombin

Thrombin, also known as coagulation factor II, is produced after the cleavage of prothrombin by factor X (Xa), converts fibrinogen into fibrin [28]. Thrombin binds to the platelet surface via the G-protein– coupled protease-activated receptors (PAR) PAR-1, PAR-3, or PAR-4, which in turn are activated by the proteolytic activity of thrombin [29]. The activation of the PAR receptor mediates the phosphorylation of phospholipase Cβ (PLCβ) after the activation of Gq and G12/13 [30] and partially Gi [31] proteins. Thereupon follows granule secretion and integrin activation through inside-out signaling [32]. Thrombin receptor-activating peptide 6 (TRAP-6) binds specifically to the PAR-1 receptor, making it an ideal synthetic thrombin agonist for in-vitro studies of thrombin-platelet interactions. It will serve as a synthetic thrombin agonist in our study [33].

1.1.1.2.2 ADP

ADP is stored in platelets in dense granules and is released upon activation [34]. ADP can dock to platelets over the Gi-coupled P2Y12 receptor and the Gq coupled-P2Y1 receptor [35]. The activation of Gi-coupled P2Y12 is followed by the activation of the Phosphoinositide 3-kinase (PI3K), supporting integrin activation [36]. Stimulation of ADP of the Gq-coupled P2Y1 receptor

is followed by the activation of PLCβ, amplifying degranulation and glycoprotein GPIIb/III surface exposure through integrin signaling [32]. Gq signaling is also discussed to play a part in platelet shape change through the mobilization of calcium [37].

1.1.1.3 Common activation markers

1.1.1.3.1 P-selectin

In 1984 McEver et al. described how the monoclonal antibody S12 recognized a membrane protein found on thrombin activated platelets. In these studies, activated platelets expressed the protein 50 times more than their inactivated counterparts. The membrane protein was first described as GMP-140 after its molecular weight of 140 kDa [38]. GMP-150, now commonly known as P-selectin (CD62p) was later identified on alpha granules of platelets and was found to be expressed on the platelets surface upon activation and degranulation [22]. P-selectin was also discovered on vascular endothelia where it is synthesized and stored in Weibel-Palade-bodies [39], and facilitates the adhesion of neutrophiles via the Syk dependent [40] P-selectin glycoprotein ligand-1 (PSGL-1)[41]. The important role of P-selectin as a signaling receptor in cell-cell interaction, vascular inflammation and platelet adhesion was elucidated and reviewed in the works of Kansas in 1996 [42]. P-selectin assumes the role of a cell adhesion molecule (CAM). After resting on the membrane of alpha-granules, P-selectin is brought to the external platelet surface. The recognition of P-selectin to monocytes and neutrophils is of central importance for inflammation and thrombus formation [43]. In monocytes binding of P- selectin onto PSGL-1 increases cytokine production [44] whereas in neutrophils it triggers NETosis. NETosis involves the projection of extracellular traps composed of neutrophil DNA, which favors platelet aggregation and activates the intrinsic coagulation cascade through factor XII [45]. Additionally, stimulation of neutrophils through P-selectin induces the secretion of tissue factor (TF), which in turn enhances fibrin formation [46]. Due to the profound impact in cell-cell interaction in vascular inflammation and thrombus formation, P-selectin has recently been targeted with crizanlizumab for the therapy of sickle cell disease [47]. P-selectin (CD62p) has established itself as a crucial player in platelet aggregation and as a reliable activation marker.

1.1.1.3.2 PAC-1

PAC-1, Is a specific activation marker for the activated fibrinogen receptor (GPIIb/IIIa) [48] after αIIbβ3 “inside out signaling”.

1.1.2 The importance of platelet apoptosis

Apoptosis, also known as programmed cell death, serves as a regulator in haemostasis by managing platelet number. Apoptotic platelets undergo structural changes that facilitate their clearance by phagocytic cells, primarily macrophages, in the spleen and liver. This process prevents the accumulation of aged or dysfunctional platelets[49]. However altered immune responses, may trigger excessive platelet apoptosis.[50]

1.1.2.1 Common markers of apoptosis

Phosphatidylserine (PS) is a phospholipid normally found on the inner side of the cell membrane. During apoptosis, Phosphatidylserine translocates from the inner to the outer membrane surface[51] . Phosphatidylserine exposure can be fluorescently labeled with Annexin V-fluorescein isothiocyanate.

The positively charged dye Tetramethylrhodamine ethyl ester (TMRE) detects mitochondrial transmembrane potential (ΔΨm) and marks the population of platelets with sustained metabolic activity and intact, viable mitochondria. TMRE will help us to differentiate alive from apoptotic cells in our study [52].

1.1.3 The notion of the "procoagulant platelet"

Central to the integrity of hemostasis is platelet activation initiated by adhesion to von Willebrand factor and collagen over GPXI and subsequent platelet aggregation over GP IIb/IIIa receptor interconnecting platelets with one another through fibrinogen bridges.

Platelet's role within thrombus formation, however, was found to extend far beyond a single mechanism. Heemskerk JW. et al. described in 1997 platelets taking on the shape of "balloon-like structures" after interaction with collagen and that, unlike platelets activated directly through fibrinogen spreading star-like shape with spreading filopodia and expressing P-Selectin, these platelets additionally expressed phosphatidylserine (PS) at their surface. The described "balloon like platelets" were found to strongly engage into thrombus formation as PS exposure increases platelet surface area, offering an opportunity for an increased activity of the intrinsic tenase and prothrombinase at the platelets surface and hence facilitating thrombin generation [53]. In 2001 Hess et al. characterized this subgroup of platelets through electron microscopy as "procoagulant platelet balloons" given their shape and size of approximately 5 μm [54]. The first images of Hess's et al. displayed the "procoagulant platelet balloons" as bloated and presenting a scarcity of cell organelles [54]. Some similarities between the PS positive platelets and platelets undergoing a process resembling necrosis became apparent [55]. Indeed, mitochondrial dysfunction and loss of membrane integrity, two common indicators of cell-necrosis [56], have been observed in platelets after over-stimulation and sustained elevation of cytosolic calcium levels [57]. In this sense, platelet necrosis occurring simultaneously to platelet activation was discussed to be a functional response [58]. 2017, Agbani and Poole proposed the term "procoagulant platelets" for phosphatidylserine (PS) and P-selectin (CD62P) expressing platelets that are facilitating thrombin generation [59]. By considering and reviewing recent findings, it becomes apparent that procoagulant platelets contribute to physiological hemostasis as well as thrombi formation and thromboinflammation [60].

1.2 The role of the Fcγ receptor IIA and Syk in platelets

Fc receptors play an important role in antigen recognition. They are commonly found lymphatic cells such as B-Lymphocytes, NK-Cells or neutrophils and take on a central role within the immune system [61]. The receptors can bind to the Fc-fragment of antibodies, facilitating antigen elimination via phagocytosis and cell-mediated cytotoxicity. Fc receptors differ from one another by their affinity to specific antibodies and immune complexes. High-affinity FcRs, like the FcγRI on activated neutrophils, can bind single antibodies, while low-affinity FcRs tend to bind immune complexes consisting of multiple antibodies [62].

Of great interest for our study is the FcγRIIa, expressed in a wide array of immune cells, including neutrophils, macrophages, monocytes, and Langerhans Cells [63]. It is also the only Fc receptor found on the platelet surface [64]. The FcγRIIA receptor is one of the low-affinity receptors and binds poorly to monomeric IgG antibodies but has a great affinity to immunoglobulin G (IgG) complexes [65].

The FcγRIIA is a single-chain receptor with an extracellular, a transmembrane and an intracellular part. The extracellular domain consists of two IgG like domains responsible for the binding IgG Immune complex through its Fc portion [66].

The receptor can recognize various immune complexes, including the H/PF4-IgG antibody complexes in the presence of heparin in HITT [67], IgG-like structures after infection with SARS-CoV-2 [68], and VITT–IgG/PF4 complexes [69];[70].

1.2.1 FcγRIIA signalling pathway and in-vitro FcγRIIA mediated platelet activation

The signaling pathway after immunoglobulin or immune complex recognition by the FcγRIIA receptor is similar to that of GPVI stimulation by collagen [71] or by that GPIb-IX stimulation by vWF [72] and is mediated via the intracellularly located immunoreceptor tyrosine-based

activation motif (ITAM) [73]. Activation of FcγRIIA can be achieved by specific receptor cross-linking with a FcγRIIA (CD32) antibody and the F(ab')2 fragment of a secondary antibody in vitro [74]. Receptor activation occurs following the cross-link formation between the CD32 monoclonal antibody initially binding to the membrane-side IgG-like domain of the receptor with its variable region (Fv) and the F(ab')2 fragment of the secondary antibody [63]. Upon activation of the FcγRIIA, tyrosine-phosphorylation of ITAM is rapidly initiated by receptor-associated Src-tyrosine kinases. The FcγRIIA associated tyrosine kinase p76/Syk auto phosphorylates and docks through its Src Homology 2 domains (SH2) to phosphorylated ITAM [75]. The Activation of Syk is key and triggers the activation of signaling proteins such as the phosphoinositide 3-kinases (PI3-K), the adaptor protein LAT (linker for activation of T cells), and of central importance: phospholipase Cγ2 (PLCγ2)[76]. PLCγ2 in turn activates the PIP2, IP3, DAG signaling cascade. PLCγ2 cleaves phosphatidylinositol bisphosphate (PIP2) into second messenger 1,2-diacylglycerol (DAG) activating protein kinase C (PKC), and inositol 1,4,5-triphosphate (IP3) which releases Ca2+ from the endoplasmic reticulum [77]. The rapid Ca2+ influx into the cytoplasm and the activation of the protein kinase C triggers integrin αIIbβ3 inside-out signaling followed by platelet shape change, exposure of P-selectin and secretion of granule contents such as ADP resulting in platelet aggregation [19].

1.2.2 The role of the tyrosine kinase Syk

Syk is a spleen tyrosine kinase that takes on a central role in immune receptor signaling as it is essential to Fc-receptors and B-cell receptor signaling [78]. It is found in a multitude of immune cells such as B- and T-lymphocytes neutrophils, macrophages but also platelets [79] where it supports Fc-signal transduction by binding its two Src homology 2 (SH2) domains to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) [80] and subsequent integrin-signaling. Furthermore, Syk is key to signaling by P-selectin glycoprotein ligand 1 (PSGL-1) [40], favoring the adhesion of leukocytes to P-selectin on activated platelets or endothelia [39], enhancing thrombus formation through cell-cell interactions such as NETosis [45].

1.2.2.1 Syk inhibition

Given the crucial role of Syk in antibody-mediated cellular responses and intracellular signaling of immune cells, Syk inhibition has been considered as a therapeutic approach in a multitude of inflammatory, autoimmune and neoplastic disorders. In particular, the transduction of activating signals within B-cells has been targeted for therapeutic intention. Syk inhibitors are mostly in clinical development, such as entospletinib for the treatment of chronic lymphocytic leukemia (CML) [81] or cerdulatinib for the treatment of therapy-resistant peripheral and cutaneous T-Cell Lymphoma [82]. In platelets, Syk inhibition prevents platelet activation via GPVI and FcγRIIA by inhibiting the ITAM receptor complex but not via G-protein coupled receptors such as ADP or thrombin receptors [79].

1.2.2.1.1 Syk inhibitor PRT-060318

PRT-060318, a novel Syk inhibitor designed for research, was shown to prevent heparin-induced thrombocytopenia and thrombosis in a transgenic mouse model [83]. In these studies, a concentration of 50 nM of PRT-060318 was found to inhibit the activity of Syk by 92%, while other kinases maintained more than 70% of their activity. Furthermore, PRT-060318 was found to inhibit chemokine secretion by Chronic lymphocytic leukemia (CLL) cells after stimulation with anti-IgM [84]. In consideration of the high specificity of the inhibitor and of the findings of Reilly et al. and Hoellenriegel et al., Syk inhibitor PRT-060318 was chosen to serve as a control to R406 in our investigations.

1.2.2.1.2 Fostamatinib (R406) in the treatment of immune thrombocytopenia.

In 2018, Syk inhibitor Fostamatinib was approved as a novel second line therapy for ITP patients by the US Food and Drug Administration (FDA) [85,86]. The response to therapy, however, largely depends on the individual state of the patient [87]. Ongoing research aims to improve our understanding of the various factors that affect drug efficacy and to develop more personalized therapy approaches. Moreover, Fostamatinib and its active metabolite R406 have been tried and tested for a great variety of inflammatory, autoimmune and neoplastic

disorders and underwent clinical trials for rheumatoid arthritis [88], non-Hodgkin lymphoma and chronic lymphocytic leukemia [89], showing promising results. Recently, as thrombotic events emerged as a significant cause of mortality after infection with SARS-CoV-2, fostamatinib has been shown to possibly prevent FcγRIIA dependent platelet activation through COVID-19 plasma [90].

1.3 Vaccine-induced thrombotic thrombocytopenia

The continuous progression of COVID-19 pandemic has led to considerable suffering and loss of human lives around the world since 2019 [91]. The development and distribution of the first vaccines against SARS-CoV2 was, however, a major step forward in the fight against the global pandemic [91]. Although vaccines that have been developed, and administered within the European Union are considered safe, severe adverse events were observed. In some rare instances, patients having received the adenoviral-based ChAdOx1 nCoV-19 vaccine (Vaxzevria: University of Oxford/AstraZeneca) reportedly developed severe cases of thrombocytopenia or thrombosis within 1 to 4 weeks following the vaccination [92]. The critical disease was later collectively defined as vaccine-induced thrombotic thrombocytopenia (VITT) [93]. In some cases, VITT lead to the thrombotic obstruction of cerebral venous sinus or to pulmonary embolisms with serious health implications for the patients [94]. Overall, VITT was attributed a mortality rate of 30% and became the focus of numerous studies pursuing an in-depth understanding of the disorder [95].

1.3.1 Pathophysiology of vaccine-induced thrombotic thrombocytopenia

First findings suggest similarities between vaccine-induced thrombotic thrombocytopenia and heparin-induced thrombotic thrombocytopenia (HIT) [95]. The clinical picture of HIT is characterized by occurrences of thrombocytopenia and thrombotic events in patients having received anti-coagulant treatment with heparin or heparin derivates, even without previous exposure to heparin. HIT has been found to be mediated by antibodies targeting platelet factor 4 (PF4) [96]. Together, heparin and PF4 form immune complexes thus activating platelets

through the FcγRIIA [97]. Similar antibodies targeting PF4 have been detected in patients suffering from VITT [98]. The discovery that VITT antibodies (VITT-Abs) induce procoagulant platelet formation via FcγRIIA and that this process can be prevented by blocking FcγRIIA with mAb IV.3 provides strong evidence that VITT antibody procoagulant platelet formation is mediated by FcγRIIA [69].

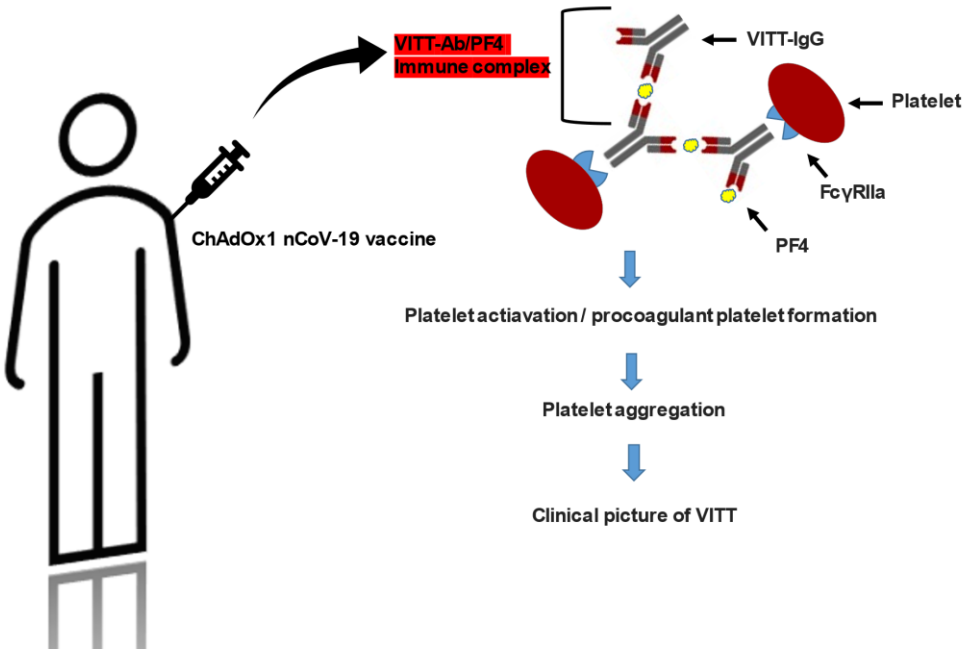

Illustration 1: Interaction between PF4, VITT-IgG and FcγRIIA (Adapted from Singh et al. 2022).

In the corresponding cases, vaccination with ChAdOx1 nCoV-19 patients developed platelet-activating antiplatelet factor 4 (PF4) antibodies thus forming VITT-ab/PF4 immune complexes that are recognized by FcγRIIA. FcγRIIA mediated platelet activation and procoagulant platelet formation favors platelet aggregation and the clinical picture of VITT[99].

These findings suggest that the FcγRIIA on platelets is of crucial importance in the pathophysiology of VITT, as it takes on the role of docking site for VITT- Ab/PF4 immune complexes and subsequently mediates platelet dysfunction through FcR activation and signaling.

1.4 Immune thrombocytopenia

Idiopathic immune thrombocytopenia (ITP), also known as morbus Werlhof, is a disorder characterized by low platelet count (below $100x10^9$/L), disturbed haemostasis and the loss of immune tolerance toward platelet precursor cells: megakaryocytes [100]. ITP has been found to be the most common cause of bleeding tendency in children [101]. The clinical picture of ITP as well as patients' response to different treatments are very heterogeneous suggesting that ITP is rather a group of disorders sharing common characteristics.

ITP patients have been found to present autoantibodies targeting glycoproteins on the platelets surface [102,103]. Targeted glycoproteins such as GPIIb/IIa and GPIb/IX were also identified on megakaryocytes resulting in impaired thrombopoiesis in ITP [104]. A decreased platelet lifespan has been observed due to excessive platelet clearance by lymphatic cells in spleen and liver through Fc-FcγR interaction between autoantibody, opsonized platelet and spleen macrophage [105]. ITP has also been observed in patients with other underlying diseases such as neoproliferative disorders, AIDS or hepatitis and was termed secondary ITP [106]. Secondary ITP will not be discussed here, and idiopathic ITP will be the sole focus of the study. The objectives of ITP treatment are to intervene in the case of an acute severe bleeding and to prevent future bleeding events [107].

1.4.1 The importance of platelet desialylation in ITP

While the targeting of GPIIb/IIa and GPIb/IX on platelets and megakaryocyte surfaces, and subsequent cell clearing in the spleen after recognition of the FC-section of the bound antibody by macrophages FcγR is crucial to the pathophysiology of ITP, some patients remained unresponsive to therapies targeting FcγR pathways or splenectomy [108]. June Li et al. suggested that anti-GPIbα antibodies seem to induce Fc-independent platelet activation, platelet surface changes, and platelet clearance in the liver via the hepatocyte Ashwell–Morell receptors[109]. After the recognition of GPIb/IX, the autoantibody (AAb) induced the release of sialidase from α granules, which in turn cleaved sialic acid at the end of glycoproteins,

exposing ß-galactose and N-acetyl glucosamine in a process characterized as desialylation[109]. Marini et al. showed that not only anti-GPIb/IX AAb, but also anti-GPIIb/IIIa AAb, were capable of triggering platelet desialylation. Desialylation of platelets and megakaryocytes glycoproteins was found to interfere with the cell's interactions with the extracellular matrix, leading to impaired platelet adhesion and megakaryocyte differentiation. In the presence of desialylation autoantibodies, a significant loss of platelet function, particularly in the ability to adhere to fibrinogen and vWF, can be observed. Furthermore, decreased platelet lifespan and a low median platelet count have been observed in patients presenting with desialylating antibodies[110]. These findings strongly suggest that the presence of desialylating antibodies affects the clinical picture in ITP. Interestingly, in vitro crosslinking of FcγRIIA triggered platelet desialylation, whereas blocking the receptor using the monoclonal antibody IV.3 strongly inhibited desialylation conferred by IgG anti-GPIIb/IIIa AAb in ITP patients[110]. The FcγRIIA is therefore discussed to be crucial to autoantibody mediated platelet impairment and clearance in ITP.

1.5 Aims of the Study

The balance within the tightly regulated balance of hemostasis can be disturbed by the presence of antibodies that induce extensive platelet clearance, for example in immune thrombocytopenia [102] or immune complexes triggering procoagulant platelet formation and increased clot formation, as occurs in Vaccine-induced thrombotic thrombocytopenia[98]. The FcγRIIA is of crucial importance in physiological platelet clearance and platelet function [65]. The tyrosine kinase Syk finds itself in the downstream signaling of the FcγRIIA [80]. We want to investigate if Syk inhibition can effectively alter the biological changes conferred by antibodies in platelets. It is important to explore if Syk inhibition can effectively prevent excessive FcγRIIA mediated platelet activation, apoptosis and procoagulant platelets formation in disease while essential platelet functions remain unaffected.

To understand the underlying mechanism of FcγRIIA conveyed platelet dysfunction and the potential benefit of Syk inhibition, we investigate VITT and ITP, two platelet disorders with

drastically different pathogenesis. Both conditions are however characterized by biological changes in platelets mediated through the FcγRIIA. We will explore the complex changes that occur on platelets conveyed by autoantibodies in VITT and ITP and hope to gain a close look upon the effects of Syk inhibition.

During the COVID-19 pandemic, our focus was drawn towards understanding VITT considering new findings in the field of immunohematology. However, at that time, the potential involvement of the tyrosine kinase Syk remained largely unexplored. By studying the interaction between VITT antibody and platelets, with and without Syk inhibition, we hope to better understand the effect of antibody-mediated platelet dysfunction and of the complex mechanisms responsible for the clinical picture of VITT.

Antibody-mediated platelet desialylation, a novel effector function of autoantibodies, has been discovered to severely impair platelet function and lifespan via involvement of Fc gamma Receptor IIA in cases of idiopathic immune thrombocytopenia[110]. In this proposal, we hypothesize that Syk inhibition can effectively alter autoantibody-mediated desialylation in ITP. Giving insight on the impact of Syk inhibition on antibody mediated platelet desialylation we hope to extend our understanding of the underlaying mechanism of this biological change and unravel potential therapeutic benefits of Syk inhibition.

In summary, the aim of this study is to shed light on the crucial role of the tyrosine kinase Syk on platelet function and explore the effect Syk inhibition for relevant haematologic diseases. Our goal is to gain a better understanding of the broad range of antibody-mediated biological effects on platelets and to provide a new perspective on potential therapies for the future.

2 Materials and Methods

2.1 Materials

2.1.1 Instruments and materials

Name	Manufacturer
100 kDa-pore sized centrifugal filters	Merck Millipore (Cork, Ireland)
BioFlux 200	Fluxion Biosciences (California USA)
Cell-Dyn Ruby hematological analyzer	Abbott (Illinois, USA)
Eppendorf-Cups 1.5; 2 ml	Eppendorf (Hamburg, Germany)
Flow cytometer tubes	Sarstedt, (Nümbrecht, Germany)
Flow-cytometer Navios	Beckman Coulter (USA)
Freezer (-80 °C)	Ilshin Europe (Ede, Netherlands)
Heraeus 37 °C incubator	Thermo Scientific (Waltham, USA)
Melon™ Gel IgG Purification Kit	Thermo Fischer (Waltham, USA)
Microscope Olympus IX73	Olympus GmbH (Hamburg, Germany)
Mikro 22R tabletop centrifuge	Hettich (Tuttlingen, Germany)
NanoDrop instrument	Thermo Fisher Scientific (Waltham, MA)
Nitril gloves	Paul Hartmann (Heidenheim, Germany)
Pasteur pipet 5 ml	Carl Roth (Karlsruhe, Germany)

Refrigerator (4 °C)	Siemens (München, Germany)
Research Plus adjustable volume pipets 10; 20; 100; 200; 1000 µl	Eppendorf AG (Hamburg, Germany)
Rotator	Neolab (Heidelberg, Germany)
Rotina 46 R Centrifuge	Hettich (Tuttlingen, Germany)
SevenCompact pH meter S210	Mettler-Toledo (Greifensee, Germany)
Shaker	Philips (Brussels, Belgium)
Single-use syringe Injekt-F 1ml	Braun (Melsungen, Germany)
S-Monovette ACD-A 8,5 ml	Sarstedt (Nümbrecht, Germany)
S-Monovette EDTA 7.5ml	Sarstedt (Nümbrecht, Germany)
S-Monovette Citrat 3,2%	
Sterican 27 g. x 1,5'' needle	Braun (Melsungen, Germany)
Sysmex XN 9000	Sysmex, Norderstedt (Germany)
Sysmex XN 9000 cell counter	Sysmex (Norderstedt, Germany)
TipOne XL Graduated Tip 10. 20; 200; 1000µl	Starlab (Hamburg, Germany)
TubeOne microcentrifuge	Starlab (Hamburg, Germany)
Tubes (15,50 ml)	Greiner bio-one (Frickenhausen, Germany)
Vortexer, Reax-Top	Heidolph, Schwabach (Germany)

2.1.2 Chemicals

Name	Manufacturer
a2-3,6,8,9-Neuraminidase	Merck (Darmstadt, Germany)
ADP	Sigma-Aldrich (Darmstadt, Germany)
Collagen	Collagen Horn Takeda (Linz, Austria)
Control immunoglobulins	Intratect (Biotest AG, Germany)
Ionomycin	Sigma-Aldrich (Darmstadt, Germany)
TRAP-6	Sigma-Aldrich (Darmstadt, Germany)

2.1.2.1 SYK-inhibitors

Name	Manufacturer
PRT-060318	Hycultec (Beutelsbach, Germany)
R406	InvivoGen (California, USA)

2.1.2.2 Antibodies

Name	Manufacturer
Annexin V-fluorescein isothiocyanate (Annexin V)	Immunotools (Frsoyhte, Germany)
Anti-CD32 (mAb IV.3)	Stemcell technologies (Vancouver, Canada)
Calcein-FITC	Thermo Fisher Scientific (Waltham, MA)
CD32 monoclonal antibody AT-10	Invitrogen (Carlsbad, USA)
CD62P antihuman	BD Biosciences (Heidelberg, Germany)
F(ab2) goat ani mouse IgG (H+L) cross absorbed secondary antibody	Invitrogen (Carlsbad, USA)
FITC-labelled ricinus communis agglutinin (RCA)	Vector Laboratories (Burlingame, USA)
Goat anti-mouse IgG	Jackson Immuno Research (West Grove, USA)
PAC-1 Monoclonal Antibody (PAC-1), FITC	Thermo Fisher (Waltham, USA).
PE-labelled Tetramethylrhodamine, ethyl ester (TMRE)	Abcam (United Kingdom)

2.1.2.3 Buffer and solutions

Name	Manufacturer, Composition
Bovine serum albumin (BSA)	Kedrion (Barga, Italy)
Hank´s balanced salt solution, Washed platelet resuspension buffer	Carl-Roth (Karlsruhe, Germany) 137 mM NaCl, 1.25 mM CaCl2, 5.5 mM glucose
Phosphate-buffered saline (PBS)	Biochrom (Berlin, Germany)
Washing solution for washed platelets	VWR international (Fontenay sous Bois, France) 10 mM TRIS, 0.9% NaCl, 1 mM $CaCl_2$, pH 7.4
IgG sample buffer	Invitrogen (Carlsbad, USA)

2.2 Methods

2.2.1 Detection of platelet activation, apoptotic markers and procoagulant platelets markers

2.2.1.1 Preparation of washed platelets

Venous cubital blood was withdrawn from healthy donors of Blood type O after obtaining written consent at the Centre for Clinical Transfusion Medicine Tübingen. The blood was collected in vacuum tubes containing anticoagulant citrate dextrose A (ACD-A). Before proceeding, the whole blood samples were allowed to rest at 37° Celsius (°C) for 30 minutes in order to minimize any pre-activation of platelets. The blood samples were then centrifuged at 120 g for 20 minutes at room temperature (RT) without a brake. This allowed the separation of platelet-rich plasma (PRP), forming the upper layer of the blood sample. The PRP was collected using a Pasteur pipette and placed into separate tubes containing 5 µl of apyrase and 333 µl ACD-A per ml of PRP. The PRP was then placed into the centrifuge (650 g, 7 min, RT, no brake) allowing the isolation of platelets (PLTs), now gathered at the bottom of the tube. The platelets were left to rest for 15 minutes in washing solution containing 10 mM TRIS, 0.9% NaCl, 1 mM CaCl2, pH 7.4 (VWR international) before a final centrifugation (650 g, 7 min, RT, no brake). The washing solution was then discarded and replaced with 2 ml of resuspension buffer which consisted of either Phosphate-buffered saline(Biochrom) or Hank´s balanced salt solution, Washed platelet (Carl-Roth) considering the experiments requirements. The platelets were then quantified via cell the counter Sysmex XN 9000 (Sysmex, Norderstedt, Germany) and adjusted to 300 $x10^3$ PLTs /µl, ensuring even samples and reliable results in further investigations.

For experiments using flow cytometer, one sample per subject was stained with PC5-labelled mouse anti-human CD41 for 30 minutes at room temperature to assess platelet viability and to set the forward and side scatter gate.

2.2.1.2 FcγRIIA activation assay

In order to achieve FcγRIIA conveyed activation of the washed platelets a cross-linking assay was established. Crosslinking is an essential tool in the study of the FcγRIIA in platelets.[63]. The crosslinking of FcγRIIA (CD32) antibody with $F(ab')_2$ fragments of a secondary antibody is expected to induce FcγRIIA platelet activation [111]

Washed platelets were first incubated for 2 minutes at room temperature with 5 µg/mL of CD32 antibody, clone AT-10, followed by an incubation with 10 µg/mL of F(ab')2 for 5 minutes at room temperature.

As a positive control it was important to explore immune receptor independent platelet activators. This is the case of Adenosine diphosphate (ADP) activating platelets over the P2Y1 receptor[112] and TRAP-6 over PAR-1 (Thrombin-receptor)[113]. Washed platelets were incubated with ADP or TRAP-6 with the respective concentration of 1 µg/mL time at room temperature was used as platelet activator instead of crosslinking the FcγRIIA. The concentration of ADP was determined through titration experiments conducted in-house by laboratory personnel.

As a negative control, monoclonal antibody IV.3 in its monomeric form in order to inhibit ligand binding to FcγRIIA and to block FcγRIIA function[111]. Washed platelets were pre-incubated for 30 minutes at 37 °C with 1 µg/mL of monoclonal antibody IV.3 before carrying out the crosslinking of the receptor, thus indirectly blocking the receptor on the platelets surface.

2.2.1.3 Characterization of platelet activation

Platelet activation was measured via Flow cytometry, determining P-selectin (CD62P) and PAC-1 on the platelets surface. Washed platelets were stained with 1 µg/mL of APC-labeled mouse Anti-Human CD62P antibody (BD Biosciences) or with 1 µg/mL FITC-labeled PAC-1 Monoclonal Antibody (Thermo Fisher, Waltham, USA) for 30 minutes at room temperature in the dark. Measurements were performed immediately after on the Flow-cytometer Navios

(Beckmann coulter, USA) after a calibration was performed according to manufacturer's specification.

2.2.1.4 Characterization of apoptotic markers in platelets

Platelet apoptosis markers Phosphatidylserine (PS) on the platelets surface and tetramethylrhodamine ethyl ester (TMRE) labeling viable mitochondria were measured in flow cytometry.

To assess Phosphatidylserine expression An Annexin V-fluorescein isothiocyanate (Annexin V) staining (Immunotools, Friesoythe, Germany) was performed. 100 µl of the washed platelet suspension collected as described previously were resuspended in Hank´s balanced salt solution (Carl-Roth, Karlsruhe, Germany) and stained with 5 µl/ml of Annexin V-FITC and incubated for 15 minutes at room temperature in the dark. The stained platelets were then analysed in flow cytometry. The platelets were gated based on their forward and side scatter characteristics, and 10,000 events were collected for each sample.

TMRE staining was performed by adding 2 µM of PE-labelled Tetramethylrhodamine, ethyl ester (TMRE) (Abcam, Cambridge, UK) to the washed platelets and incubating for 15 minutes at room temperature in the dark. The platelets were gated based on their forward and side scatter characteristics, and 10,000 events were collected for each sample. The level of TMRE fluorescence correlates with the mitochondrial membrane potential of the platelets, with higher levels indicating a more polarized mitochondrial membrane potential and more viable mitochondria. Lower levels of TMRE fluorescence will suggest a decrease in mitochondrial membrane potential, reflecting reduced metabolic activity and potentially indicating apoptotic or less viable platelets within the population under study.

To establish a positive control, washed platelets were washed and then incubated with ionomycin at a concentration of 5 µM for 15 minutes at room temperature before being stained with Annexin V or TMRE as described above.

2.2.1.5 Characterization of procoagulant platelets and procoagulant platelet markers

To detect procoagulant platelet formation, the washed platelet sample was stained with respectively 5 µl/ml and 1 µl/ml of Annexin V-FITC and CD62p for 15 minutes at room temperature. Double CD62p/Phosphatidylserine (PS) positive events measured in flow-cytometry were considered to represent procoagulant platelets. To establish a positive control, washed platelets were subjected to incubation with 5 µM of ionomycin for 15 minutes at room temperature, and 10 µM of TRAP-6 for 30 minutes at room temperature.

2.2.1.6 Assessment of the impact of Syk inhibition on of FcγRIIA mediated biological effects in healthy platelets

In order to assess the impact of Syk inhibition, washed platelets were pre-incubated with respectively 1 µM and 5 µM PRT-060318 or R406 for 30 minutes at 37 °C before resuming with the crosslinking assay and flow cytometry measurements as described above. The concentration of PRT-06318 was determined by self-performed titration experiments and in accordance with the findings of[83]. The concentration of R406 was determined considering findings concerning the pharmacokinetics of Fostamatinib [114] and up-to-date dosage recommendations. With the dilutions of PRT-060318 and R406 was performed with PBS.

This protocol remained unchanged with investigations regarding the interaction between FcγRIIA mediated biological effect and Syk inhibition when working with washed platelets only as described above.

2.2.2 Assessment of the impact of Syk inhibition in vaccine-induced thrombotic thrombocytopenia

2.2.2.1 VITT Patient cohort and IgG preparation

Included in the VITT patient cohort were Patients diagnosed in accordance with the International Society on Thrombosis and Haemostasis (ISTH) guidelines and were tested positive for anti-PF4 IgG antibodies. Moreover, in the presence of PF4, the patients' sera induced platelet aggregation in the HIPA assay [115].

Isolation of IgG fractions was achieved using the MelonTM-Gel IgG Spin Purification Kit from Thermo Fisher Scientific (Waltham, USA). Heat-inactivated serum was diluted 1:10 in purification buffer and then mixed with the kit's Gel IgG Purification Support in four cycles of 10 minutes each. After each cycle, centrifugation steps were performed through a 10 µm pore size filter for 1 minute at 5000 g. The resulting flow-through was collected into 100 kDa-pore sized centrifugal filters from Merck Millipore (Cork, Ireland) and concentrated back to the initial volume of the serum sample through centrifugation (10-15 min, 2000g, 4°C). The concentration of IgG was determined using a NanoDrop instrument from Thermo Fisher Scientific. To assess the purity and architecture of the isolated IgG fractions, equal amounts of IgG isolates were solubilized in reducing sample buffer (Invitrogen, Carlsbad, USA) and boiled for 5 minutes at 95°C. As a positive control, commercially available immunoglobulins (Intratect, Biotest AG, Germany) were used.

Diagnosis, selection of donors and IgG preparation of the VITT patient cohort has been performed by physicians and laboratory personnel of the Institute Clinical and experimental Transfusion Medicine Tübingen (IKET) other than me.

2.2.2.2 VITT IgG induced procoagulant platelet detection

Washed platelets prepared as described above were stained with respectively 5 µl/ml and 1 µl/ml of Annexin V-FITC and CD62p for 15 minutes at room temperature after a 30 minute incubation with VITT IgG at room temperature in the dark under lightly rotating conditions. Double CD62p/Phosphatidylserine (PS) positive events measured in flow-cytometry. Where indicated the platelet sample was incubated with Syk-inhibitor PRT-060318 or R406 with respectively 1 µM and 5 µM for 30 minutes at 37 °C.

2.2.2.3 Assessment of ex-vivo clot formation

The effect of VITT IgGs and Syk inhibition was further analyzed in an ex-vivo flow model using a microfluidic system (BioFlux 200; Fluxion Biosciences, Alameda, CA).

2.2.2.3.1 Preparation of the flow chamber

The microfluidic channels were coated by perfusing the channel with 100 µg/mL collagen (Collagen Horn; Takeda, Linz, Austria) and left to rest overnight at 4°C. Before performing the experiment the channels where blocked using 2.5% of human serum albumin (Kedrion, Barga, Italy) following recommendations of the International Society on Thrombosis and Haemostasis standardization committee for biorheology[116].

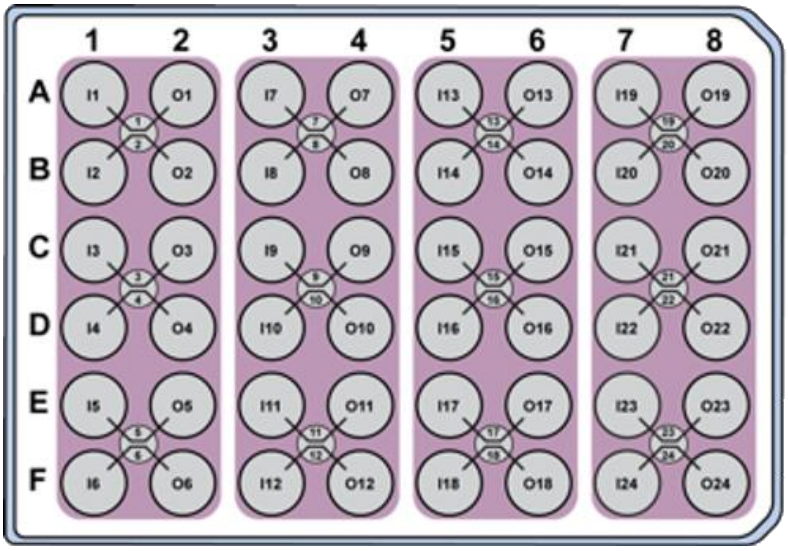

Illustration 2: Bioflux plate schema

BioFlux plates consist of an array of microfluidic flow cells. A pressure interface is connected to the top of the inlet well plate (I1 on the schema) containing the blood sample and applies a controlled pneumatic pressure forcing the fluid at a specified flow rate through the coated channel (fluid is forced from I1 to O1). The observation area (here marked with 1) find itself at half way between inlet and outlet well and consists of a viewing channel that is 350µm wide and 75µm deep. In our study a 48-well high shear plate was used.

2.2.2.3.2 Preparation of the blood sample

Healthy individuals with blood group O were selected, and their citrated whole blood samples were collected using S-Monovette, Citrat 3,2% (Sarstedt) after obtaining written consent. These samples were allowed to rest for 30 minutes at room temperature. Afterward, the whole blood was divided into aliquots of 200 µL and platelet-rich plasma (PRP) was obtained through centrifugation (20 minutes, 120 g, at RT, without a break). Next, 45 µL of the supernatant PRP was gently separated and incubated with 5 µL of the VITT IgG fraction or a healthy control sera, specifically AB-Serum collected under the same conditions. The incubation took place for 90 minutes at RT under rotating conditions. Platelets were fluorescently labelled with 4 µM Calcein-FITC (Thermo Fisher Scientific, Waltham, MA) for 15 minutes before the end of the incubation period. Following this, the PRP was gently

reintroduced to reconstitute the whole blood samples. Were indicated, the PRP sample was further incubated with Syk inhibitors PRT-060318 or R406 at concentrations of 1 µM and 5 µM, respectively, for 15 minutes at room temperature before incubation with the VITT IgG fraction or healthy control sera.

2.2.2.3.3 Image analysis

In ImageJ, standard operating scripts were utilized to automatically and manually adjust the threshold setting on fluorescence microscopic images (Illustration 3). By using threshold tool in ImageJ (Illustration 3) the image is converted from a grayscale image into a binary image. A binary image contains only two values: black and white (or 0 and 1). This allows to separate the surface area covered by the thrombus from the background of the image and perform various image analysis tasks such as measuring surface area coverage by the thrombi.

Images were loaded into the software by an operator blinded to the experimental setting. After adjusting bit-depth, contrast and visibility the images were processed identically. In a few cases the automated threshold setting had to be adjusted manually by 1–2 grey values because of the incorrect filling of gaps between thrombi.

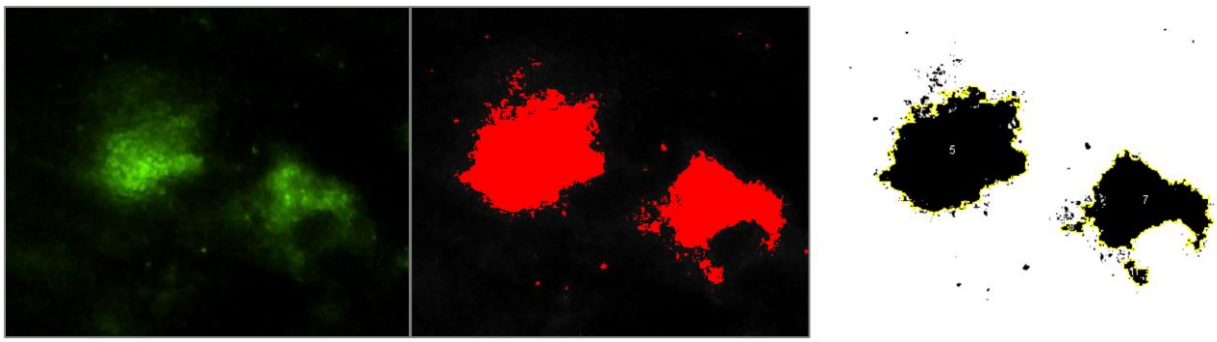

Illustration 3: Analysis of Surface of "area covered" in ImageJ using a binary image.

The measurement of percent of Surface area covered (%SAC) was performed in ImageJ while excluding particles of less than 80 µm² to reduce error. Out of focus images and pictures with artefacts were excluded from the analysis.

2.2.3 Assessment of the Impact of Syk inhibition on auto-antibody mediated desialylation in immune thrombocytopenia

2.2.3.1 ITP patient cohort and Sera

Experienced physicians other than I included willing ITP Patients into this cohort according to the latest ITP diagnostic guidelines. Patients suffering from accompanying diseases that can lead to thrombocytopenia were rigorously removed from the study. Experiments were conducted using leftover serum material from ITP patients. Patient serum samples were stored at the Department for Transfusion Medicine Tübingen at -80° C. Control sera with the blood-group AB were obtained from healthy blood donors with written consent at the Institute for Clinical and experimental Transfusion Medicine Tübingen (IKET). To exclude any unspecific effects such as the activation of platelets all sera were heat-inactivated at 56° C for 25 minutes, followed by centrifugation at 5000 g separating supernatant from a discarded pellet.

2.2.3.2 Detection of platelet sialylation

To determine platelet sialylation, 25µl of washed platelets prepared as described above were incubated with 25µl of ITP serum for two hours at room temperature in rotating conditions. The samples were then fixed with 2% paraformaldehyde (PFA) for 20 minutes at room temperature and washed twice with PBS at 650 g for 7 minutes at room temperature with a brake. The platelet sialylation was then determined in flow cytometry using 1 µg/mL of FITC-labelled ricinus communis agglutinin (RCA), which specifically binds to ß-Galactose. Where indicated, washed platelets were incubated with Syk inhibitors PRT-060318 or R406 at respectively 1µM and 5µM for 15 minutes at room temperature.

2.2.4 Statistical analysis

Statistical analysis was performed using a T-test for normally distributed results. When data failed to show a normal distribution, a nonparametric test (Mann-Whitney test) was used. All statistical analysis has been carried out in GraphPad Prism 9 (La Jolla, USA). P values in figures were summarized as follow: *p < 0.05, **p < .01, ***p < .001 and****p < 0.0001.

2.3 Ethics

All patients and donors involved in this project were informed in detail about the aims of the study and provided written consent after an appropriate time of consideration. The Project has furthermore been approved by the Ethics Committee of Faculty of Medicine, Eberhard Karls University of Tübingen and conducted in strict accordance with the declaration of Helsinki.

3 Results

3.1 The impact of Syk inhibition in human platelets

In order to elucidate the impact of Syk inhibition in human platelets we proceeded according to a fixed scheme: First, we assessed the biological effect mediated by FcγRIIA activation in healthy platelets to later investigate the same effect with additional inhibition of tyrosine kinase Syk. Used Syk inhibitors are the experimental Syk inhibitor PRT-060318 and Syk inhibitor R406, the active metabolite of Fostamatinib.

3.1.1 The effect of Syk inhibition on FcγRIIA mediated platelet activation

Central to the platelet's role is their activation status. In this section we assessed platelet activation through FcγRIIA with and without Syk inhibition via the read-out PAC -1 surface expression, marker for the activated fibrinogen receptor and P-selectin (CD62P) surface exposure in flow cytometry.

3.1.1.1 Crosslinking of the FcγRIIA induces platelet activation in healthy platelets

Washed platelets of nine healthy donors were incubated with anti CD32 clone AT-10, followed by incubation with secondary F(ab')2 antibody to crosslink and activate the FcγRIIA receptor. Platelets have been fluorescently marked with FIT-C labeled PAC-1 monoclonal antibody and analyzed via flow cytometry. The crosslinking of FcγRIIA induced the expression of PAC -1 a specific marker for αIIbβ3 inside-out signaling in platelets (Figure 1).

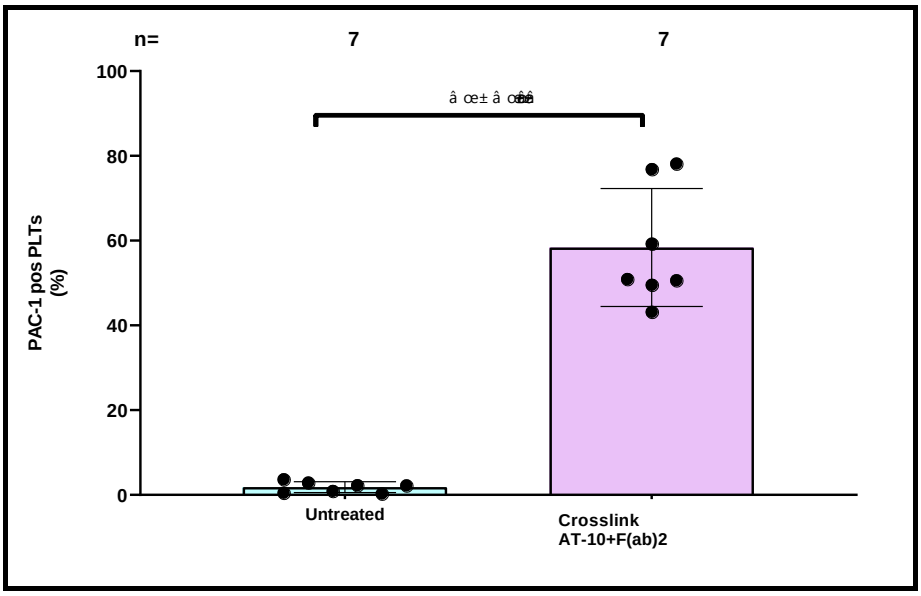

Figure 1: Crosslinking the FcγRIIA induces PAC-1 expression in healthy platelets

Cross-linking the FcγRIIA receptor with monoclonal antibodies AT-10 and F(ab')2 fragments led to a significant increase in the expression of PAC-1 on the surface of platelets. We found that the mean percentage ± Standard deviation (SD) of platelets positive for PAC-1 increased from 1.853% ± 1.281% in the untreated control group to 58.38% ± 13.90% in the cross-linked group. Additionally, the difference between the means of the two groups, "Crosslink AT-10+F(ab')2" and "Untreated," was 56.53% ± a standard error of mean (SEM) of 5.275%, which was statistically significant with a p-value of less than 0.0001. These results were obtained from seven healthy donors.

The crosslinking and activation of the FcγRIIA also induced the surfacing of P-selectin (Figure 2), marker for the degranulation of alpha granules upon platelet activation. The detection of P-selectin was achieved by marking platelets of seven healthy donors with APC-labelled mouse anti-human CD62P antibody and measured in flow cytometry.

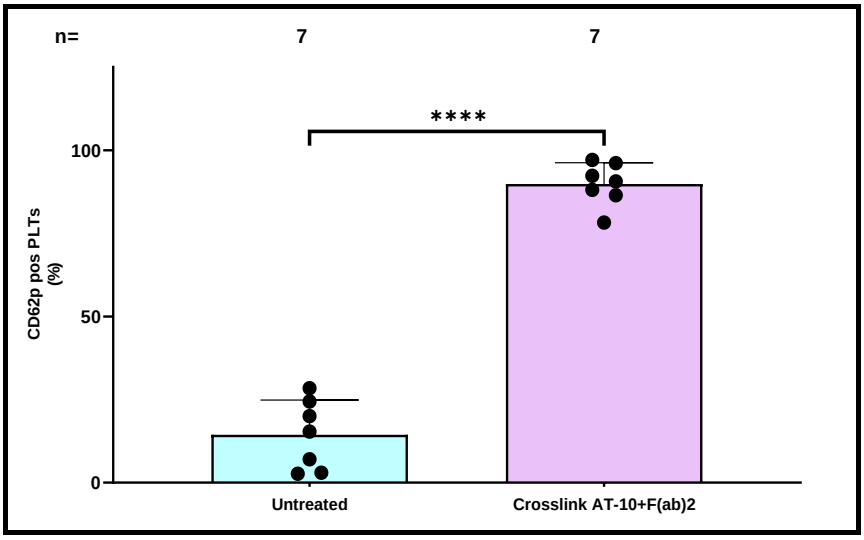

Figure 2 Crosslinking the FcγRIIA induces P-selectin expression in healthy platelets

Cross-linking the FcγRIIA receptor with monoclonal antibodies AT-10 and F(ab')2 fragments resulted in a significant increase in the expression of P-Selectin (CD62p) on the surface of platelets. We observed that the mean percentage ± SD of platelets positive for CD62p increased from 14.42% ± 10.42% in the untreated control group to 89.83% ± 6.420% in the cross-linked group. The difference between means ± SEM of the two groups, "Crosslink AT-10+F(ab')2" and "Untreated," was 75.41% ± 2.095% which was statistically significant with a p-value of less than 0.0001. These results were obtained from seven healthy donors.

In order to determine whether platelet activation through incubation with anti CD32 clone AT-10 and crosslinking with F(ab)2 antibody was FcγRIIA specific, the washed platelets of three healthy donors were pre-incubated with anti-human CD32 antibody clone IV.3. competitively blocking the receptor. Platelet activation status after FcγRIIA crosslink was again determined via P-selectin (CD62P) surface exposure in flow cytometry (Figure 3).

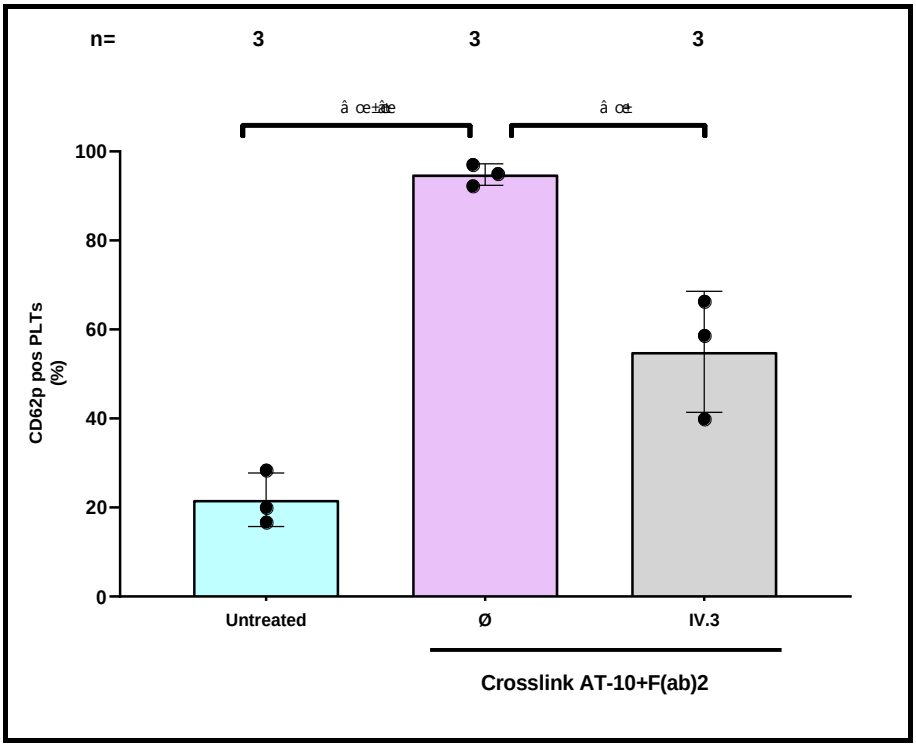

Figure 3 Platelet activation mediated via crosslinking is FcγRIIA specific

The crosslinking of FcγRIIA induced CD62p positive platelets as shown previously. In "Crosslink AT-10 + F(ab')2" the mean percentage of CD62p positive platelets ± SD was 94.81 ± 2.410% vs. 21.74 ± 6.014% in untreated. The difference between means ± SEM of "Crosslink AT-10 + F(ab')2 + Ø" and "Untreated" was 73.07 ± 2.813% with a p-value of 0.0064.

Blocking of the FcγRIIA with monoclonal antibody IV.3 significantly inhibited FcγRIIA mediated formation CD62p positive platelets. The difference between means ± SEM "Crosslink AT-10+F(ab')2 + IV.3" and "Crosslink AT-10+F(ab')2 + Ø" was -39.84 ± 9.177% with a p-value of 0.0492. Here the sample size was respectively three healthy donors.

We found that pre-incubation of the washed platelets with monoclonal antibody IV.3 was able to competitively inhibit P-selectin expression indicating that platelet activation via cross-linking of the FcγRIIA receptor with monoclonal antibodies AT-10 and F(ab')2 fragment is FcγRIIA specific.

In this section we were able to show that the cross-linking of FcγRIIA significantly induces platelet activation.

3.1.1.2 Syk inhibition prevents FcγRIIA mediated platelet activation

In order to assess the impact of Syk inhibition on FcγRIIA mediated platelet activation, the washed platelets of seven healthy donors were pre-incubated with Syk inhibitor PRT-060318 and Syk inhibitor R406 for 30 minutes before proceeding to the cross-linking of the FcγRIIA following identical protocol as above. We found that the inhibition of tyrosine kinase Syk with inhibitor PRT-060318 but not R406 significantly inhibited the expression of PAC -1 after FcγRIIA crosslinking (Figure 4).

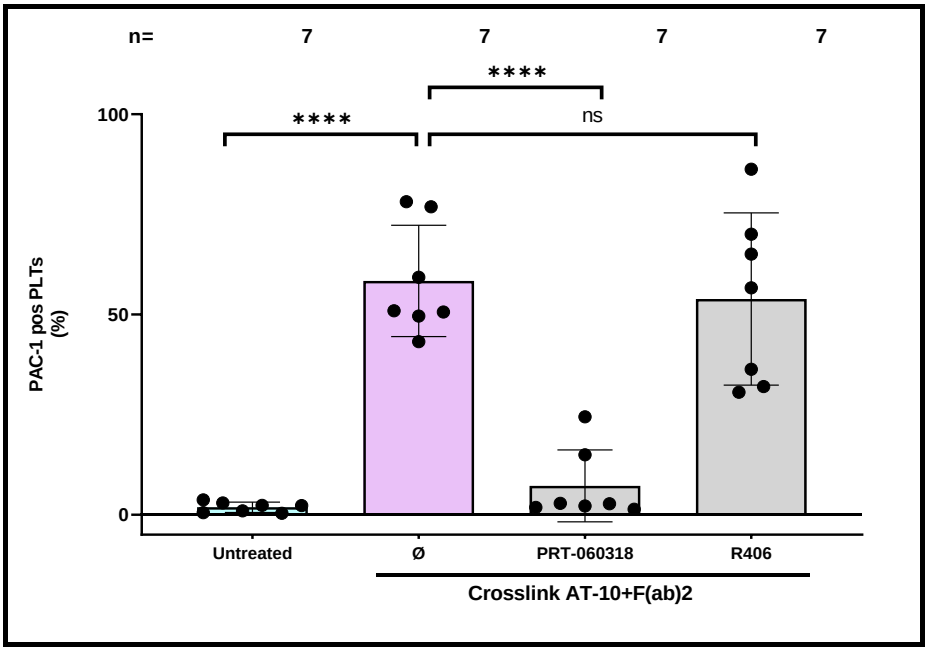

Figure 4 FcγRIIA mediated induced PAC-1 expression can be reduced by Syk inhibition in healthy platelets

The mean percentage ± SD of PAC-1 positive platelets in the untreated sample is 1.853 1281%. We observe that the mean percentage ± SD of PAC-1 positive platelets decreased from 58.38 ± 13.90% in the cross-linking group to 7.197 ± 8.993% in the cross-linking group with PRT-060318 inhibition. Moreover, the difference between means ± SEM of "Crosslink

AT-10 + F(ab')2 + PRT-060318" and "Crosslink AT-10 + F(ab')2 + Ø" was -51.18 ± 6.257% which is statistically significant with a p-value of less than 0.0001. In contrast, the difference between means ± SEM in "Crosslink AT-10 + F(ab')2 + R406" and " Crosslink AT-10 + F(ab')2 + Ø," was -4.529 ± 9.677%, which is not statistically significant with a p-value of 0.6482 based on a sample size of seven healthy donors.

Furthermore, we found that the inhibition of tyrosine kinase Syk with inhibitor PRT-060318 but not R406 significantly inhibited the P-selectin surfacing after FcγRIIA crosslinking (Figure 5).

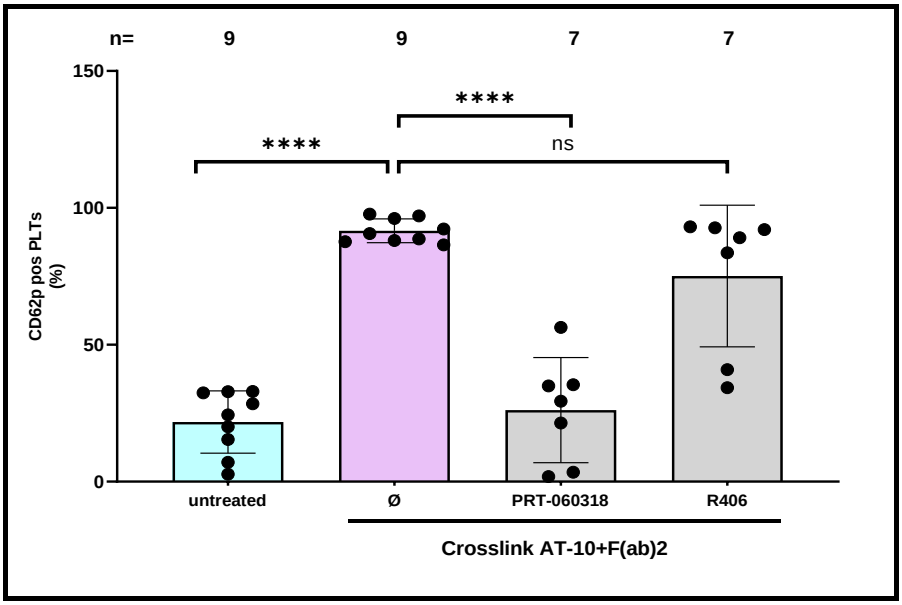

Figure 5 FcγRIIA mediated induced P-selectin expression can be reduced by Syk inhibition in healthy platelets

Here our results from a sample size of seven healthy donors, indicate that Syk inhibition with PRT-060318 effectively inhibited FcγRIIA-mediated expression of P-selectin in platelets. The mean percentage of CD62p positive platelets ± SD was 21.80 ± 3.794% in the "Untreated" group, 91.62 ± 1.453% in the "Crosslink AT-10 + F(ab')2 + R406" group, 26.11 ± 7.264% in the "Crosslink AT-10 + F(ab')2 + PRT-060318" group and 75.10 ± 9.785% in the "Crosslink AT-10 + F(ab')2" group. The difference in means ± SEM between the "Crosslink AT-10 + F(ab')2 + PRT-060318" group and the "Untreated" group was -66.50 ± 6.686%, which was

statistically significant with a p-value of less than 0.0001. However, Syk inhibition with R406 did not significantly affect P-selectin expression. The difference in means ± SEM between the "Crosslink AT-10 + F(ab')2 + R406" group and the "Untreated" group was -17.77 ± 8.516%, which was not statistically significant with a p-value of 0.0820.

These findings show that the inhibition of tyrosine kinase Syk with inhibitor PRT-060318 was able to significantly inhibit CD62p and PAC-1 surface expression after stimulation of the FcγRIIA. Interestingly the inhibition of Syk with inhibitor R406 did not alter platelet activation status.

3.1.2 The effect of Syk inhibition on FcγRIIA mediated expression of apoptotic markers in platelets

As platelet apoptosis takes on a central role in platelet lifespan, viability and function. We here assessed the FcγRIIA mediated expression of apoptotic markers in platelets. We will determine phosphatidylserine (PS) surface exposure and mitochondrial membrane potential as a viability marker by labeling active mitochondria with cell permeant PE-labelled Tetramethylrhodamine, ethyl ester (TMRE) in flow cytometry.

3.1.2.1 Crosslinking of the FcγRIIA increases platelet apoptotic markers in healthy platelets

Washed platelets of nine healthy donors were incubated with anti CD32 clone AT-10, followed by incubation with secondary F(ab')2 antibody to crosslink and activate the FcγRIIA receptor. The activation of the FcγRIIA resulted in enhanced apoptotic markers. We noticed increased phosphatidylserine (PS) externalization (Figure 6) via fluorescently marked by FIT-C labelled Annexin V is observed in flow cytometry.

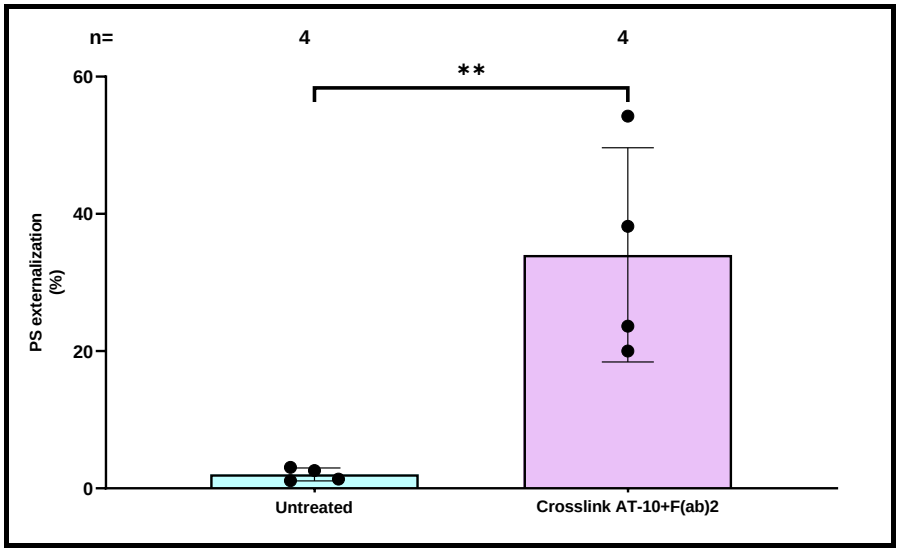

Figure 6 Crosslinking the FcγRIIA induces PS externalization in healthy platelets

The cross-linking of FcγRIIA using monoclonal antibodies AT-10 and F(ab')2 fragment significantly increased the expression of phosphatidylserine (PS) on the surface of platelets, as detected using FIT-C labeled Annexin V in flow cytometry. Specifically, the mean percentage ± SD of Annexin V positive platelets was 2.035 ± 0.4693% in the "Untreated" group, and 34.03 ± 7.806% in the "Crosslink AT-10+F(ab')2" group. The difference in means ± SEM between the "Crosslink AT-10+F(ab')2" group and the "Untreated" group was 31.99 ± 7.820%, which was statistically significant with a p-value of 0.0064. These results were obtained in a sample size of four healthy donors.

The crosslinking and activation of the FcγRIIA was also found to induce mitochondrial-transmembrane potential depolarization in platelets of three healthy donors after marking the platelets with PE-labelled Tetramethylrhodamine, ethyl ester in flow cytometry (figure 7).

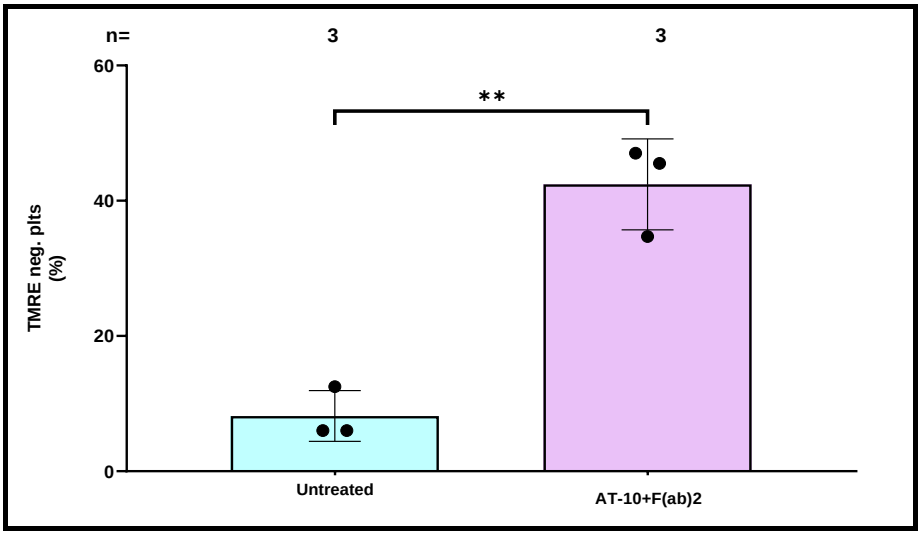

Figure 7 : Crosslinking the FcγRIIA induces the loss of mitochondrial transmembrane potential (ΔΨm)

The cross-linking of the FcγRIIA receptor using monoclonal antibodies AT-10 and F(ab')2 fragment led to a significant reduction in the TMRE (PE) signal detected in flow cytometry, indicating a halt in metabolic activity and initiation of apoptosis. Specifically, the mean percentage ± SD of TMRE negative platelets increased from 8.173 ± 3.747% in the untreated group to 42.42 ± 6.714% in the cross-linking group. Moreover, the difference between means ± SEM of the cross-linking group and the untreated group was 34.25% ± 4.439%, which was statistically significant with a p-value of 0.0015.

In order to determine whether the expression of apoptotic markers after the incubation with anti CD32 clone AT-10 and crosslinking with F(ab)2 antibody was FcγRIIA specific, washed platelets of four healthy donors were pre-incubated with anti-Human CD32 Antibody Clone IV.3. After FcγRIIA crosslink the apoptotic marker phosphatidylserine externalization was determined via flow cytometry (Figure 8).

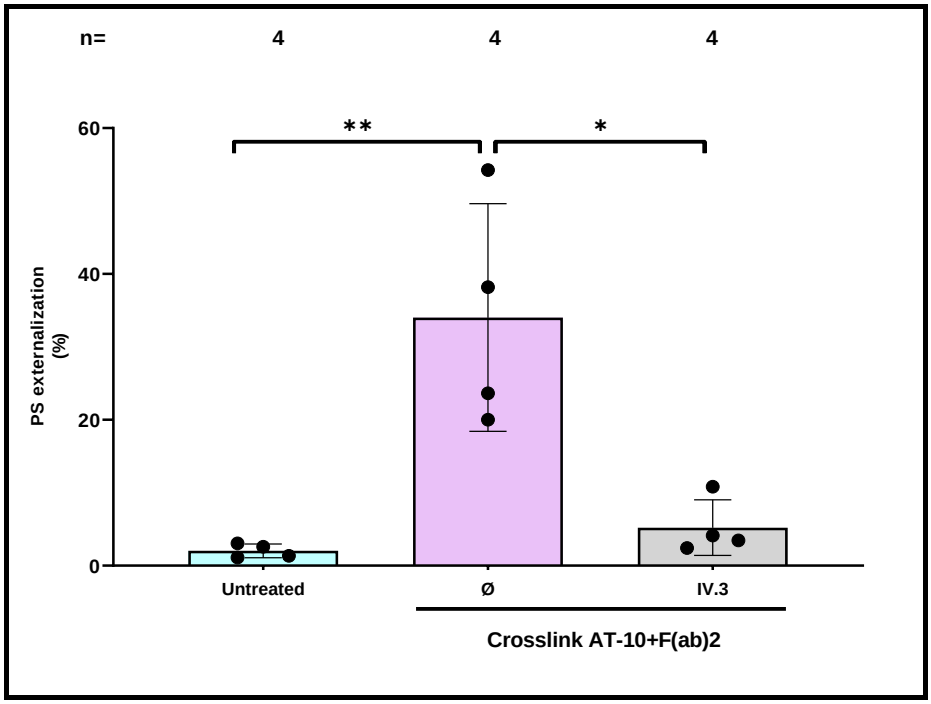

Figure 8 Phosphatidylserine externalization mediated via crosslinking of FcγRIIA is FcγRIIA specific

As shown above, the crosslinking of FcγRIIA induced Phosphatidylserine (PS) externalization. In “Crosslink AT-10 + F(ab’)2” the mean percentage of PS positive platelets ± SD was 34.03 ± 15.61% vs. 2.035 ± 0.9386% in the untreated group. The difference between means of the “Crosslink AT-10 + F(ab’)2 + Ø” group and the “Untreated” group ± SEM was 31,99 ± 7,820% with a p-value of 0.0064. The additional blocking of the FcγRIIA with anti-Human CD32 Antibody Clone IV.3 showed a mean percentage of PS positive platelets ± SD was 5.213 ± 3.803% after crosslink.

Blocking of the FcγRIIA with monoclonal antibody IV.3 significantly inhibited FcγRIIA mediated formation PS positive platelets. The difference between means ± SEM of the “Crosslink AT-10+F(ab’)2 + IV.3” group and the “Crosslink AT-10+F(ab’)2 + Ø” group was -28.82 ± 8,034% based in a sample size of four healthy donors.

We found that the pre-incubation of the washed platelets with monoclonal antibody IV.3 was able to competitively inhibit phosphatidylserine exposure indicating that the expression of

apoptotic markers via cross-linking of the FcγRIIA receptor with monoclonal antibodies AT-10 and F(ab')2 fragment is FcγRIIA specific.

These results indicate that after the specific activation of the FcγRIIA, platelets present a significant increase in apoptotic markers such as phosphatidylserine exposure and suffer a significant loss of mitochondrial transmembrane potential. This data suggests that the activation of FcγRIIA could induce platelet apoptosis.

3.1.2.2 Syk inhibition prevents FcγRIIA mediated expression of apoptotic markers in platelets

To assess the impact of Syk inhibition on FcγRIIA mediated apoptotic marker expression in platelets, washed platelets of eight healthy donors were pre-incubated with Syk inhibitor PRT-060318 and washed platelets of six healthy donors were pre-incubated Syk inhibitor R406 (Fostamatinib) for 30 minutes before proceeding to the crosslinking of the FcγRIIA following identical protocol. We found that the inhibition of tyrosine kinase Syk with inhibitor PRT-060318 as well as R406 significantly inhibited the phosphatidylserine externalization (Figure 9).

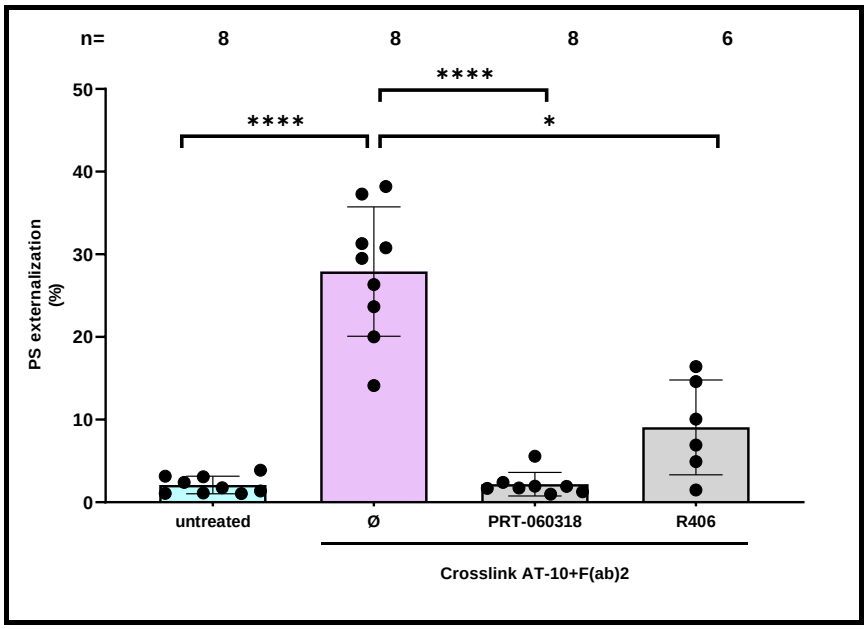

Figure 9 FcγRIIA mediated induced Phosphatitylserin (PS) externalization can be reduced by Syk inhibition in healthy platelets

Here, Syk inhibition with PRT-060318 and Syk inhibition with R406 significantly inhibited FcγRIIA-mediated phosphatidylserine externalization. Specifically, the mean percentage ± SD of annexin V positive platelets decreased from 27.91 ± 7.820% in the cross-linking group to 9.065 ± 5.742% with Syk inhibition via R406 and to 2.188 ± 1.434% in the cross-linking group with Syk inhibition via PRT-060318. The difference between means ± SEM of the cross-linking group with PRT-060318 inhibition, "Crosslink AT-10 + F(ab')2 + PRT-060318" and the "Crosslink AT-10 + F(ab')2 + Ø" was -26.71 ± 8.297%, which was statistically significant with a p-value of less than 0.0001. The difference between means of the cross-linking group with R406 inhibition ± SEM "Crosslink AT-10 + F(ab')2 + R406" and "Crosslink AT-10 + F(ab')2 + Ø," was -16.45 ± 4.775%, which was statistically significant with a p-value of 0.0183. The sample size for each group was eight and six healthy donors, respectively.

Moreover, we found that the inhibition of tyrosine kinase Syk with inhibitor PRT-060318 as well as R406 significantly inhibited mitochondrial-transmembrane potential depolarization in platelets of 3 healthy donors (Figure 10).

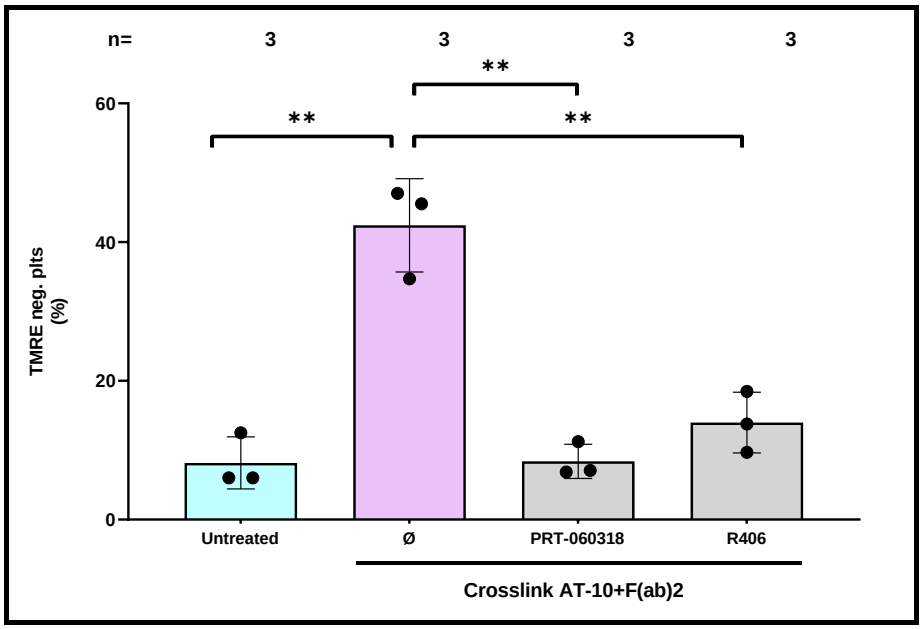

Figure 10 FcγRIIA mediated induced loss of mitochondrial transmembrane potential (ΔΨm) can be restored by Syk inhibition in healthy platelets

The difference between mean percentages ± SEM of "Crosslink AT-10 + F(ab')2 + PRT-060318" and "Crosslink AT-10 + F(ab')2 + Ø" was -34.04 ± 4.128%, with a p-value of 0.0012. The difference between means ± SEM for "Crosslink AT-10 + F(ab')2 + R406" and "Crosslink AT-10 + F(ab')2 + Ø" was -28.44 ± 4.629%, with a p-value of 0.0036. The sample size for this experiment was three healthy donors. It appears that both Syk inhibition with PRT-060318 and Syk inhibition with R406 can prevent the loss of TMRE (PE) signal detected in flow cytometry indicating a decrease in mitochondrial membrane potential suggesting the arrest of metabolic activity and ongoing platelet apoptosis mediated via FcγRIIA signaling.

The results demonstrate that inhibiting the tyrosine kinase Syk using inhibitors PRT-060318 and R406 effectively interrupts FcγRIIA-mediated expression of apoptotic markers in platelets.

3.1.3 The effect of Syk inhibition on FcγRIIA mediated expression of procoagulant platelet markers

Procoagulant platelets, defined as phosphatidylserine (PS) and P-selectin (CD62P) expressing platelets, are discussed to be central actors in thrombus formation. In this section we will assess the expression of procoagulant platelet markers after FcγRIIA activation with and without Syk inhibition.

3.1.3.1 Crosslinking of the FcγRIIA induces the expression of procoagulant platelet markers

Washed platelets of nine healthy donors were incubated with anti CD32 clone AT-10, followed by incubation with secondary F(ab')2 antibody to crosslink and activate the FcγRIIA receptor. Platelets have been fluorescently marked with FIT-C labelled Annexin V and CD62P-APC in order to detect respectively Phosphatidylserine (PS) and P-selectin (CD62p) on the platelets surface via flow cytometry. After the crosslinking of the receptor, the number of platelets presenting both P-selectin (CD62P) as well as phosphatidylserine (PS) significantly increased (Figure 11). The activation of FcγRIIA induced the expression of procoagulant platelet markers.

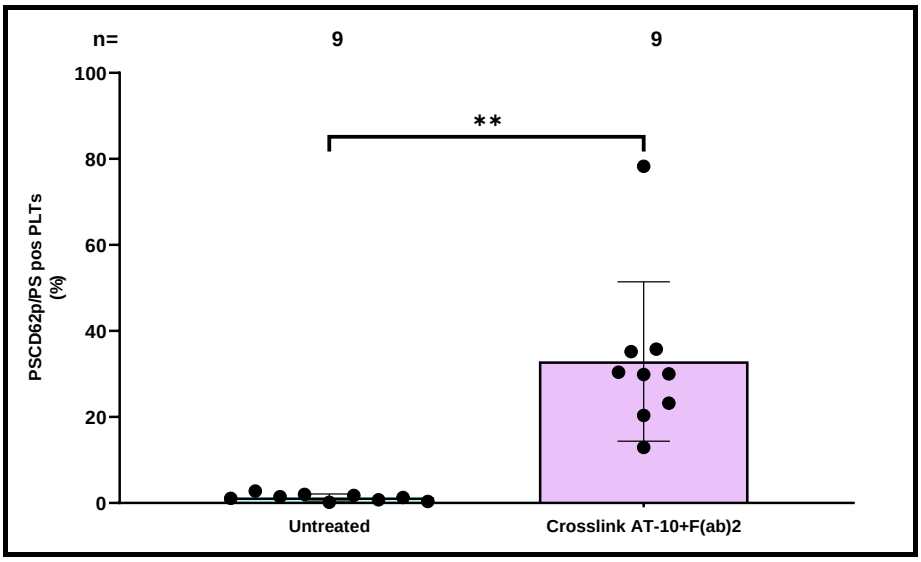

Figure 11 Crosslinking the FcγRIIA induces the expression of procoagulant platelet markers

The cross-linking of the FcγRIIA receptor with monoclonal antibodies AT-10 and F(ab')2 fragments induced the formation of both CD62p and Phosphatidylserine positive platelets. The mean percentage ± SD of CD62p/PS positive platelets was significantly higher crosslink sample with 32.90 ± 18.52% compared to the untreated samples with 1.291 ± 0.8159%. The difference between means ± SEM of the "Crosslink AT-10+F(ab')2" and the "Untreated" samples was 31.61 ± 6.363%, with a p-value of less than 0.0011. This data was gathered from 9 healthy donors.

In order to determine whether the expression of procoagulant platelet markers after the incubation with anti CD32 clone AT-10 and crosslinking with F(ab)2 antibody was FcγRIIA specific, the washed platelets of four healthy donors were pre-incubated with anti-Human CD32 Antibody Clone IV.3. and labeled with FIT-C labelled Annexin V and CD62p-APC for analysis in flow cytometry (Figure 12).

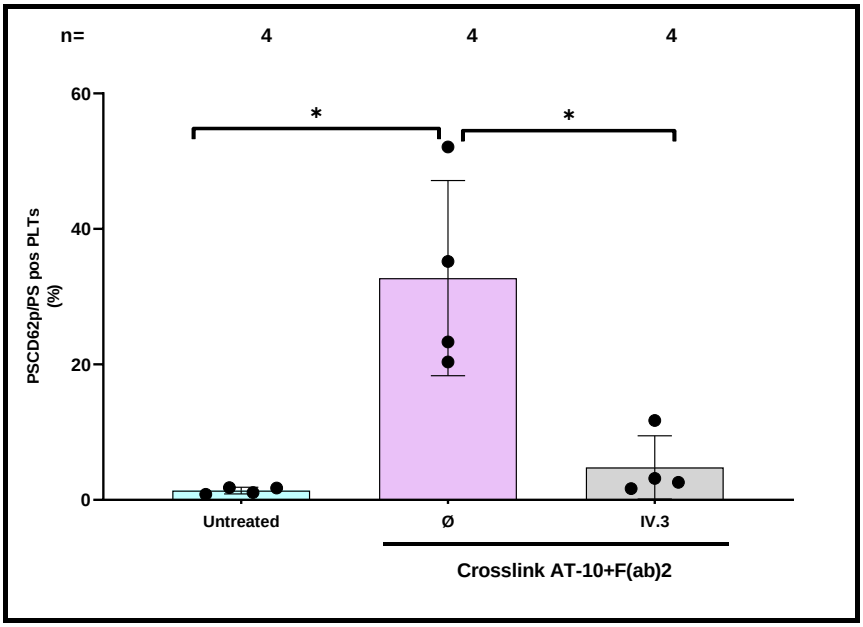

Figure 12 The expression of procoagulant platelet markers mediated via crosslinking is FcγRIIA specific

The crosslinking of FcγRIIA induced the formation of CD62p/PS positive platelets. In the untreated group the mean percentage of CD62p/PS positive platelets ± SD was 1.373 ± 0.4844% in contrast to 32.75 ± 14.41% in the FcγRIIA crosslink group. The difference between means of "Crosslink AT-10 + F(ab')2 + Ø" and "Untreated" ± SEM was 14.00 ± 6.998% which was significant with a p-value of 0.0207.

Blocking the FcγRIIA with monoclonal antibody IV.3 significantly inhibited FcγRIIA mediated formation of CD62p/PS positive platelets. The mean percentage of CD62p/PS positive platelets ± SD was 4.790 ± 4.661% The difference between means of "Crosslink AT-10+F(ab')2 + IV.3" and "Crosslink AT-10+F(ab')2 + Ø" ± SEM was -27.96 ± 8.536% with a p-value of 0.0466. Here the sample size was four healthy donors.

We found that the pre-incubation of the washed platelets with monoclonal antibody IV.3 was able to abrogate the formation of phosphatidylserine (PS) and P-selectin (CD62P) positive platelets, indicating that the procoagulant platelet marker expression after cross-linking of the FcγRIIA receptor with monoclonal antibodies AT-10 and F(ab')2 fragment is FcγRIIA specific.

In this section our findings showed that the activation of the FcγRIIA specifically induces a significant expression procoagulant platelet markers.

3.1.3.2 Syk inhibition prevents FcγRIIA mediated expression of procoagulant markers

To assess the impact of Syk inhibition on FcγRIIA mediated platelet activation, the washed platelets of seven healthy donors were pre-incubated with Syk inhibitor PRT- 060318 and the washed platelets of five healthy donors with Syk inhibitor R406 (Fostamatinib) for 30 minutes before proceeding to the cross linking of the FcγRIIA following identical protocol. We found that the inhibition of tyrosine kinase Syk with inhibitor PRT-060318 as well as R406 significantly inhibited the expression of PAC -1 after FcγRIIA crosslinking (Figure 13).

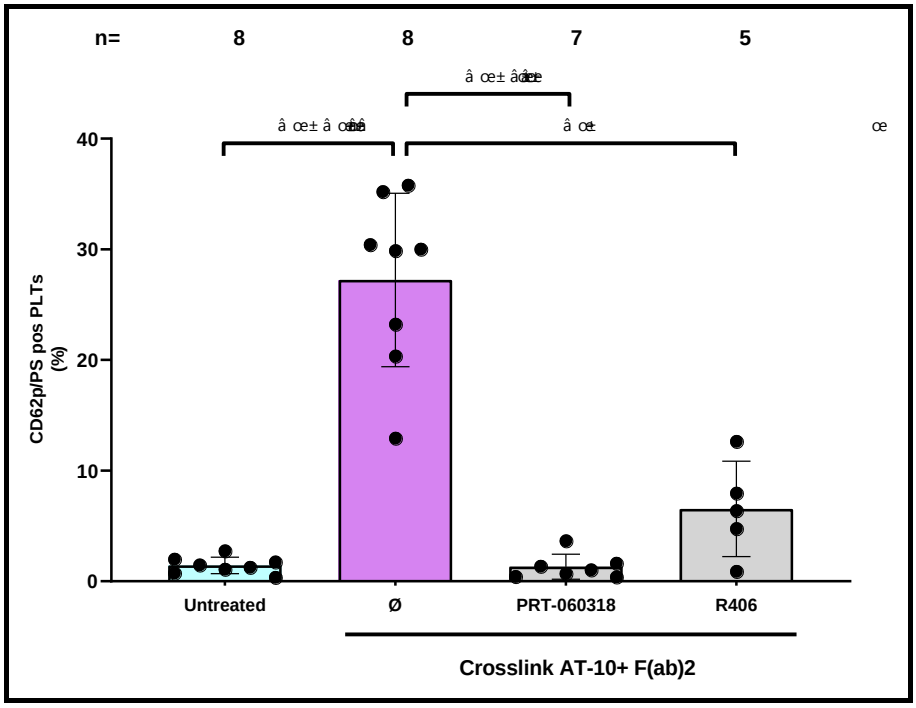

Figure 13 FcγRIIA mediated espression of procoagulant platelet markers is reduced by Syk inhibition in healthy platelets

The inhibition of Syk with PRT-060318 or R406 was found to effectively inhibit the formation of CD62p/PS positive platelets induced by FcγRIIA crosslinking. Preincubation with PRT-060318 resulted in a mean percentage of CD62p/PS positive platelets ± SD of 1.430 ±

0.2651%, compared to 27.23 ± 2.769% without preincubation. Similarly, preincubation with R406 resulted in a mean percentage of CD62p/PS positive platelets of 1,319 ± 0.4282% compared to 6.534 ± 1.928% without preincubation. The mean difference ± SEM between the "Crosslink AT-10 + F(ab')2 + PRT-060318" and "Crosslink AT-10 + F(ab')2 + Ø" samples was -26.89 ± 3.222%, with a p-value of less than 0.0002 for a sample size of seven healthy donors. Likewise, the mean difference ± SEM between the "Crosslink AT-10 + F(ab')2 + R406" and "Crosslink AT-10 + F(ab')2 + Ø" samples was -17.90 ± 4.995%, with a p-value of 0.0231 for a sample size of five. These results show that both PRT-060318 and R406 significantly reduced the formation of CD62p/PS positive platelets induced by FcγRIIA crosslinking. We can conclude that the inhibition of Syk interrupts FcγRIIA-mediated expression of procoagulant platelet markers.

3.1.4 The effect of Syk inhibition on basic platelet function

In this section we assess if despite the inhibition of tyrosine kinase Syk, platelets are still capable of fulfilling basic functions, in particular their activation. to achieve this, we will test the capacity of platelets to activate after exposure platelets with APC-labelled mouse anti-human CD62P antibody and measured in flow cytometry. When indicated, samples were pre-incubated with Syk inhibitor PRT-06018 and R406. We observed that Syk inhibition did not alter platelet activation status after TRAP-6 exposure (Figure 14)

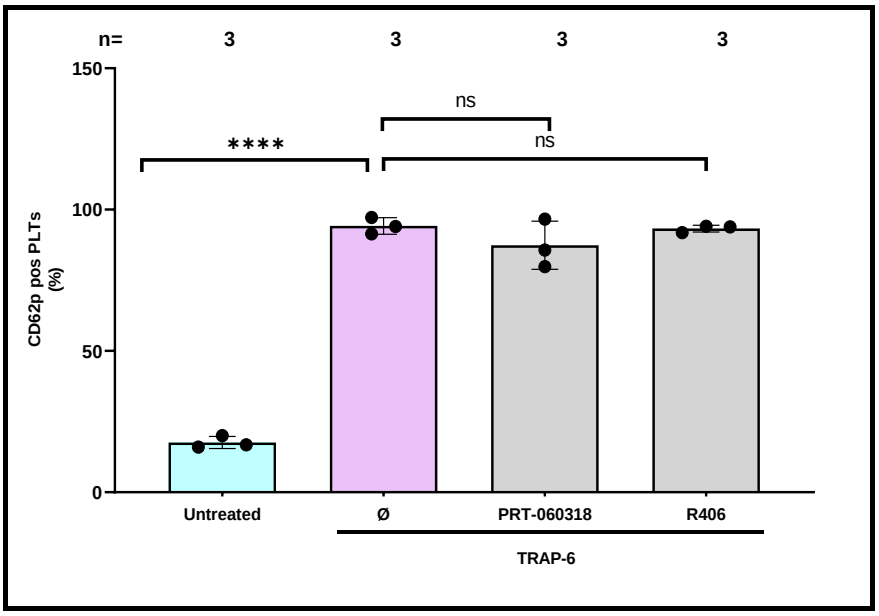

Figure 14 Syk inhibition does not affect platelet activation over thrombin pathway.

The mean percentage of CD62p positive platelets ± SD without TRAP-6 was 17.59 ± 2.149% compared to 94.22 ± 2.934% in the presence of TRAP-6. After the preincubation with PRT-060318 and TRAP-6 the mean percentage of CD62p positive platelets ± SD was 87.37 ± 8.528%. Similarly, the mean percentage of CD62p positive platelets ± SD with R406 and TRAP-6 was 93.27 ± 1.208%. Syk inhibition with either PRT-060318 or R406 did not impede platelet activation via TRAP-6, suggesting that the downstream activation signaling of Syk remained unaffected.

ADP stimulates Gi-coupled P2Y12 receptor and the Gq coupled-P2Y1 eventually leading to granule secretion and subsequent surfacing of P-selectin. Activation status after stimulation with ADP, with and without Syk inhibition, has also been tested by marking P-selectin on the surface platelets surface. We observed, in the washed platelets of three healthy donors, Syk inhibition did not impair platelet activation status after ADP exposure (Figure 15)

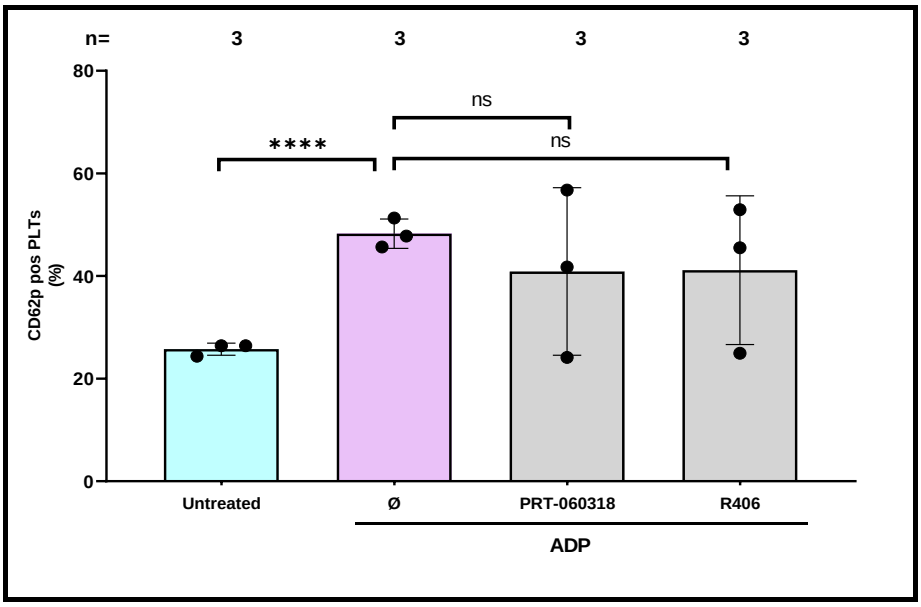

Figure 15 Syk inhibition does not affect platelet activation over ADP pathway

The mean percentage of CD62p positive platelets ± SD in the untreated sample was 25.75 ± 1.184 % compared to 48.25 ± 1.650% in the presence of ADP. Similarly, the mean percentage ± SD of CD62p positive platelets in the presence of ADP after preincubation with PRT-060318 and R406 was respectively 40.25 ± 16.31% and 41.12 ± 14.49 %. The difference between means ± SEM of "ADP + PRT-060318" and "ADP + Ø" samples was -7.360 ± 9.562%, with a p-value of 0.4844 for a sample size of n=3. Likewise, the difference between means of the "ADP + R406" and "ADP + Ø" samples was -7.123 ± 8.528%, with a p-value of 0.4506 for a sample size of three. Therefore, it appears that Syk inhibition with either PRT-060318 or R406 did not influence platelet activation via ADP, indicating that the activation signaling downstream of Syk was not affected.

The platelets' ability to activate after stimulation of the PAR-1 receptor via TRAP-6 or the Gi-coupled P2Y12- and Gq-coupled P2Y1 receptor via ADP was not significantly impaired by Syk inhibition with either PRT-060318 or R406.

3.2 The role of Syk inhibition in vaccine induced thrombocytopenia

3.2.1 Syk inhibition effectively prevents the expression of procoagulant platelet markers conferred by VITT antibodies

Recent studies have shown how procoagulant platelet formation is a important aspect in the pathophysiology of VITT[69]. In this section, we investigate the impact of Syk inhibition on vaccine-induced immune thrombotic thrombocytopenia (VITT) IgG-mediated procoagulant platelet formation. Specifically, we compare the expression of procoagulant platelet markers with and without Syk inhibition.

Platelet rich plasma (PRP) of healthy donors were incubated with VITT IgG.
Where indicated, the sample was pre-incubated with Syk inhibitors PRT-060318 and R406.

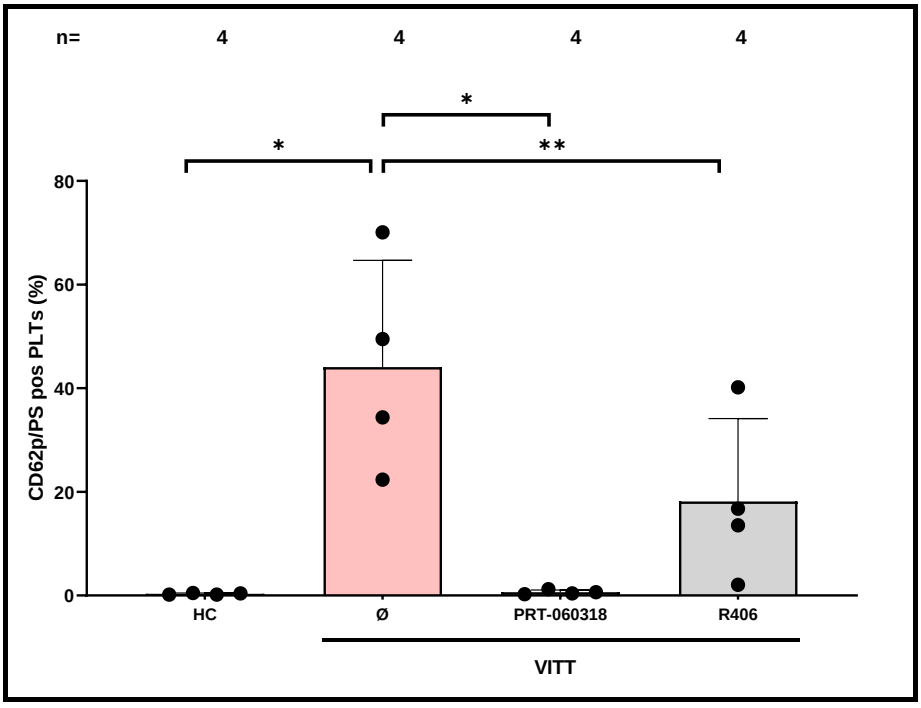

Figure 16 SYK inhibtion reduces VITT- Antibody incuced the expression of procoagulant platelet markers

The mean percentage ± SD of CD62p/PS positive platelets was of 44.08 ± 20.59% after incubation with VITT IgG compared to 0.3100 ± 0.17633% in healthy controls. The difference between means ± SEM between the “VITT + Ø” and the healthy control was 43.77 ± 10.32% with a p-value of 0.0240 in a sample size of four donors. VITT IgG induced the expression of procoagulant platelet markers CD62p and PS. Interestingly, when Syk inhibition was introduced with either PRT-060318 or R406, it was found that the expression of procoagulant platelet markers conferred by VITT-IgG was inhibited. Specifically, the mean percentage ± SD of CD62p/PS positive PLTS decreased to 0.6863± 0.4285% when preincubated with PRT-060318 and 18.13 ± 16.00% when preincubated with R406, compared to 44.08 ± 20.59% in the absence of inhibitors. The difference between means ± SEM between “VITT + PRT-060318” and “VITT+ Ø” was -43.45 ± 10.38% with a p-value of 0.0249. The difference between means (“VITT + R406”-“VITT + Ø”) ± SEM was -25.95 ± 3.155% with a p-value of 0.0038 in a sample size of respectively four donors.

These findings suggest Syk inhibition can effectively alter procoagulant platelet marker expression conveyed by VITT-IgG.

3.2.2 Syk inhibition effectively prevents clot formation conferred by VITT antibodies in a ex vivo flow model.

In this section we investigate whether Syk inhibition can effectively prevent VITT IgG mediated thrombus formation in a ex-vivo flow model.

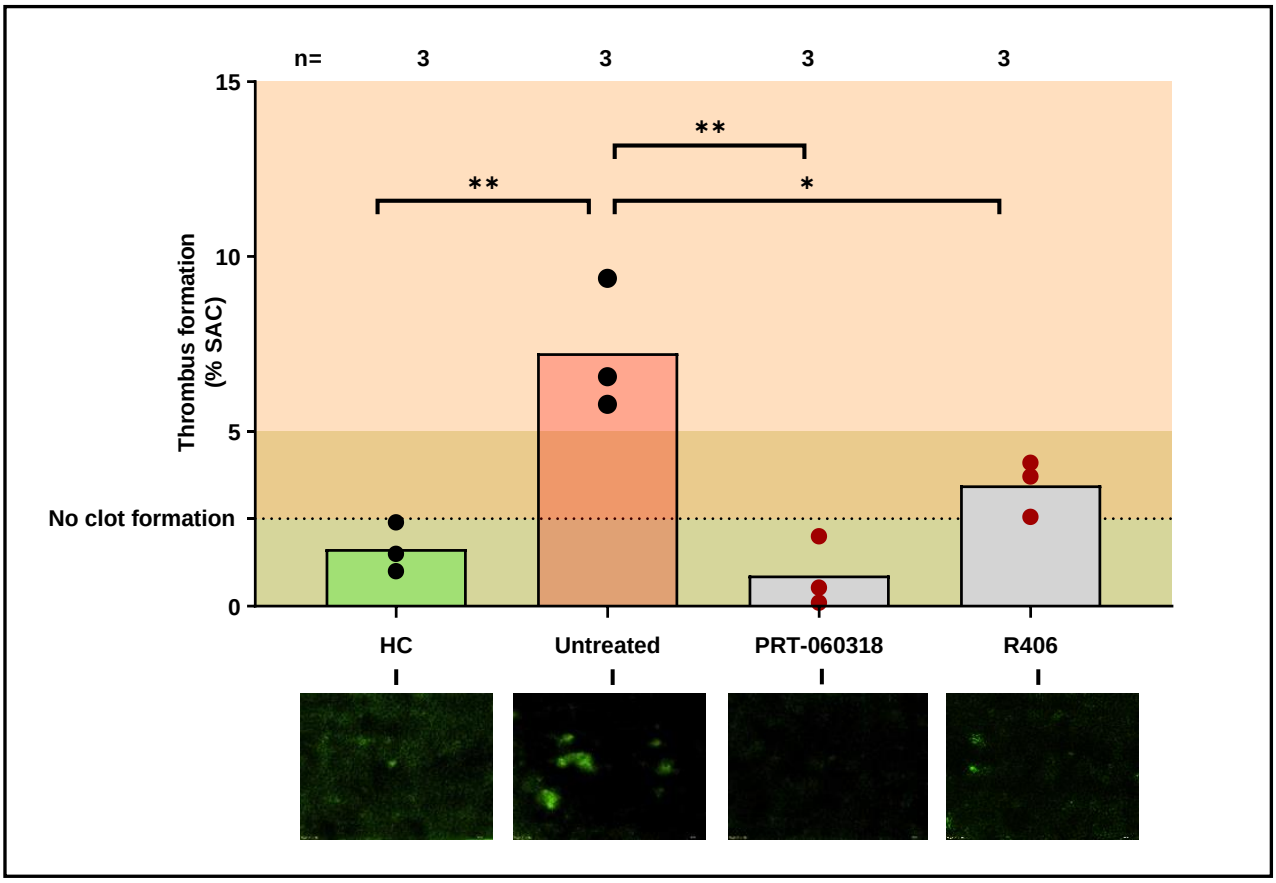

Figure 17 Syk inhibition prevents VITT- Antibody incuced thrombus formation in ex-vivo flow model (HC=healthy control)

VITT IgG induced thrombus formation in an ex-vivo flow chamber. The mean percentage ± SD of surface area covered by thrombus in the field of view (%SAC) was 7.237 ± 1.892% in the VITT IgG. In healthy control group the mean percentage ± SD of surface area covered by thrombus in the field of view (%SAC) was 1.633 ± 0.7095%. The difference between means ± SEM of the "Untreated" sample, meaning the sample with VITT IgG without Syk inhibition and the healthy control "HC" was 5.604 ± 1.167% and significant with a p-value of 0.0086. The sample size consisted of three donors. Thrombus formation in the ex-vivo flow chamber induced by VITT IgG was inhibited by Syk inhibition with PRT-060318 and R406. The mean percentage ± SD of surface area covered by thrombus in the field of view in the PRT-060318 preincubated blood sample was 0.8767 ± 0.9963% and with R406 3.455 ± 0.8022%. The difference between means ± SEM between the "PRT-060318" sample and the "Untreated" sample was -6.360 ± 1.235%, with a p-value of 0.0067. The difference between means

between the "R406" sample and the “Untreated" sample was -3.782 ± 1.187%, also significant with a p-value of 0.033 in a sample size of three.

This data suggests that Syk inhibition can effectively alter thrombus formation conveyed by VITT-IgG in an ex-vivo flow model.

3.3 The role of Syk inhibition in immune thrombocytopenia

The works of Marini et al. showed that platelet desialylation in immune thrombocytopenia is conveyed by antibodies and is FcγRIIA dependent[110]. In this section we searched to investigate whether the activation of the receptor through crosslink induces platelet desialylation in healthy platelets and if so, if Syk inhibition can be considered as a possible target. Healthy washed platelets were incubated with anti CD32 clone AT-10, followed by the crosslinking with the secondary F(ab)2 antibody to activate the FcγRIIA. Desialylation was determined as lectin binding RCA, compared to untreated samples. Where indicated, platelets were pre-incubated with Syk inhibitors PRT-060318 and R406.

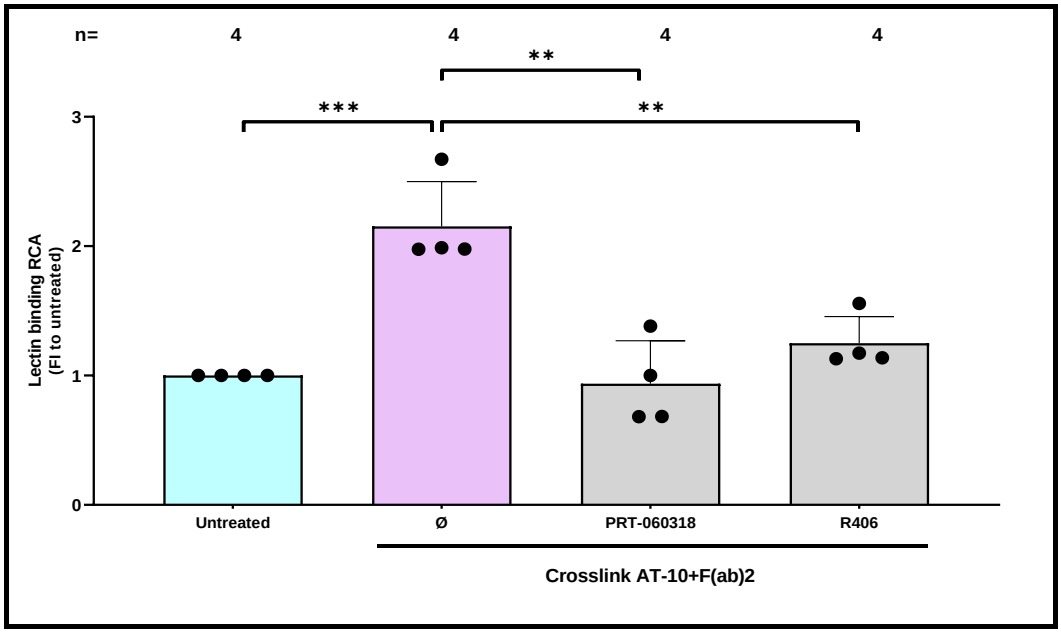

Figure 18 FcγRIIA mediated platelet desialylatlation can be reduced by Syk inhibition in healthy platelets

In our study, we observed that crosslinking of the FcγRIIA receptor resulted in platelet desialylation in vitro. Specifically, we found a significant increase in the percentage of desialylated platelets (% FI) in the crosslinked group, with a fold increase ± SD of 2.153 ± 0.3457% compared to the untreated group. This difference was statistically significant, with a p-value of 0.0005 and a difference between means ± SEM of 1.153 ± 0.1729%. Furthermore, we investigated the effect of Syk inhibition with PRT-060318 and R406 on FcγRIIA-mediated platelet desialylation. Our results showed that both PRT-060318 and R406 effectively inhibited platelet desialylation induced by FcγRIIA crosslinking. Specifically, we observed a significantly lower fold increase ± SD of desialylated platelets in the PRT-060318 sample with 0.9367 ± 0.3328% and the R406 sample with 1.25 ± 0.2061% compared to the crosslinked group. The differences between means ± SEM of desialylated platelets in the PRT-060318 and R406 samples compared to the crosslinked group were statistically significant, with p-values of 0.0023 and 0.0041, respectively. The difference between means ± SEM of desialylated platelets in the PRT-060318 and R406 samples was also statistically significant, with a p-value of 0.0278. The sample sample size was four.

3.3.1 Syk inhibition can effectively prevent antibody mediated desialylation in ITP

Considering the results of the previous experiment in which Syk inhibition was able to undo platelet desialylation conferred by FcγRIIA activation we extended our in-vitro investigations to Syk inhibition in ITP. Healthy washed platelets were incubated with sera isolated from four ITP patients that were previously found to present desialylating autoantibodies. Desialylation was determined as lectin binding RCA, compared to healthy control (HC). Where indicated, platelets were pre-incubated with Syk inhibitor PRT-06018 and R406.

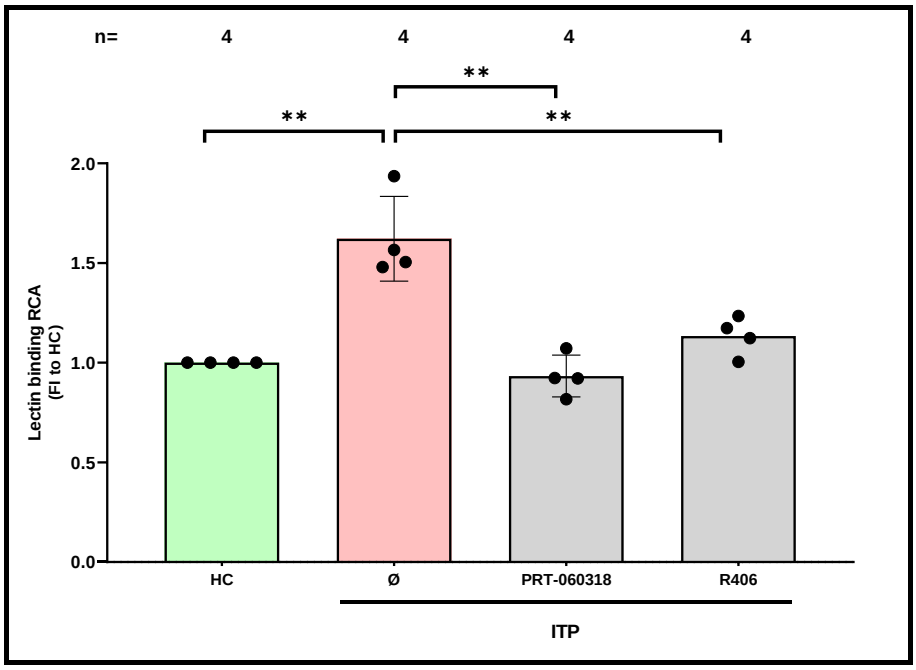

Figure 19 SYK inhibtion reduces ITP- Antibody mediated Lectin binding in healthy platelets

W observed that patients with immune thrombocytopenia (ITP) had increased platelet desialylation in-vitro compared to healthy individuals. Specifically, the ITP group had a significant fold increase ± SD in desialylated platelets of 1.622 ± 0.1063%, which was higher than the healthy control group. The difference between the means of the two groups was 0.6216 ± 0.2126% and the p-value was 0.0100. Furthermore, we investigated the effects of Syk inhibition with PRT-060318 and R406 on ITP-antibody mediated platelet desialylation in-

vitro. Our results showed that both inhibitors significantly reduced the fold increase ± SD of desialylated platelets compared to the untreated ITP group. Specifically, the PRT-060318 sample had a fold increase ± SD of 0.9332 ± 0.1047%, while the R406 sample had a fold increase ± SD of 1.134 ± 0.09739%. The difference between means ± SEM between the "ITP+ PRT" sample and the "ITP+ Ø" sample was -0.6884 ± 0.07236% with a p-value of 0.0025, and the difference between means ± SEM between the "ITP + R406" sample and the "ITP+ Ø" sample was -0.4878 ± 0.08214% with a p-value of 0.0095.

Syk inhibition with PRT-060318 and R406 inhibited ITP Antibody-mediated platelet desialylation in-vitro.

4 Discussion

In our study, we investigated the role of Syk in platelet function and the potential therapeutic benefits of inhibiting Syk in the prevention and treatment of platelet disorders. Our results showed that the inhibition of Syk significantly inhibited platelet activation, expression of apoptotic markers, and expression of procoagulant platelet markers in vitro, conveyed through the Fc gamma receptor IIa. These findings were highly significant as they underline the crucial role of Syk in these processes. Importantly, basic platelet function was maintained, indicating that inhibiting Syk can selectively target specific platelet functions while leaving other functions intact. This selective targeting is particularly noteworthy because it suggests a therapeutic approach that minimizes adverse effects on essential platelet functions.

The inhibition of Syk leads to a reduction in platelet activation, which can be beneficial in conditions such as thrombosis and cardiovascular diseases where excessive platelet activation can lead to pathological conditions. Our finding that the inhibition of Syk significantly inhibited platelet activation agrees with previous studies, thereby reinforcing the validity of our results. This concurrence with prior research solidifies the potential of Syk inhibitors as a reliable therapeutic strategy. In addition, we found that the inhibition of Syk prevented FcγRIIA mediated expression of apoptotic markers in platelets. This finding suggests that inhibiting Syk may have a potential protective effect on platelets and may be beneficial in the prevention of thrombotic diseases. This protective effect is particularly important in clinical settings where platelet preservation is crucial, and could lead to better management of thrombotic risks. Moreover, the study demonstrated that Syk inhibition can prevent the FcγRIIA mediated expression of procoagulant platelet markers. Procoagulant platelets have been implicated in a range of diseases, including deep vein thrombosis, myocardial infarction, and stroke. This suggests that Syk inhibition could abrogate FcγRIIA mediated platelet activation, apoptosis, and procoagulant platelet formation without affecting other physiological processes and may have significant therapeutic potential. The therapeutic potential of Syk inhibitors in these contexts could revolutionize the treatment protocols for

these conditions, offering new hope for patients with these serious health issues. Additionally, by focusing on a specific signaling pathway, the risk of unintended side effects might be reduced, making Syk inhibitors a more attractive option for long-term management of platelet-related disorders.

Recent research efforts have focused on vaccine-induced thrombotic thrombocytopenia (VITT) and highlighted the involvement of antibodies targeting the FcγRIIA receptor. This study aimed to investigate whether Syk inhibition can prevent the biological changes mediated by VITT antibodies in platelets. Our experiments revealed that Syk inhibition was able to prevent the expression of procoagulant platelet markers in vitro and disrupt clot formation in an ex-vivo flow chamber model. These findings suggest that Syk inhibition may have therapeutic potential in preventing the thrombotic events associated with VITT. This is a critical insight given the current focus on understanding and mitigating the side effects of vaccines. The ability to counteract vaccine-related thrombotic complications could enhance vaccine safety and public confidence, which is particularly relevant in the context of global vaccination campaigns.

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by low platelet counts and an increased risk of bleeding. Some patients with ITP develop autoantibodies that lead to desialylation of surface glycoproteins and platelet dysfunction via the involvement of FcγRIIA. Working with a small number of ITP patients presenting desialylating autoantibodies, Syk inhibition was able to prevent ITP antibody mediated platelet desialylation in our in-vitro study. Since the etiology of ITP varies between patients, the ability to tailor treatment options for specific individuals may significantly improve patient outcomes. These results suggest that Syk inhibition may be a promising approach for the personalized treatment of patients with ITP. This personalization of treatment is particularly promising in achieving better clinical outcomes. Tailoring treatments based on individual patient profiles not only enhances efficacy but also minimizes adverse effects, leading to improved patient quality of life.

The varying effects of different Syk inhibitors may also have significant implications for personalized medicine. Depending on a patient's individual genetic and molecular profile, certain Syk inhibitors may be more effective or have fewer side effects than others. The ability to match specific inhibitors to patient profiles could lead to more effective and safer treatments. Overall, this study provides valuable insights into the complex and multifaceted role of Syk inhibition in platelet disorders and highlights the need for further research in this area to fully understand its potential as a therapeutic approach. Continued investigation is crucial to unravel the nuances of Syk signaling and to optimize the use of Syk inhibitors in clinical practice, ultimately aiming to improve patient outcomes in platelet-related diseases.

In conclusion, the study suggests that Syk inhibition has significant potential in preventing platelet activation and thrombosis in patients with antibody-mediated disorders, including VITT. While Syk inhibition shows promise as a therapeutic approach, further research is needed to fully understand its mechanism of action and efficacy in larger patient populations. The use of Syk inhibitors may provide a personalized approach to treatment for patients with different platelet disorders. Our study also highlighted that different Syk inhibitors may have varying effects on FcγRIIA-mediated platelet activation, expression of apoptotic and procoagulant platelet markers. Specifically, the Syk inhibitor R406 was found to be less effective in inhibiting these effects compared to the Syk inhibitor PRT-060316. This suggests that the effectiveness of Syk inhibition may depend on the specific phosphorylation site targeted on Syk. This finding adds further complexity to the potential use of Syk inhibitors as a therapeutic approach for platelet disorders. The choice of Syk inhibitor may be critical in determining its efficacy and potential side effects. It is possible that the specific phosphorylation site targeted by a Syk inhibitor may be more important in certain platelet disorders than others, and further research may be needed to elucidate these differences.

5 Summary

Spleen tyrosine kinase (Syk) inhibitors have been shown to inhibit signal transduction of Fc-activating receptors, which play a key role in antibody-mediated disorders. In the proposed study, the elucidation of various aspects of Syk inhibition in human platelets was pursued. By analyzing in detail platelet behavior in flow cytometry, it was possible to demonstrate that Syk inhibition is able abrogate FcγRIIA mediated platelet activation and interrupts the expression of apoptotic and procoagulant platelet markers in-vitro.

In the context of the COVID-19 pandemic, recent research efforts centered around vaccine induced thrombotic thrombocytopenia (VITT) highlighted the involvement antibodies targeting the FcγRIIA inducing the formation of procoagulant platelets. The effect of Syk inhibition as a therapeutic approach for thrombotic thrombocytopenic remained largely unexplored. This study aimed to unravel whether the inhibition of Syk can fend off biological changes mediated by VITT antibodies in platelets. Presented results show that Syk inhibition is in fact able to prevent procoagulant platelet marker expression in vitro. Syk inhibition is furthermore able to disrupt clot formation conferred by VITT antibodies in an ex-vivo flow chamber model.

Considering these promising results, the effect of Syk inhibition on other biological changes mediated by antibodies was examined. In some patients with Immune thrombocytopenia (ITP) autoantibody mediated desialylation leads to platelet dysfunction via involvement of FcγRIIA. In this study, working with a small number of voluntary ITP patients presenting desialylating autoantibodies, after stimulation antibody stimulation could be prevented by Syk-inhibition in ex-vivo model using flow cytometry. These results suggest that Syk inhibition may be a promising approach in the sense of personalized treatment of patients with ITP.

By exploring the opportunities of Syk inhibition in healthy platelets, different effects of Syk-inhibition on platelet function were elucidated. While investigating the effect of Syk inhibition

in ex-vivo models of VITT and ITP, two platelet disorders with drastically different pathogenesis, it became apparent how the inhibition of Syk can prevent negative biological effects in platelets conveyed by antibodies. These results encourage further research with the outlook for new therapy approaches.

6 Zusammenfassung

Es konnte in vorangegangenen Studien gezeigt, dass Tyrosinkinase Syk-Inhibitoren die Signaltransduktion von Fc-aktivierenden Rezeptoren hemmen, die eine Schlüsselrolle bei Antikörper-vermittelten Erkrankungen spielen. In dieser Studie wurde versucht sich ein besseres Verständnis über verschiedene Aspekte der Syk-Hemmung bei menschlichen Blutplättchen zu verschaffen. Durch eine ausführliche Untersuchung der Thrombozyten in der Durchflusszytometrie konnte gezeigt werden, dass Syk-Hemmung in der Lage ist, die FcγRIIA-vermittelte Thrombozytenaktivierung und Expression von apoptotischen und prokoagulanten Blutplättchen-Marker in vitro zu unterbinden.

Im Zusammenhang mit der COVID-19-Pandemie hoben jüngste Forschungsanstrengungen rund um die impfstoffinduzierte thrombotische Thrombozytopenie (VITT) die Beteiligung von Antikörpern hervor, die über den FcγRIIA die Überexpression von prokoagulaten Blutplättchen-Markern vermitteln. Syk-Hemmung als Therapieansatz für VITT blieb bislang größtenteils unerforscht. Diese Studie zielte darauf ab, herauszufinden, ob die Hemmung von Syk biologische Veränderungen abwehren kann, die durch VITT Antikörper in Blutplättchen vermittelt werden. Die vorgestellten Ergebnisse zeigen, dass Syk-Hemmung in der Lage ist die VITT antikörpervermittelte Expression von prokoagulanten Blutplättchen-Markern in vitro zu verhindern. Syk-Hemmung ist darüber hinaus in der Lage, die Gerinnselbildung durch VITT-Antikörper in einem Ex-vivo Flussmodel zu unterbrechen.

Unter Berücksichtigung dieser vielversprechenden Ergebnisse wurde die Wirkung von Syk-Inhibitoren auf andere biologische Veränderungen, die durch Antikörper vermittelt werden,

untersucht. Bei einigen Patienten mit Immunthrombozytopenie (ITP) führt die Autoantikörper-vermittelte Desialylierung der Blutplättchen über die Beteiligung von FcγRIIA zu einer Thrombozytenfunktionsstörung. Durch die Zusammenarbeit mit ITP-Patienten, die dialysierende Autoantikörper präsentierten, konnte in der Durchflusszytometrie gezeigt werden wie die Hemmung von Syk autoantikörpervermittelte desialylierung verhindert. Diese Ergebnisse deuten darauf hin, dass Syk-Hemmung ein vielversprechender Ansatz im Sinne einer personalisierten Behandlung von Patienten mit ITP sein könnte.

Durch die Erforschung der Möglichkeiten der Syk-Hemmung in gesunden Thrombozyten wurden verschiedene Auswirkungen dieser auf die Thrombozytenfunktion gezeigt. Bei der Untersuchung der Wirkung der Syk-Hemmung in Ex-vivo-Modellen von VITT und ITP, zwei Thrombozytenerkrankungen mit drastisch unterschiedlicher Pathogenese, wurde deutlich, wie die Hemmung von Syk negative biologische Effekte in Thrombozyten verhindern kann, die durch Antikörper vermittelt werden. Diese Ergebnisse ermutigen zu weiteren Forschungen mit der Aussicht auf neue Therapieansätze.

7 Bibliography

1. Virchow R. Gesammalte abhandlungen zur wissenschaftlichen medtzin. *Medinger Sohn & Co*. 1856:219–732.

2. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. *Physiol Rev*. Jan 2013;93(1):327-58. doi:10.1152/physrev.00016.2011

3. Holinstat M. Normal platelet function. *Cancer Metastasis Rev*. Jun 2017;36(2):195-198. doi:10.1007/s10555-017-9677-x

4. Machlus KR IJ, Jr. The incredible journey: From megakaryocyte development 1638 to platelet formation. *Cell Biol*. 2013;201(6):785-96.

5. Bizzozero. On a new blood particle and its role in thrombosis and blood coagulation. *Huber*. 1982;90

6. Lefrançais E, Ortiz-Muñoz G, Caudrillier A, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*. 2017;544 (7648):105–109.

7. Harker LA, Roskos LK, Marzec UM, et al. Effects of megakaryocyte growth and development factor on platelet production, platelet life span, and platelet function in healthy human volunteers. *Blood*. Apr 15 2000;95(8):2514-22.

8. Slichter S. Relationship between platelet count and bleeding risk in thrombocytopenic patients. *Transfus Med Rev*. 2004;18(3):153-167.

9. Jurk K. Analysis of platelet function and dysfunction. *Hamostaseologie*. 2015;35(1):60-72. doi:10.5482/HAMO-14-09-0047

10. Jackson S. Arterial thrombosis--insidious, unpredictable and deadly. *Nat Med*. 2011;17(11):1423-1436.

11. Ahmad FB, Anderson RN. The Leading Causes of Death in the US for 2020. *JAMA*. May 11 2021;325(18):1829-1830. doi:10.1001/jama.2021.5469

12. Neunert C NN, Norman G, et al. Severe bleeding events in adults and children with primary immune thrombocytopenia: a systematic review. *J Thromb Haemost*. 2015;13(3):457-464.

13. Gawaz M, Vogel S. Platelets in tissue repair: control of apoptosis and interactions with regenerative cells. *Blood*. Oct 10 2013;122(15):2550-4. doi:10.1182/blood-2013-05-468694

14. Ewelina M. Golebiewska AWP. Platelet secretion: From haemostasis to wound healing and beyond. *Blood Reviews*. 2015;29(3):153-162.

15. Ghoshal K, Bhattacharyya M. Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. *ScientificWorldJournal*. 2014;2014:781857. doi:10.1155/2014/781857

16. Estevez B, Du X. New Concepts and Mechanisms of Platelet Activation Signaling. *Physiology (Bethesda)*. Mar 2017;32(2):162-177. doi:10.1152/physiol.00020.2016

17. Varga-Szabo D, Braun A, Nieswandt B. Calcium signaling in platelets. *J Thromb Haemost*. Jul 2009;7(7):1057-66. doi:10.1111/j.1538-7836.2009.03455.x

18. Tsuboi S. Calcium integrin-binding protein activates platelet integrin alpha IIbbeta 3. *J Biol Chem*. Jan 18 2002;277(3):1919-23. doi:10.1074/jbc.M110643200

19. Collen D, Lu HR, Stassen JM, et al. Antithrombotic effects and bleeding time prolongation with synthetic platelet GPIIb/IIIa inhibitors in animal models of platelet-mediated thrombosis. *Thromb Haemost*. Jan 1994;71(1):95-102.

20. Coller BS, Shattil SJ. The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood*. Oct 15 2008;112(8):3011-25. doi:10.1182/blood-2008-06-077891

21. Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying thrombus formation. *J Thromb Haemost*. Jul 2003;1(7):1602-12. doi:10.1046/j.1538-7836.2003.00267.x

22. Stenberg PE MR, Shuman MA, Jacques YV, Bainton DF. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol*. 1985;101(3):880-886.

23. GH van Zanten SdG, PJ Slootweg, HF Heijnen, TM Connolly, PG de Groot, JJ Sixma. Increased platelet deposition on atherosclerotic coronary arteries. *J Clin Invest*. 1994;93:615.

24. Kessler CM, Floyd CM, Rick ME, Krizek DM, Lee SL, Gralnick HR. Collagen-factor VIII/von Willebrand factor protein interaction. *Blood*. Jun 1984;63(6):1291-8.

25. Reininger AJ, Heijnen HF, Schumann H, Specht HM, Schramm W, Ruggeri ZM. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. *Blood*. May 1 2006;107(9):3537-45. doi:10.1182/blood-2005-02-0618

26. Jarvis GE, Atkinson BT, Snell DC, Watson SP. Distinct roles of GPVI and integrin alpha(2)beta(1) in platelet shape change and aggregation induced by different collagens. *Br J Pharmacol*. Sep 2002;137(1):107-17. doi:10.1038/sj.bjp.0704834

27. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin alphaIIb beta3 signaling in platelets. *J Thromb Haemost*. Aug 2005;3(8):1752-62. doi:10.1111/j.1538-7836.2005.01429.x

28. Gilberto R. Sambrano EJW, Yao-Wu Zheng, Wei Huang & Shaun R. Coughlin. Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature*. 2001;413(6851):74-78.

29. Kahn ML, Zheng YW, Huang W, et al. A dual thrombin receptor system for platelet activation. *Nature*. Aug 13 1998;394(6694):690-4. doi:10.1038/29325

30. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost*. Aug 2005;3(8):1800-14. doi:10.1111/j.1538-7836.2005.01377.x

31. Kim S FC, Lecchi A, et al. Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. *Blood*. 2002;99(10):3629-3636.

32. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha(q)-deficient mice. *Nature*. Sep 11 1997;389(6647):183-6. doi:10.1038/38284

33. Gremmel T CA, Steiner S, et al. Is TRAP-6 suitable as a positive control for platelet reactivity when assessing response to clopidogrel? *Platelets*. 2010;21(7):515-521.

34. Fogelson AL, Wang NT. Platelet dense-granule centralization and the persistence of ADP secretion. *Am J Physiol*. Mar 1996;270(3 Pt 2):H1131-40. doi:10.1152/ajpheart.1996.270.3.H1131

35. Offermanns S. Activation of platelet function through G protein-coupled receptors. *Circ Res*. 2006;99(12):1293-1304.

36. Hirsch E, Bosco O, Tropel P, et al. Resistance to thromboembolism in PI3Kgamma-deficient mice. *FASEB J*. Sep 2001;15(11):2019-21. doi:10.1096/fj.00-0810fje

37. Vogt S, Grosse R, Schultz G, Offermanns S. Receptor-dependent RhoA activation in G12/G13-deficient cells: genetic evidence for an involvement of Gq/G11. *J Biol Chem*. Aug 1 2003;278(31):28743-9. doi:10.1074/jbc.M304570200

38. McEver RP, Martin MN. A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J Biol Chem*. Aug 10 1984;259(15):9799-804.

39. McEver RP BJ, Moore KL, Marshall-Carlson L, Bainton DF. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J Clin Invest*. 1989;84(1):92-99.

40. Urzainqui A, Serrador JM, Viedma F, et al. ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. *Immunity*. Oct 2002;17(4):401-12. doi:10.1016/s1074-7613(02)00420-x

41. DA Guyer KM, EB Lynam, CM Schammel, S Rogelj, RP McEver, LA Sklar. P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand for L-selectin in neutrophil aggregation. *Blood*. 1996;88(7):2415–2421.

42. Kansas G. Selectins and their ligands: current concepts and controversies. *Blood*. 1996;88(9):3259-3287.

43. Furie B, Furie BC. Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation. *Trends Mol Med*. Apr 2004;10(4):171-8. doi:10.1016/j.molmed.2004.02.008

44. Thomas MR, Storey RF. The role of platelets in inflammation. *Thromb Haemost*. Aug 31 2015;114(3):449-58. doi:10.1160/TH14-12-1067

45. Brühl Mv, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz Mea. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *The Journal of experimental medicine*. 2012;209(4):819–835.

46. Lisman T. Platelet-neutrophil interactions as drivers of inflammatory and thrombotic disease. *Cell and tissue research*. 2018;371(3):567–576.

47. Ataga KI, Kutlar A, Kanter J, et al. Crizanlizumab for the Prevention of Pain Crises in Sickle Cell Disease. *N Engl J Med*. Feb 2 2017;376(5):429-439. doi:10.1056/NEJMoa1611770

48. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. *J Biol Chem*. Sep 15 1985;260(20):11107-14.

49. McArthur K, Chappaz S, Kile BT. Apoptosis in megakaryocytes and platelets: the life and death of a lineage. *Blood*. 2018;131(6):605-610. doi:10.1182/blood-2017-11-742684

50. Goette NP, Glembotsky AC, Lev PR, et al. Platelet Apoptosis in Adult Immune Thrombocytopenia: Insights into the Mechanism of Damage Triggered by Auto-Antibodies. *PLoS One*. 2016;11(8):e0160563. doi:10.1371/journal.pone.0160563

51. Bevers EM WP. Getting to the Outer Leaflet: Physiology of Phosphatidylserine Exposure at the Plasma Membrane. *Physiol Rev*. 2016;96(2):605-645.

52. Crowley LC, Christensen ME, Waterhouse NJ. Measuring Mitochondrial Transmembrane Potential by TMRE Staining. *Cold Spring Harb Protoc*. Dec 1 2016;2016(12)doi:10.1101/pdb.prot087361

53. Heemskerk JW VW, Feijge MA, Reutelingsperger CP, Lindhout T. Collagen but not fibrinogen surfaces induce bleb formation, exposure of phosphatidylserine, and procoagulant activity of adherent platelets: evidence for regulation by protein tyrosine kinase-dependent Ca2+ responses. *Blood*. 1997;90:2615–2625.

54. Hess MW, Siljander P. Procoagulant platelet balloons: evidence from cryopreparation and electron microscopy. *Histochem Cell Biol*. May 2001;115(5):439-43. doi:10.1007/s004180100272

55. Jackson SP SS. Procoagulant platelets: are they necrotic? *Blood*. 2010;116(12):2011-2018.

56. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol*. Jan 1995;146(1):3-15.

57. Remenyi G, Szasz R, Friese P, Dale GL. Role of mitochondrial permeability transition pore in coated-platelet formation. *Arterioscler Thromb Vasc Biol*. Feb 2005;25(2):467-71. doi:10.1161/01.ATV.0000152726.49229.bf

58. Mason KD, Carpinelli MR, Fletcher JI, et al. Programmed anuclear cell death delimits platelet life span. *Cell*. Mar 23 2007;128(6):1173-86. doi:10.1016/j.cell.2007.01.037

59. EO. Agbani APA. Procoagulant platelets: generation, function, and therapeutic targeting in thrombosis. *Blood*. 2017;130(20):2171-2179.

60. Denorme F, Campbell RA. Procoagulant platelets: novel players in thromboinflammation. *Am J Physiol Cell Physiol*. Oct 1 2022;323(4):C951-C958. doi:10.1152/ajpcell.00252.2022

61. Takai T. Roles of Fc receptors in autoimmunity. *Nature Reviews Immunology*. 2002/08/01 2002;2(8):580-592. doi:10.1038/nri856

62. Daeron M. Fc receptor biology. *Annu Rev Immunol*. 1997;15:203-34. doi:10.1146/annurev.immunol.15.1.203

63. Arman M, Krauel K. Human platelet IgG Fc receptor FcgammaRIIA in immunity and thrombosis. *J Thromb Haemost*. Jun 2015;13(6):893-908. doi:10.1111/jth.12905

64. Qiao J, Al-Tamimi M, Baker RI, Andrews RK, Gardiner EE. The platelet Fc receptor, FcgammaRIIa. *Immunol Rev*. Nov 2015;268(1):241-52. doi:10.1111/imr.12370

65. Fridman WH. Fc receptors and immunoglobulin binding factors. *FASEB J*. Sep 1991;5(12):2684-90. doi:10.1096/fasebj.5.12.1916092

66. Ravetch JV, Bolland S. IgG Fc receptors. *Annu Rev Immunol*. 2001;19:275-90. doi:10.1146/annurev.immunol.19.1.275

67. Kelton JG, Sheridan D, Santos A, et al. Heparin-induced thrombocytopenia: laboratory studies. *Blood*. Sep 1988;72(3):925-30.

68. Chakraborty S, Gonzalez J, Edwards K, et al. Proinflammatory IgG Fc structures in patients with severe COVID-19. *Nat Immunol*. Jan 2021;22(1):67-73. doi:10.1038/s41590-020-00828-7

69. Althaus K, Moller P, Uzun G, et al. Antibody-mediated procoagulant platelets in SARS-CoV-2-vaccination associated immune thrombotic thrombocytopenia. *Haematologica*. Aug 1 2021;106(8):2170-2179. doi:10.3324/haematol.2021.279000

70. Montague SJ, Smith, C. W., Lodwick, C. S., Stoneley, C., Roberts, M., Lowe, G. C., Lester, W. A., Watson, S. P., & Nicolson, P. Anti-platelet factor 4 immunoglobulin G levels in vaccine-induced immune thrombocytopenia and thrombosis: Persistent positivity through 7 months. Research and practice in thrombosis and haemostasis. 2022;6(3):e12707.

71. Watson SP, Herbert JM, Pollitt AY. GPVI and CLEC-2 in hemostasis and vascular integrity. *J Thromb Haemost*. Jul 2010;8(7):1456-67. doi:10.1111/j.1538-7836.2010.03875.x

72. Sullam PM, Hyun WC, Szollosi J, Dong J, Foss WM, Lopez JA. Physical proximity and functional interplay of the glycoprotein Ib-IX-V complex and the Fc receptor FcgammaRIIA on the platelet plasma membrane. *J Biol Chem*. Feb 27 1998;273(9):5331-6. doi:10.1074/jbc.273.9.5331

73. Indik ZK, Salehuddin M, McKenzie SE, Kelly C, Levinson AI, Schreiber AD. Human Fc gamma RII: the structure of the Fc gamma RII cytosolic domain governs phagocytic function. *Trans Assoc Am Physicians*. 1992;105:214-21.

74. Rosenfeld SI LR, Leddy JP, Phipps DC, Abraham GN, Anderson CL. Human platelet Fc receptor for immunoglobulin G. Identification as a 40,000-molecular-weight membrane protein shared by monocytes. *J Clin Invest*. 1985;76(6):2317-2322.

75. Yanaga F, Poole A, Asselin J, et al. Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor. *Biochem J*. Oct 15 1995;311 (Pt 2):471-8. doi:10.1042/bj3110471

76. Blake RA AJ, Walker T, Watson SP. Fc gamma receptor II stimulated formation of inositol phosphates in human platelets is blocked by tyrosine kinase inhibitors and associated with tyrosine phosphorylation of the receptor. *FEBS Lett*. 1994;342(1):15–18.

77. Moroi AJ, Watson SP. Impact of the PI3-kinase/Akt pathway on ITAM and hemITAM receptors: haemostasis, platelet activation and antithrombotic therapy. *Biochem Pharmacol*. Apr 1 2015;94(3):186-94. doi:10.1016/j.bcp.2015.02.004

78. Mocsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol*. Jun 2010;10(6):387-402. doi:10.1038/nri2765

79. Poole A, Gibbins JM, Turner M, et al. The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J*. May 1 1997;16(9):2333-41. doi:10.1093/emboj/16.9.2333

80. Turner M, Schweighoffer E, Colucci F, Di Santo JP, Tybulewicz VL. Tyrosine kinase SYK: essential functions for immunoreceptor signalling. *Immunol Today*. Mar 2000;21(3):148-54. doi:10.1016/s0167-5699(99)01574-1

81. Sharman J, Di Paolo J. Targeting B-cell receptor signaling kinases in chronic lymphocytic leukemia: the promise of entospletinib. *Ther Adv Hematol*. Jun 2016;7(3):157-70. doi:10.1177/2040620716636542

82. Steven M. Horwitz TAF, Brian T. Hess, Michael S. Khodadoust, Youn H. Kim, Javier Munoz, Manish R. Patel, Tycel J. Phillips, Stephen D. Smith, Sonali M. Smith, Ryan A. Wilcox, Matt R. Birrell, Janet M. Leeds, Pamela B. Conley, Glenn C. Mich. A Phase 2 Study of the

Dual SYK/JAK Inhibitor Cerdulatinib Demonstrates Good Tolerability and Clinical Response in Relapsed/Refractory Peripheral T-Cell Lymphoma and Cutaneous T-Cell Lymphoma. *Blood*. 2019;134

83. Reilly MP, Sinha U, Andre P, et al. PRT-060318, a novel Syk inhibitor, prevents heparin-induced thrombocytopenia and thrombosis in a transgenic mouse model. *Blood*. Feb 17 2011;117(7):2241-6. doi:10.1182/blood-2010-03-274969

84. Hoellenriegel J, Coffey GP, Sinha U, et al. Selective, novel spleen tyrosine kinase (Syk) inhibitors suppress chronic lymphocytic leukemia B-cell activation and migration. *Leukemia*. Jul 2012;26(7):1576-83. doi:10.1038/leu.2012.24

85. Newland A, Lee EJ, McDonald V, Bussel JB. Fostamatinib for persistent/chronic adult immune thrombocytopenia. *Immunotherapy*. Jan 2018;10(1):9-25. doi:10.2217/imt-2017-0097

86. Connell NT, Berliner N. Fostamatinib for the treatment of chronic immune thrombocytopenia. *Blood*. May 9 2019;133(19):2027-2030. doi:10.1182/blood-2018-11-852491

87. Bussel JB, Arnold DM, Boxer MA, et al. Long-term fostamatinib treatment of adults with immune thrombocytopenia during the phase 3 clinical trial program. *Am J Hematol*. May 2019;94(5):546-553. doi:10.1002/ajh.25444

88. Weinblatt ME, Kavanaugh A, Burgos-Vargas R, et al. Treatment of rheumatoid arthritis with a Syk kinase inhibitor: a twelve-week, randomized, placebo-controlled trial. *Arthritis Rheum*. Nov 2008;58(11):3309-18. doi:10.1002/art.23992

89. Friedberg JW SJ, Sweetenham J, et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood*. 2010;115(13):2578-2585.

90. Sokratis A Apostolidis AS, Heather M Giannini, Rishi R Goel, Divij Mathew, Aae Suzuki, Amy E Baxter, Allison R Greenplate, Cécile Alanio, Mohamed Abdel-Hakeem, Derek A Oldridge, Josephine Giles, Jennifer E Wu, Zeyu Chen, Yinghui Jane Huang, Aj. Signaling through FcγRIIA and the C5a-C5aR pathway mediates platelet hyperactivation in COVID-19. *bioRxiv*. 2021;

91. Callaway E, Ledford H, Viglione G, Watson T, Witze A. COVID and 2020: An extraordinary year for science. *Nature*. Dec 2020;588(7839):550-552. doi:10.1038/d41586-020-03437-4

92. Schultz NH, Sorvoll IH, Michelsen AE, et al. Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. *N Engl J Med*. Jun 3 2021;384(22):2124-2130. doi:10.1056/NEJMoa2104882

93. Greinacher A, Thiele T, Warkentin TE, Weisser K, Kyrle PA, Eichinger S. Thrombotic Thrombocytopenia after ChAdOx1 nCov-19 Vaccination. *N Engl J Med*. Jun 3 2021;384(22):2092-2101. doi:10.1056/NEJMoa2104840

94. See I SJ, Lale A, et al. US Case Reports 371 of Cerebral Venous Sinus Thrombosis With Thrombocytopenia After Ad26.COV2.S Vaccination. *JAMA*. 2021;325(24):2448-2456.

95. Scully M, Singh D, Lown R, et al. Pathologic Antibodies to Platelet Factor 4 after ChAdOx1 nCoV-19 Vaccination. *N Engl J Med*. Jun 10 2021;384(23):2202-2211. doi:10.1056/NEJMoa2105385

96. Amiral J BF, Dreyfus M, et al. Platelet factor 4 complexed to heparin is the target for antibodies generated in heparin-induced thrombocytopenia. *Thromb Haemost*. 1992;68(1):95-96.

97. John G. Kelton DS, Aurelio Santos, James Smith, Karen Steeves, Carol Smith, Claudia Brown, William G. Murphy. Heparin-Induced Thrombocytopenia: Laboratory Studies. *Blood*. 1988;72(3):925-930.

98. Huynh A, Kelton JG, Arnold DM, Daka M, Nazy I. Antibody epitopes in vaccine-induced immune thrombotic thrombocytopaenia. *Nature*. Aug 2021;596(7873):565-569. doi:10.1038/s41586-021-03744-4

99. Singh A, Toma F, Uzun G, et al. The interaction between anti-PF4 antibodies and anticoagulants in vaccine-induced thrombotic thrombocytopenia. *Blood*. 2022;139(23):3430-3438. doi:10.1182/blood.2021013839

100. Provan D, Arnold DM, Bussel JB, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *Blood Adv*. Nov 26 2019;3(22):3780-3817. doi:10.1182/bloodadvances.2019000812

101. Zeller B, Rajantie J, Hedlund-Treutiger I, et al. Childhood idiopathic thrombocytopenic purpura in the Nordic countries: epidemiology and predictors of chronic disease. *Acta Paediatr*. Feb 2005;94(2):178-84. doi:10.1111/j.1651-2227.2005.tb01887.x

102. Harrington WJ, Minnich V, Hollingsworth JW, Moore CV. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J Lab Clin Med*. Jul 1951;38(1):1-10.

103. McMillan R, Tani P, Mason D. The demonstration of antibody binding to platelet-associated antigens in patients with immune thrombocytopenic purpura. *Blood*. Dec 1980;56(6):993-5.

104. Iraqi M, Perdomo J, Yan F, Choi PY, Chong BH. Immune thrombocytopenia: antiplatelet autoantibodies inhibit proplatelet formation by megakaryocytes and impair platelet production in vitro. *Haematologica*. May 2015;100(5):623-32. doi:10.3324/haematol.2014.115634

105. McMillan R. The pathogenesis of chronic immune thrombocytopenic purpura. *Semin Hematol*. Oct 2007;44(4 Suppl 5):S3-S11. doi:10.1053/j.seminhematol.2007.11.002

106. George JN. Definition, diagnosis and treatment of immune thrombocytopenic purpura. *Haematologica*. Jun 2009;94(6):759-62. doi:10.3324/haematol.2009.007674

107. Neunert C, Lim W, Crowther M, et al. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. *Blood*. Apr 21 2011;117(16):4190-207. doi:10.1182/blood-2010-08-302984

108. Vianelli N, Galli M, de Vivo A, et al. Efficacy and safety of splenectomy in immune thrombocytopenic purpura: long-term results of 402 cases. *Haematologica*. Jan 2005;90(1):72-7.

109. Li J vdWD, Zhu G, et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. *Nat Commun*. 2015;6:7737.

110. Marini I, Zlamal J, Faul C, et al. Autoantibody-mediated desialylation impairs human thrombopoiesis and platelet lifespan. *Haematologica*. Jan 1 2021;106(1):196-207. doi:10.3324/haematol.2019.236117

111. Rosenfeld SI LR, Leddy JP, Phipps DC, Abraham GN, Anderson CL. Human platelet Fc receptor for immunoglobulin G. Identification as a 40,000-molecular-weight membrane protein shared by monocytes. *J Clin Invest*. 1985;76:2317–22.

112. Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem*. Jan 23 1998;273(4):2030-4. doi:10.1074/jbc.273.4.2030

113. Ceruso MA, McComsey DF, Leo GC, et al. Thrombin receptor-activating peptides (TRAPs): investigation of bioactive conformations via structure-activity, spectroscopic, and computational studies. *Bioorg Med Chem*. Nov 1999;7(11):2353-71. doi:10.1016/s0968-0896(99)00180-7

114. Baluom M, Grossbard EB, Mant T, Lau DT. Pharmacokinetics of fostamatinib, a spleen tyrosine kinase (SYK) inhibitor, in healthy human subjects following single and multiple oral dosing in three phase I studies. *Br J Clin Pharmacol*. Jul 2013;76(1):78-88. doi:10.1111/bcp.12048

115. Nazy I SU, Arnold DM, et al. Recommendations for the clinical and laboratory diagnosis of VITT against COVID-19: Communication from the ISTH SSC Subcommittee on Platelet Immunology. . *J Thromb Haemost* 2021;19(6):1585-1588.

116. Roest M RA, Zwaginga JJ, King MR, Heemskerk JW; Biorheology Subcommittee of the SSC of the ISTH. Flow chamber-based assays to measure thrombus formation in vitro: requirements for standardization. . *J Thromb Haemost* 2011;9(11):2322-2324. doi:10.1111/j.1538-7836.2011.04492.x

8 Author contribution

The completion of the thesis was carried out in the Institute Clinical and experimental Transfusion Medicine Tübingen (IKET) under the supervision and guidance of Prof. Dr. T. Bakchoul as my habilitated supervisor and of Dr. A. Singh as my doctoral supervisor.

The design of the study was carried out in collaboration with Prof. Dr. med. T. Bakchoul and Dr. rer. nat. A. Singh. All experiments were carried out by me (if not indicated otherwise) after receiving training by laboratory personnel. The statistical evaluation was carried out under the guidance of Dr. rer. nat. A. Singh. I can ensure that I have written the manuscript independently and that I have not used any sources other than those I have indicated.

9 Publications

I am pleased to acknowledge that parts of this dissertation have been published in an article entitled “The interaction between anti-PF4 antibodies and anticoagulants in vaccine-induced thrombotic thrombocytopenia” which was co-authored by Anurag Singh, Filip Toma, Günalp Uzun, Teresa R. Wagner, Lisann Pelzl, Jan Zlamal, Verena Freytag, Karoline Weich, Stefanie Nowak-Harnau, Ulrich Rothbauer, Karina Althaus, and Tamam Bakchoul. The article was published in the journal Blood, volume 139, issue 23, pages 3430-3438 in 2019. I am grateful for the opportunity to contribute to this publication and for the valuable experience it has provided me. Additionally, I also contributed to the manuscript entitled “Platelet spleen tyrosine kinase is a key regulator of anti-PF4-induced immunothrombosis,” which is currently under review.

10 Acknowledgments

I would like to express my deep appreciation to all those who have supported me throughout this project, particularly Prof. Dr. med. T. Bakchoul for his outstanding mentorship and for welcoming me into a team of dynamic, competent, and kind-hearted individuals whose collaborative efforts have made my dissertation a meaningful and enjoyable experience. I am also grateful to my project lead, Dr. Anurag Singh, for sharing his extensive theoretical and practical expertise, guiding me through the development of my thesis, and providing valuable advice that will undoubtedly shape my professional career. Furthermore, I was grateful to have been invited to present my work regarding the assessment of the impact of Syk inhibition on antibody-mediated desialylation and therapy for immune thrombocytopenia in September 2021 at the 54th meeting of the German Society for Transfusion Medicine and Immunohematology (DGTI 2021).