Aus dem

Institut für Klinische und Experimentelle Transfusionsmedizin

der Universität Tübingen

Autoantibody-induced desialylation of platelets in immune thrombocytopenia: Potential impact on platelet function and survival

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Zlamal, Jan

2023

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Tag der Disputation: 24.05.2023

For my wife

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Abbreviations

AAb	Autoantibody
Ab	Antibody
ACD-A	Anticoagulant citrate dextrose solution
ADP	Adenosine diphosphate
AML	Acute myloid leukemia
AMR	Ashwell-Morrell-receptor
APC	Antigen presenting cell
ASH	American Society for Haematology
BAFF	B-cell activating factor
B-cell	B lymphocyte
B-reg	B-regulatory cell
BSA	Bovine serum albumin
C1q	Complement component 1q
C2	Complement component 2
C3	Complement component 3
C4	Complement component 4
C5b-9	Complement membrane attack complex
CD	Cluster of differentiation
CD154	CD40 ligand
CD16a	Fc-gamma-receptor IIIa
CD19	B-lymphocyte antigen CD19
CD20	B-lymphocyte antigen CD20
CD24	Signal transducer CD24
CD25	Interleukin-2 receptor alpha chain
CD3	Cluster of differentiation 3
CD32a/c	Fc-gamma-receptor IIa/c
CD38	Cyclic ADP ribose hydrolase
CD4	Cluster of differentiation 4
CD40L	Cluster of differentiation 154
CD41	GP IIb

CD42	GP Ib
CD62P	P-selectin
CD64	Fc-gamma-receptor I
CD8	Cluster of differentiation 8
DAF	Complement decay accelerating factor
ECL	Erythrina crista galli lectin
ECM	Extracellular matrix protein
EDTA	Ethylenediaminetetraacetic acid
F(ab)	Antigen-binding fragment
FC	Flow cytometry
Fc	Fragment crystallizable region
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) Phenylhydrazone
FcγR	Fc-gamma-receptor
FI	Fold increase
FITC	Fluorescein isothiocyanate
Foxp	Forkhead box P
FSC	Forward scatter
GP IIb/IIIa	Fibrinogen receptor
GP	Glycoprotein
GPIb-IX-V	von-Willebrand-receptor
GPVI	Collagen receptor
HC	Healthy control
HIPA	Heparin induced platelet aggregation assay
HSA	Human serum albumin
IgG	Immunoglobulin G
IL-10	Interleukin-10
IL-2	Interleukin-2
ITAM	Immuno receptor tyrosine-based activation motif
ITIM	Immuno receptor tyrosine-based inhibitory motif
ITP	Immune thrombocytopenia
IV.3	Anti-human CD32a monoclonal antibody

IVIG	Intravenous immunoglobulin
JAK1	Janus kinase 1
LBA	Lectin binding assay
MAIPA	Monoclonal antibody-specific immobilization of platelet antigen
MCP	Membrane cofactor protein
MDS	Myelodysplastic syndrome
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIRL	Membrane inhibitor of reactive lysis
moAb	Monoclonal antibody
NaCl	Sodium chloride
NSG	NOD scid gamma
PBS	Phosphate buffered saline
PC5	Phycoerythrin Cyanin 5
PE	Phycoerythrin
PFA	Paraformaldehyd
PGE1	Prostaglandin E1
PLT	Platelet
PMA	Phorbol myristrate-acetate
PMP	Platelet microparticle
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RBC	Red blood cell
RCA	Ricinus communis agglutinin
RT	Room temperature
SEM	Standard error of the mean
Src	Sarcoma
SSC	Sideward scatter
STAT3	Signal transducer and activator of transcription 3
Syk	Spleen tyrosine kinase

TBS	Tris buffered saline
Tc1	Cytotoxic T-cell type 1
Tc2	Cytotoxic T-cell type 2
T-cell	T lymphocyte
Th1	T helper type 1 cell
Th17	T helper 17 cell
Th2	T helper type 2 cell
TMB	Tetramethylbenzidine
TMRE	Tetramethylrhodamine, ethyl ester
TPO	Thrombopoietin
TRAP	Thrombin receptor activating protein
T-reg	T-regulatory lymphocyte
vWF	von Willebrand factor
vWFr	von Willebrand factor receptor
wPLT	Washed platelet

1 Introduction

1.1 Definition of immune thrombocytopenia

Human beings produce up to 10^{11} platelets (PLTs) per day and maintain a physiologic peripheral PLT-count between 150-400x10^9 PLTs/L (1-3). An adequate PLT-mass in the circulation is pivotal to prevent bleeding, maintain wound healing and different immunological functions like host defense and cytokine secretion (4, 5). Instead of being solely restricted to their role in haemostasis, PLTs are progressively identified as versatile reporters of various physiological and pathological processes in the human body (6, 7). To maintain these important functions, a fine regulated balance between PLT-production and depletion is required. Alterations in this tightly regulated system can lead to various pathologic conditions like severe bleeding, vessel occlusion and inflammation (8). Thrombocytopenia, defined as the decrease in PLT-count under 100x10^9 PLTs/L, is a common result of such alterations and accompanies several disease states, reaching from infections with different pathogens, hereditary syndromes as well as autoimmune disease (9, 10).

Immune thrombocytopenia (ITP), previously known as idiopathic immune thrombocytopenic purpura, represents an autoimmune disease that is characterized by a low PLT-count and higher bleeding tendency (11-13). While primary ITP is defined as a diagnosis of exclusion without any other underlying cause, secondary ITP is characterized by thrombocytopenia that is concomitant to disease states like the infection with human immunodeficiency virus (HIV), hepatitis or several neoplastic conditions (14-17). Current guidelines of the American Society for Haematology (ASH) distinguish different stages of ITP. These include acute (<3 months), persistent (3-12 months) and chronic ITP (>12 months) (12). The incidence of ITP is reported to be 1.6-3.9 per 100,000 in adults and 1.9-6.4 per 100,000 in children (18-20). In paediatric ITP, short courses with spontaneous remissions are typically observed and an association to proceeding viral infections is evident (21). Contrary, the majority of adult ITP patients face a more severe course with a higher tendency of chronification, bleeding events in the skin and mucous membranes like petechiae, purpura and in 1% of cases,

intracranial bleeding (22). Moreover, disturbances of health-related life quality like persistent fatigue with associated depression are frequently reported in ITP patients (23-26). The avoidance of these clinical manifestations with the major concern of bleeding prevention represents the main aim for the ITP treating physician (11). Unfortunately, treatment still remains challenging and often unsatisfactory, most likely due to the various and not fully clarified pathogenic mechanisms that are leading to an imbalance of PLT production and destruction in the disease course of ITP (16).

1.2 Pathogenesis of ITP

The exact mechanisms that result to the breakdown of self-tolerance in ITP are not fully clarified yet (27). Nonetheless, the heterogeneity of treatment responses and patient outcomes opened the field for various research approaches that identified perturbations in central (T- and B-cell immunity) as well as in peripheral immunity (Fc-gamma-receptor dependent mechanisms) as causing factors that lead to low PLT-counts and higher bleeding tendency in ITP.

1.2.1 T-cell perturbations in ITP

T-cells are crucial to conduct immune reactions like cell mediated tissue destruction as well as antibody (Ab) production through their interaction with B-cells (28). Under physiological conditions, self-tolerance is maintained by the depletion of self-reactive T-cells during the complex maturation process in the thyme or through rendering self-reactive T-cells quiescent. The latter is mediated by the lack of costimulatory signals, control by T-regulatory cells (Tregs) or peripheral depletion (29-31). Alterations in this fine-tuned system can lead to different pathogenic immune effector mechanisms including the loss of self-tolerance, namely autoimmunity (32-34).

Similar as observed in other organ specific autoimmune disease, the loss of T-cell mediated immune tolerance plays an important role in the pathogenesis of ITP (15, 35). Till now, numerous research efforts focused on the function of different T-cell subsets and investigated their impact regarding the regulation and

maintenance of immune tolerance to PLTs as well as their ability to induce autoimmunity in ITP.

The presence of PLT-reactive T-cells in ITP-patients as well as the antigenic potential of PLTs was first identified by Semple and Freedman. They observed an increased expression of IL-2 by ITP CD4+ T-helper-cells after the coculture with PLTs from healthy individuals in vitro (36).

Based on these data, several authors reported an imbalance of the T-helper cell subsets type 1 (Th1) and 2 (Th2) with an increased Th1/Th2 ratio in acute ITP patients that was previously described to be associated with other autoimmune diseases (37-39). Additionally, Th17 cells, that were lately characterized and are a high proinflammatory effector cell subset, are increasingly identified to play an important role in the pathogenesis of ITP via the promotion of a Th1 driven autoimmune response (40, 41).

Furthermore, Sakakura et al. recognized that another earlier mentioned member of the T-cell family, namely Tregs (Th4+CD25highFoxp+), are involved in the pathogenesis of ITP (42). Tregs, that make up to 5-10% in the whole T-cell population, are characterized by their suppressive function towards the initiation of immune reactions. Nowadays, it is well accepted that Tregs are impaired in their number and suppressive activity in ITP patients (42, 43). Of note, a rise in the number of Tregs analogue to PLT-counts was reported in ITP patients receiving treatment with thrombopoietin-receptor (TPO) agonists (44). This finding points towards a non-expected immune-suppressive effect of these standard ITP therapeutic agents and future research approaches that clarify the immune-suppressive mechanisms might be of great interest.

Controversial to the fact that ITP is characterized due to the presence of specific anti-PLT AAbs, it is well accepted that 50-60% of ITP patients do not have any detectable anti-PLT AAbs in their serum (45-47). Till now, this circumstance is raising the question of alternative PLT destruction mechanisms in ITP. The early characterization of a Th1 specific cytokine milieu and the finding that a subgroup of ITP patients expresses MHC class I molecules on the PLT surface made the

involvement of cytotoxic T-cells feasible (48). Of note, this T-cell involvement was first verified, as Olsson et al. detected a gene profile that is typical for the involvement of cytotoxic T-cells (CD3+CD8+) as well as an increased cytotoxic T-cell-induced destruction of radiolabelled autologous PLTs in six out of eight patients with active ITP (49). These results were further confirmed as increased Tc1/Tc2 ratios were observed in the blood and spleen of ITP patients by different research groups (50, 51). Interestingly, the involvement of cytotoxic T-cells in PLT destruction supports the observations of other groups where ITP was refractory despite of the depletion of Ab producing B-cells via the anti-CD20 depleting Ab Rituximab (52).

1.2.2 B-cell abnormalities in ITP

B-cells are the main site of Ab production and play a crucial role in the pathogenesis of ITP (53, 54). It is well recognized that B-cells produce an oligoclonal pattern of immunoglobulin G (IgG) class anti-PLT AAbs that target several antigenic epitopes residing on the PLT surface (55, 56). These include, next to others, mainly the PLT glycoproteins (GPs) IIb/IIIa (fibrinogen receptor) and Ib-IX-V (von Willebrand receptor) (57, 58). F(ab) mediated binding of these anti-PLT AAbs leads to the consumption of PLTs by the reticuloendothelial system via their Fc-domain, mainly in the spleen and through phagocytes residing elsewhere (59). Despite this evidence about the PLT's fate after AAb opsonization, the exact mechanisms that initiate the production of ITP anti-PLT AAbs are not fully clarified yet. In an early work, a higher frequency of autoreactive B-cells, yielding anti-PLT AAb producing plasma cells was observed in the red pulp of the spleen (60). These findings were extended, as a more recent work confirmed the occurrence of autoreactive B-cells not only in the spleen but also in the peripheral blood of ITP patients (61). Complementary, there is an increasing gain of evidence focusing on the potential role of the B-cell activating factor BAFF (a member of the tumor necrosis factor family) that is essential for the proliferation and activation of B-cells (62). Previously, excessive BAFF amounts were associated with different systemic autoimmune disease like systemic lupus erythematosus or rheumatic arthritis (63, 64). Similarly, increased BAFF levels as well as an increased promoter-activity in the BAFF gene-region

were found in different ITP-patient cohorts suggesting that an excess of BAFF may contribute to the induction of autoreactive B-cells in ITP (65). However, the expected consequences, namely elevated levels of anti-PLT AAbs that are produced by these self-reactive B-cells were not observed in a cohort of 57 ITP patients and let the authors conclude that an engagement with activated T-cells might be more likely to initiate increased anti-PLT AAb production (66). Just recently, this association was also suggested by Kuwana et al. who presumed that B-cells need to interact with activated self-reactive T-helper cells for triggering anti-PLT AAb formation (67).

The role of B-regulatory cells (Bregs, [$CD19^{+}CD24^{high}CD38^{high}$]), a subset of naive B-cells, was recently demonstrated to have regulatory effects on autoimmune responses. In general, it is assumed that the regulatory effects are mainly executed by the modulation of T-cell effector activation through the secretion of anti-inflammatory IL-10 and differentiation-control of antigen presenting cells (68, 69). In parallel to the findings of an impaired T-reg function, Li et al. reported a defective Breg-activity in ITP. This was confirmed by a decreased Breg-count, impaired IL-10 expression as well as decreased monocyte suppression. Interestingly, these alterations correlated with low PLT-counts in patients of this ITP cohort (70).

Additionally, not only different B-cell subsets but also the direct interactions of B-cells with CD40L (CD154) were recently identified to play a role in the complex pathophysiology of ITP. Interestingly, activated T-cells were reported to stimulate B-cells via the interaction of CD40L (CD154) and the surface B-cell receptor CD40 resulting in B-cell proliferation and differentiation (71). Furthermore, it was shown that activated PLTs from a cohort of 22 ITP patients expressed increased levels of CD40L on their surface. Notably, this PLT-subset was able to elicit a sustained activation of B-cells with subsequent formation of anti-PLT AAbs targeting GP IIb/IIIa in vitro (72). Additionally, the blockade of the PLT and B-cell interaction via a humanized monoclonal Ab was sufficient to increase PLT-counts and inhibit the emergence of anti-PLT AAbs in a subgroup of refractory ITP patients in a phase 1 multicentre study (73).

1.3 Role of autoantibodies in ITP

Since the report of an ITP patient with an improving PLT-count after splenectomy and the ground-breaking association that the spleen might play a major role in the destruction of PLTs by Kaznelson in 1916, great efforts have been performed to further clarify the pathogenesis of ITP (**Figure 1**) (74). The experiments of Harrington and Hollingsworth in the 1950s brought deeper insights by their characterization of a thrombocytopenic and to healthy recipients transmittable factor, that was identified to be dose dependent (75). Almost ten years later Schulmann et al. identified AAbs of the immunoglobulin G (IgG) class (IgG1 subclass) as the ITP inducing thrombocytopenic factor (76). Later, Van Leeuwen et al. provided first evidence, that these AAbs were primarily directed against the glycoprotein (GP) IIb/IIIa, as 32 out of 42 ITP patient serum AAbs bound to PLTs of healthy individuals but not to PLTs of GP IIb/IIIa deficient Glanzmann thrombasthenia patients (58).

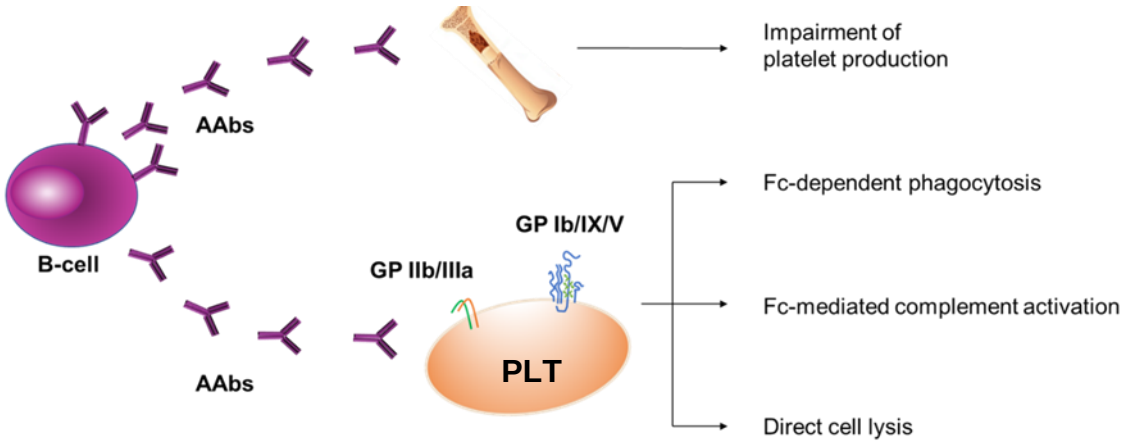

Figure 1: Autoantibody-mediated mechanisms leading to thrombocytopenia in ITP

PLT targeting ITP AAbs affect PLT-counts via the impairment of thrombopoiesis in megakaryocytes residing in the bone marrow niche or via different Fc-dependent PLT-depletion mechanisms. AAbs, autoantibodies; GP, glycoprotein; GP IIb/IIIa, fibrinogen receptor; GP Ib/IX/V, von Willebrand factor receptor; PLT, platelet. Adapted from Iraqui et al., 'Antiplatelet autoantibodies inhibit proplatelet formation by megakaryocytes and impair platelet production in vitro', Haematologica, 2015; McMillan et al., 'In vitro platelet phagocytosis by splenic leukocytes in idiopathic thrombocytopenic purpura', N Engl J Med., 1974; Bakchoul et al., 'Glycosylation of autoantibodies: insights into the mechanisms of immune thrombocytopenia', Thromb Haemost., 2013; Olsson et al., 'T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura', Nat Med., 2003.

GP IIb/IIIa (αIIβ3) is a cation dependent, noncovalently associated heterodimer that binds under physiologic conditions to fibrinogen, von Willebrand factor (VWF) as well as fibronectin. With copy numbers of 50, 000-80, 000/PLT, GP IIb/IIIa represents the most abundantly expressed surface receptor on human PLTs (77, 78). Despite this quantity, the integrity of this receptor is highly fragile as EDTA cation absorption leads to the dissociation of the heterodimeric complex (79). Investigations by Kiyomizu et al. revealed that PLT bound anti-IIb/IIIa ITP AAbs bind mainly to specific epitopes expressed on the extracellular N-terminal-globular head (L1-W235) of the IIb subunit which is formed by a 7 bladed (W1-7) beta propeller domain. More specifically, the authors demonstrated that the highly restricted regions W1: 1-2, W1: 2-3 and W2: 3-4 in the beta propeller domain are the main recognition sites of anti-IIb ITP AAbs (80). Additionally, it was confirmed that AAbs against GP IIb/IIIa show a restricted κ/λ light chain usage indicating that anti-GP IIb/IIIa AAbs derive from a monoclonal or oligoclonal subset of plasma cells. Studies that investigated the differences of PLT associated and non-PLT associated ITP AAbs further revealed that there are major differences in these types of AAbs. Non-PLT associated ITP anti-PLT AAbs that are directed towards GP IIb/IIIa were identified to mainly recognize binding epitopes in the C-terminal cytoplasmatic domain of the receptor's subunit IIIa. These cytoplasmatic binding sites were suggested not to be accessible under physiological conditions. The authors further conclude that the amount of in plasma detectable anti-IIb/IIIa AAbs is independent of the amount of PLT-bound AAbs. Hence, it has been suggested that in plasma detectable ITP anti-PLT AAbs do not represent a fraction of oversaturated PLT bound AAbs that reside under non-saturated conditions on the PLT-surface (81, 82).

These previous findings and the fact that PLTs harbour a myriad of potential antigenic epitopes on their surface, set up the stage for the identification of ITP anti-PLT AAbs that are targeting another highly expressed GP on the PLT surface, namely GP Ib-IX-V (83, 84). GP Ib-IX-V encodes for the von Willebrand receptor (vWFr) and is expressed on the surface of PLTs with a copy number of up to 50, 000 per PLT (85). In early attempts, Woods et al. showed that ITP anti-PLT AAbs that are targeting GPIb-IX-V are able to induce an increased PLT-

clearance (86). Following analyses identified that the major epitope of these AAbs is the extracellular GPIbα subunit within the GPIb-IX-V complex as recombinant fragments of the 485 amino acid containing Ibα domain were primarily targeted by anti-GP Ib-IX AAbs (87, 88).

Anti-GP IIb/IIIa and anti-GP Ib-IX are the most frequently detected and well characterized anti-PLT AAbs in ITP. The observation of alternative target epitopes on the PLT surface opened the field for ongoing research approaches. Just recently, the group of Sachs et al. showed a common prevalence of ITP anti-PLT AAbs that are solely targeting the GPV subunit of the PLT GP Ib-IX-V complex. These AAbs from a low avidity type were characterized to induce an increased uptake of PLTs by macrophages in vitro and increased PLT destruction in a NOD/SCID mouse model. This finding point towards an underestimated role of anti-GPV PLT AAbs, giving it a relevant role in routine testing and therapeutic implications (89). Additionally, AAbs against GPVI, the major receptor for collagen on the PLT surface that is associated with an intracellular immunotyrosin activation motif (ITAM) were described to be detected in the serum of ITP patients. In detail, anti-GPVI PLT AAbs were identified to activate as well as to affect PLT function and correlated with bleeding symptoms despite moderate PLT-counts (90-92).

1.4 Platelet clearance mechanisms in ITP

1.4.1 Fc-gamma-receptor mediated phagocytosis

The findings of Kaznelson and the hypothesis that the spleen might play a central role in the pathophysiology of ITP opened the stage for a myriad of investigations in the following decades (74, 93). Soon it became clear, that one of the major PLT destruction mechanisms in ITP might be mediated via the reticuloendothelial system through phagocytic active cells, that are mainly located in the spleen as well as in the liver. This theory was underlined by imaging studies of Tavassoli and McMillan who identified a high abundance of PLT-like cell debris in red pulp macrophages, also called splenocytes, of ITP patient spleens (94). Via the use of phagocytic assays, these findings were reinforced by the same group as radioactive chromium labelled homologous PLTs were internalized through ITP

splenocytes in vitro (59). Later works that focused on the kinetics of PLT-turnover in ITP patients in vivo showed that not only splenocytes but also macrophages in the liver represent the main destruction sites of PLTs. Nevertheless, a correlation amongst the site of PLT destruction and the response to splenectomy was not observed and the existence of alternative PLT destruction pathways was prospected in these studies (95, 96).

Tissue macrophages as well as monocytes are able to internalize AAb-coated PLTs or immune complexes trough their phagocytic activity and represent a major site of PLT destruction in ITP (97). Additionally, these cells have an important role in the modulation of the adaptive immune response based on their role as antigen presenting cells (98). The internalization and destruction of ITP AAb-coated PLTs by tissue macrophages is mainly mediated by different subsets of Fc-gamma-receptors (FcγRs) that reside on the surface of different phagocytic active cell-subsets (99, 100). Due to their ability to engage IgGs and based on their expression on different cells of the immune system, FcγRs form an interface between target antigens and immune effector cells (101).

In general, two main subsets of FcγRs can be distinguished. Binding to activating immunoreceptor tyrosine associated activating motif (ITAM) FcγRs results in activating intracellular signals with subsequent phagocytosis. These activating receptors include FcγRI (CD64), FcγRIIa/c (CD32a/c) and FcγRIIIa (CD16a) that are further characterized due to their binding affinity to the Fc-domains of Abs (102). Fc-mediated binding to the activating receptor's extracellular domain initiates the phosphorylation of intracellular ITAM domains via Src-family kinases Lyn and Fyn. This phosphorylation is followed by subsequent binding and phosphorylation of the spleen tyrosine kinase (Syk), a pivotal mediator of intracellular activating signalling processes (101).

A specific involvement of FcγRs in the pathophysiology of ITP was shown in different research attempts. In their important work Kuwana et al. showed that FcγRI (CD64) expressed on splenocytes plays a major role in the phagocytosis of ITP AAb-coated PLTs. This was confirmed as the inhibition via a monoclonal anti-CD64 Ab diminished an increased uptake of PLTs. Furthermore, a critical

role of these APCs in the activation of quiescent autoreactive T-cells via the presentation of cryptic GP IIb/IIIa epitopes through MHC-class II, was suggested (103). An additional involvement of another FcγR, namely FcγRIIA (CD32a) in the complex pathophysiology of ITP was postulated as an increased expression of this low avidity FcγR was detected on blood monocytes in a cohort of 23 patients. Interestingly, the levels of inhibitory FcγRIIb were significantly reduced in this patient cohort and completely restored after a four-day course of high dose dexamethasone therapy (104).

Contrary to activating FcγRs, the inhibitory FcγRIIb (CD32b) is associated with an intracellular immunoreceptor tyrosine associated inhibitory motif (ITIM) and represents an opponent of the above-mentioned activating FcγRs (105). The balance in the expression of activating and inhibitory FcγR-subsets might be of importance for a homeostatic state of the immune system and affect the onset of ITP (104). Nevertheless, these theories are controversial as no significant alterations in the expression of FcγRs were detected on splenocytes of ITP patients when compared with healthy controls (106).

1.4.2 ITP and the role of complement

Similar to red blood cells, PLTs express different receptors for the activating components (C1q, C2, C4) of the complement system on the surface (107). The presence of these complement binding receptors makes PLTs susceptible to inflammatory and immunological responses (108). To prevent the latter, PLTs are equipped with a repertoire of inhibitory complement proteins (MIRL, MCP, DAF) that either inhibit or modulate these immunologic reactions (109). An increasing body of evidence suggests that the imbalance of activating and inhibitory factors contributes to the onset of different diseases as well as is important for the physiological process of PLT ageing (110). In this context it is fairly interesting that the expression of activating as well as inhibitory proteins on the PLT surface shows distinct heterogeneities, especially within women and men (111). In addition to inhibitory proteins, a very versatile process to escape from membrane target complex (C5b-9) induced lysis is the formation of small C5b-9 loaded PLT-microparticles (PMPs) (108).

Early works that focused on the role of complement in ITP suggest that ITP anti-PLT AAbs targeting GP IIb/IIIa as well as GP Ib-IX are able to fix complement factor C3 and C4 in an AAb dose-dependent manner that correlated with an impaired PLT survival (112). Decades later, Najaoui et al. reported, that nearly 50% of sera from an ITP patient cohort that enrolled 240 individuals induced the binding of the complement factor C1q with subsequent activation of the classical complement pathway (113). Here the authors suggest that the fixation of complement is mainly dependent on the presence of AAbs targeting GP IIb/IIIa or GP Ib-IX as the lack of these target antigens resulted in the absence of complement factor binding. Further investigations by the same group identified that the amount of glycosylation of the ITP AAb Fc-domain is pivotal for the potential to bind complement. Of note, the enzymatically cleavage of N-glycans within the Fc-domain led to a significant reduction of complement binding in vitro as well as in vivo (114, 115).

1.5 Predictors of bleeding in ITP

1.5.1 Human platelet-counts in ITP

Different studies focused on the association between human PLT-counts and bleeding tendency. Soon it became clear that a PLT-mass of 7-8x10^9/L seems to be adequate to prevent bleeding in thrombocytopenic individuals with different underlying causes (5, 116). Similiar to these results, PLT-counts below 10-20x10^9/L were later assessed to be a benchmark for bleeding prediction in patients with lymphoproliferative disease (117, 118).

Current guidelines define isolated thrombocytopenia with PLT-counts below 100x10^9 PLTs/L as one of the main criteria for the diagnosis of ITP (14). Nevertheless, it seems not to be reasonable to set this PLT-count as predictive for the determination of outcomes in ITP patients (22). Especially emerging bleeding as the most important complication and its consequences on morbidity, mortality and life quality cannot be derived from this quantitative marker (22). Lacey et al. revealed that severe bleeding is rarely seen in ITP patients that are presenting with PLT-counts above 10x10^9/L (119). Later, these results were further confirmed by Khelaf et al. who identified only a mild correlation (r = -0.40;

p<0.01) between the presence of bleeding and low PLT-counts. In particular, 82% of patients with PLT-counts lower than $10x10^9$/L did not show any signs of severe bleeding complications (120). Additionally, another study was not able to find a significant correlation of marked thrombocytopenia and severe bleeding when compared to patients with moderate thrombocytopenia. These described findings indicate that the prediction of severe bleeding is not inversely correlated to low PLT-counts in ITP (121).

Contradictive to this evidence, the analysis of a big retrospective ITP cohort performed by Cohen et al. revealed that PLT-counts lower than $30x10^9$/L are related to increased non-fatal and fatal haemorrhages in an age dependent manner (122). The results of this work were further confirmed by a systemic review that evaluated 10,908 ITP patients that were included in a total of 118 studies. Here the authors report an increased tendency of bleeding in ITP patients with PLT-counts that were either less than $10x10^9$/L or $20x10^9$/L. Remarkably and of high clinical relevance, a significant decrease in the overall life expectancy and increased 5-year mortality in non-treated ITP patients with low PLT-counts was observed (123).

Due to the controversial findings regarding the role of PLT-counts, the estimation of emerging bleeding in ITP patients still remains challenging. It is intriguing that the prevalence of bleeding in ITP seems to be such heterogenous while other diseases that are associated with thrombocytopenia and rapid falls of PLT-counts or the presence of PLT-targeting alloantibodies show higher bleeding tendency (22). Hence, the needs of reliable and validating biomarkers for the risk stratification of bleeding in ITP remains highly important and the establishment of such predictors is more than desirable.

1.5.2 Platelet function and relevance on bleeding

The tendency of bleeding in ITP is variable and the amount of PLTs in the circulation is a marker that seems not to be appropriate to identify patients that are at high risk of severe bleeding events (22, 124). AAbs that target PLT GP IIb/IIIa and Ib-IX are typically observed in ITP and have been described to interfere with PLT function as well as PLT formation and maturation (125).

Nevertheless, there is a lack of data regarding the question whether the assessment of PLT function in ITP could be of predictive value to determine the onset of bleeding events. One of the reasons for the scant data might be the fact that the detection of functional defects still remains a burden in ITP. On the one hand, the detection of ITP anti-PLT AAbs with the commonly performed detection assays is of low sensitivity (126). On the other side, well established PLT-functionality tests like the transmission light aggregometry after Born requires PLT-counts in patients being greater than $150x10^9$ PLTs/L and high volumes of PLT rich plasma (PRP) for reliable measurements (127, 128). These methodological aspects lead to limitations of this test in thrombocytopenic ITP patients and unfortunately, especially in children (128, 129).

Facing these limitations, different approaches that utilized new methods, have been performed to get further insights into the impaired PLT-function in ITP. Using a cohort of 41 chronic ITP patients, Panzer et al. systemically assessed the activation level and the ability of PLTs to adhere and form aggregates under high shear conditions via flow cytometry (FC) and an adhesion-assay, respectively. Notably, a significant correlation of an impaired PLT adhesion ability on a polystyrene surface and a clinically increased bleeding tendency of ITP patients that was independent of PLT-counts, was observed. Additionally, the investigators detected an increase in the size of PLT aggregates that correlated with the expression-levels of the surface PLT-activation marker P-selectin (CD62P). These findings suggest that PLTs of thrombocytopenic ITP-patients might have a higher reactivity and ability to form aggregates in order to compensate for low PLT-counts (130). Further flow-cytometric approaches performed by Psaila et al. revealed that PLTs of ITP patients showed an increased response to agonist stimulation when compared to patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). This study confirms the previous findings of Panzer et al. and suggests the presence of a high reactive PLT-population that compensates for the detected low PLT-counts in ITP patients. Interestingly, contrary to individuals with AML or MDS, patients with ITP that experienced bleeding, showed less exposure of activated GP IIb/IIIa and CD62P in response to agonist stimulation on their surface. This observation

was explained either to in vivo preactivation or exhaustion of PLTs in this patient subgroup (131).

Via the establishment of two new whole blood PLT-functionality assays, namely a micro-aggregation and a PLT-reactivity assay, van Bladel et al. detected altered PLT functions in a cohort of 33 children with chronic ITP. In detail, whole blood of children with ITP that presented with severe bleeding symptoms showed a decreased co-aggregation potential after stimulation with the GP IIb/IIIa activating agonist phorbol-myristate-acetate (PMA) when compared to controls. The authors interpreted these findings as that the inhibition of activation might be due to the presence of PLT bound anti-GP IIb/IIIa AAbs that interfere with the binding site of PMA. Additionally, and as described in former publications, paediatric ITP patients with a severe bleeding phenotype presented with a decreased agonist induced PLT-activation response compared with non-bleeders (131, 132). In another study, performed by Frelinger et al. whole blood samples from a cohort of 57 paediatric ITP patients were analyzed before and subsequently after agonist stimulation. Of note, PLTs of children with severe bleeding presented with increased expression levels of CD62P and activated GPIIb/IIIa in unstimulated as well as with decreased PLT-reactivity following the activation with thrombin receptor activating protein (TRAP) and ADP. In contrast, GP Ib-IX, that is physiologically internalized after PLT activation, was markedly elevated in unstimulated PLTs from patients with a high bleeding tendency. The authors conclude that there might be an impaired detection of activated GP IIb/IIIa du to interaction of the binding Ab with anti-GP IIb/IIIa AAbs but prefer the assumption that PLTs might be desensitized at the bleeding site in circulation (133).

1.6 The role of platelet sialylation

The average lifetime of a human PLT lasts around 7-11 days. Following the release from megakaryocyte pro-PLT protrusions into the bone-marrow sinusoids, PLTs mature and reside in the circulation until their clearance is initiated (134). In ITP and massive bleeding events, AAb-opsonized PLTs are eliminated via different Fc-dependent clearance mechanisms as well as due to the consumption of PLTs at the bleeding site, respectively (135). Despite

increasing evidence of the PLT faith in different diseases, the knowledge about their physiological depletion or in other words, about the exact mechanisms that are initiating the clearance of PLTs in healthy individuals is limited (136).

Further insights about the physiological PLT life cycle were gained as it became evident that PLTs carry a significant amount of sugar residues on the surface. These include oligosaccharides composed of glucose, mannose and galactose that are linked together (137). On PLTs, these oligosaccharides, also called glycans are predominantly located on the surface of the vWFr GPIbα subunit. Attached to the mucin and ligand binding domain via an O- (oxygen) or N- (nitrogen) atom-linkage, they represent a highly glycosylated region on the surface of PLT GPIbα (138). From high biological relevance is the fact that the attached glycans are capped with terminal sialic acids (134). Sialic acids represent a group of negatively charged nine-carbon-containing monosaccharides which contain neuraminic-acid as core molecule. Together, they comprise a family of up to eighty different derivates with N-Acetylneuraminic acid as the most frequent one and play an important role in the mediation of a various cell-cell and cell-matrix interactions (139-141). Of note, increasing evidence suggests that the loss of terminal sialic acid content, namely desialylation due to blood borne sialidases contributes to the clearance of senescent PLTs. Specifically, sialidase-induced PLT desialylation has been identified to result in the exposure of underlying oligosaccharides that are recognized by different cells as “eat me” signal (137).

Sialidases that are found in mammals include the subclasses neuraminidase 1-4. Together, they belong to the family of metalloproteinases and are residing mainly in the inner-compartment or membrane associated on PLTs (142). Additionally, pathogens like the influenza virus or the bacterium streptococcus pneumoniae utilize surface associated neuraminidase as a pathogenic factor (143). Sialidase/neuraminidase is able to cleave terminal sialic acids at their α2,3-linkage from the underlying glycan structures (144). Consequently, desialylated glycans are recognized and internalized by the Ashwell-Morrell-receptor (AMR) residing on the surface of hepatocytes in the liver (145, 146).

Belonging to the C-type lectin family, AMR is a transmembrane hetero-oligodimeric GP-complex that is able to recognize desialylated glycans, also called asialoglycoproteins. The recognition of asialoglycoproteins triggers the internalization of such modified, desialylated glycan structures (147). Four decades of research were necessary to further investigate the physiologic function of the in mammals highly conserved AMR. In their elegant study Grozovsky et al. identified that PLT desialylation and the production of thrombopoietin (TPO) are directly linked together. Using in vitro and in vivo approaches the authors demonstrated that PLT-ageing in the circulation initiates desialylation by blood borne sialidases that leads to the uptake of aged PLTs by the AMR. This uptake of desialylated PLTs via the AMR is followed by the upregulation of the JAK1-STAT3 signaling pathway in hepatocytes in a feedforward dependent manner. As a consequence, TPO is produced and released which results in stimulation of MK maturation as well as increased PLT production (148). Prior to these observations, Grewal et al. identified that the removal of terminal sialic acid on GPIbα is also induced via the bacterium streptococcus pneumoniae. Using a sepsis mouse-model it was shown that PLTs were desialylated via the bacteria's membrane associated sialidase. Additionally, the authors observed that the clearance of desialylated PLTs was associated with low PLT-counts in sepsis patients. From potential translational relevance is the fact, that low PLT-counts were identified as being protective regarding coagulopathy. Of note, in this study, the clearance of desialylated PLTs was associated with lower incidences of disseminated intravascular coagulopathy (149). Following investigations that utilized sialyl-transferase depleted mice and cold stored PLTs further confirmed the role of sialic acid loss and the impact of desialylation on the survival of PLTs (145, 146).

Interestingly, next to the role of PLT desialylation in physiologic PLT-ageing, the role of sialic acid loss was increasingly identified to play a role in the pathophysiology of ITP. Alternative PLT clearance mechanisms in ITP were first suggested as a subgroup of ITP patients were observed for being non-responsive to FcγR targeting therapies like the administration of intravenous IgG (IVIG) or splenectomy. Of note, in these studies the investigators revealed that patients

with detectable ITP anti-PLT AAbs against the GP Ib-IX are often refractory to FcγR targeting treatment approaches (150-153). These findings and considering that the GPIbα subunit is harbouring up to 80% of the PLT's sialic acid content, made alternative FcγR-independent PLT clearance mechanisms conceivable (154).

First evidence for this assumption was demonstrated in the early 2000s in a murine model where monoclonal mouse-antibodies (moAbs) that are targeting the PLT GPIbα-subunit showed the capability to induce PLT-clearance in a Fc-independent manner (155). Human AAb-mediated desialylation as such a Fc-independent PLT clearance was later progressively identified in patients with detectable ITP anti-GP Ib-IX AAbs that were refractory to standard therapeutics and successfully treated with a neuraminidase inhibitor (154, 156).

Finally, in 2015, the group of Ni et al. was able to further identify the mechanisms of anti-GPIbα Ab-mediated desialylation. Via the utilization of moAbs and human ITP plasma with detectable anti-GPIbα ITP AAbs, the authors detected an increased activation of murine as well as of human PLTs. Remarkably, in parallel to PLT activation, a significant translocation of PLT neuraminidase from the PLT inner- compartment was detected. This release of neuraminidase resulted in the cleavage of terminal sialic acids on GPIbα that triggered subsequent internalization of desialylated PLTs by the hepatic AMR that was significantly inhibited in Ashwell–Morrell-deficient (Aspgr2−/−) mice (**Figure 2**). Contrary to these findings, no significant desialylation was induced via anti GP IIb/IIIa moAbs or human ITP plasma samples with detectable ITP AAbs targeting GPIIb/IIIa, respectively. These findings suggest that moAbs and human ITP AAbs targeting PLT GPIbα or GP IIb/IIIa induce different PLT clearance mechanisms, namely Fc-independent PLT clearance via GPIbα targeting AAbs and Fc-dependent clearance mechanisms via anti-GP IIb/III AAbs, respectively (157). The identification of a new Fc-independent PLT clearance mechanism that is induced via PLT GPIbα targeting moAbs as well as human ITP AAbs in these studies revealed a new potential therapeutic target in ITP.

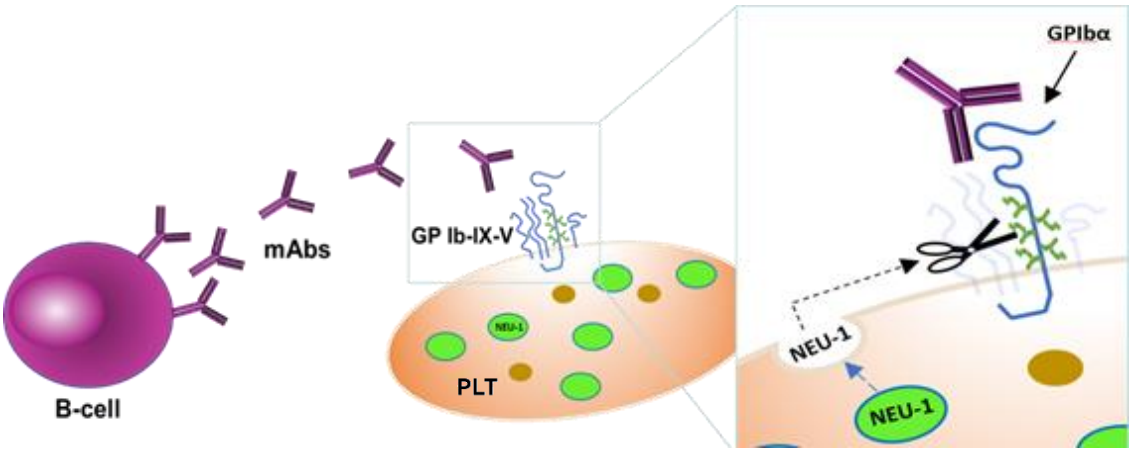

Figure 2: Antibody-induced desialylation in ITP

mAbs have the capability to induce PLT desialylation via the ligation of the GPIbα subunit of the GP Ib-IX-V complex. GPIbα engagement leads to intracellular signalling mechanisms that result in the release of sialidase, namely neuraminidase form the PLT's intracellular compartment. The released sialidase has the ability to cleave terminal sialic acid that cape different sugar residues on the surface of GPIbα. This loss of terminal sialic acid, namely desialylation enables the recognition of underlying sugar residues via the AMR located on hepatocytes in the liver with subsequent phagocytosis of desialylated PLTs. GP, glycoprotein; GP Ib-IX-V, von Willebrand factor receptor; mAbs, monoclonal antibodies; NEU-1, neuraminidase-1. Adapted from Li J. et al., 'Desialylation is mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia', Nat. commun., 2015.

1.7 Aims of the study

Despite the interesting findings of previous research attempts that focus on the role of desialylation in ITP, there are some experimental and clinical limitations (155, 157). First, most of the performed experiments utilized moAbs. Due to interspecies differences, variabilities in moAb induced effects in murine and human PLTs have to be considered (157, 158). Additionally, experiments with human ITP sera were primarily performed using ITP plasma samples from patients with detectable AAbs against GP Ib-IX. As only a low number of ITP plasma samples with detectable AAbs against PLT GP IIb/IIIa were utilized in these studies, it cannot be concluded whether anti-GP IIb/IIIa AAbs might induce desialylation or not. This hypothesis is further emphasized as therapy refractoriness with severe courses is also observed in ITP patients with detectable AAbs against GP IIb/IIIa. Hence, it might take a higher representative number of ITP patients with this AAb subset to conclude whether desialylation is solely restricted to patients with anti-GP Ib-IX AAbs or might also be observed in ITP patients with AAbs targeting different PLT epitopes (155, 157).

In this study, we sought to investigate whether:

I) Desialylation of PLTs is a pathomechanism that frequently occurs in human ITP

II) Changes in the glycan pattern of PLTs can also be induced via human anti-GP IIb/IIIa ITP AAbs as well as sera without ITP AAbs

III) Desialylation might contribute to an increased bleeding tendency and impaired PLT function

IV) ITP AAb-induced desialylation affects the survival of PLTs in vivo as well as PLT apoptosis in vitro

2 Materials and Methods

2.1 Materials

Table 1 lists used chemicals. Devices and laboratory materials are listed in **Table 2** and **Table 3**, respectively. The utilized antibodies/lectin and buffers are listed in **Table 4** and **Table 5**, respectively.

Table 1: Chemicals

Chemical	Company
Apyrase	Sigma Aldrich, St. Louis, USA
Bovine serum albumin (BSA)	Serva, Heidelberg, Germany
Calcium chloride	Gbiosciences, St. Louis, USA
Carbonyl cyanide 4-(trifluoromethoxy) Phenylhydrazone (FCCP)	Abcam, Cambridge, United Kingdome
Distilled water	Fresenius Kabi, Bad Homburg, Germany
Dulbecco´s phosphate buffered saline (PBS)	Biochrom, Berlin, Germany
Exogenous sialidase (α2-3,6,8,9-Neuraminidase)	Merck, USA
Fibrinogen	Sigma Aldrich, Munich, Germany
Glucose 10%	Braun, Melsungen, Germany
Hirudin	DSM, Aesch BL, Switzerland
Human serum albumin (HSA)	Kedrion, Barga, Italy
Hydrochloric acid	VWR international, Fontenay sous Bois, France
Magnesium chloride	Merck, Hohenbrunn, Germany

Chemical	Company
Melon^{TM}-Gel IgG Spin Purification Kit	Thermo Fisher Scientific, Waltham, USA
Oseltamivir acid	Santa Cruz Biotechnology, Dallas, USA
Paraformaldehyd (PFA)	Morphisto, Frankfurt am Main, Germany
PerFix-non centrifugation assay Kit	Beckman Coulter, Marseille, France
Prostaglandin E1 (PGE1)	Sigma Aldrich, St. Louis, USA
Sodium azide	Serva, Heidelberg, Germany
Sodium carbonate	Biochrom, Berlin, Germany
Sodium hydrogen carbonate	Merck, Darmstadt, Germany
Sodium-chloride 0.9% (NaCl)	Braun, Melsungen, Germany
Sulfuric acid	Merck, Darmstadt, Germany
Tetramethylbenzidine (TMB)	Kementec, Taastrup, Denmark
Tris Buffer	VWR international, Fontenay sous Bois, France
Triton X-100	Merck, Darmstadt, Germany
Tween-20	Sigma Aldrich, St. Louis, USA
von Willebrand factor	Baxalta, Vienna, Austria

Table 2: Devices

Device	Company
Cell-Dyn Ruby hematological	Abbott, USA

Device	Company
analyzer	
Flow-cytometer Navios	Beckman Coulter, USA
Freezer (-80°C)	ilshin Europe, Ede, Netherlands
Heraeus 37°C incubator	Thermo Scientific, Waltham, USA
Infrared lamp	Beurer, Ulm, Germany
Microscope Olympus IX73	Olympus GmbH, Hamburg, Germany
Mikro 22R tabletop centrifuge	Hettich, Tuttlingen, Germany
Mouse restrainer	Braintree Scientific, Braintree, USA
Photometer infinite F50	Tecan Group Ltd., Männedorf, Switzerland
Refrigerator (4°C)	Siemens, 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
Test Tube Thermostat Model TCR100	Carl Roth, Karlsruhe, Germany
TubeOne microcentrifuge	Starlab, Hamburg, Germany
Vortexer, Reax-Top	Heidolph, Schwabach, Germany

Table 3: Laboratory Materials

Laboratory Material	Company
50ml tubes	Greiner bio-one, Frickenhausen, Germany
Anticoagulant citrate dextrose solution (ACD-A) Vaccutainer	Becton Dickinson, Plymouth, United Kingdom
Chamber-slides	Corning, New York, USA
S-Monovette EDTA 7.5ml	Sarstedt, Nümbrecht, Germany
Eppendorf-Cups 1.5; 2ml	Eppendorf, Hamburg, Germany
Flow cytometer tubes	Sarstedt, Nümbrecht, Germany
Microplate 96 well	Greiner bio-one, Frickenhausen, Germany
Nitril gloves	Paul Hartmann, Heidenheim, Germany
Pasteur pipet 5ml	Carl Roth, Karlsruhe, Germany
Single-use syringe Injekt-F 1ml	Braun, Melsungen, Germany
Sterican 27 gauge needles	Braun, Melsungen, Germany
TipOne XL Graduated Tip 10; 20; 200; 1000µl	Starlab, Hamburg, Germany

Table 4: Antibodies and Lectins

Antibody / Lectin	Company
Anti-Human CD32-Antibody, Clone IV.3	Stemcell technologies, Canada
FITC-labelled Rat anti-Mouse CD41, Clone MWReg30	Becton Dickenson, USA
FITC-labelled Rat IgG1, κ Isotype Control, Clone R3-34	Becton Dickenson, USA
FITC-labelled erythrina crista galli lectin (ECL)	Vector laboratories, Burlingame, USA
FITC-labelled ricinus communis agglutinin (RCA)	Vector laboratories, Burlingame, USA
Goat anti-mouse IgG	Jackson Immuno Research, West Grove, USA
Monoclonal mouse anti-human CD41 antibody, Clone P2	Beckman Coulter, Marseille, France
Monoclonal mouse anti-human CD42 antibody, Clone SZ1	Beckman Coulter, Marseille, France
Mouse anti-human IgG1-FITC/IgG1 -PE/IgG1-PC5 Antibody Cocktail	Beckman Coulter, Marseille, France
PC5-labelled mouse anti-human CD41, Clone P2	Beckman Coulter, Brea, USA
Peroxidase-conjugated goat anti-human IgG	Jackson Immuno Research, West Grove, USA
PE-labelled Tetramethylrhodamine, ethyl ester (TMRE)	Abcam, United Kingdom

Table 5: Buffers and Solutions

Buffer / Solution	Components
Magnesium chloride-solution	Magnesium-chloride 0.2M
	Distilled water
Calcium chloride-solution	Calcium-chloride 0.2M
	Distilled water
Coating-buffer	Sodium carbonate 0.015M
	Sodium hydrogen carbonate 0.035M
	Sodium azide 0.0031M
	Distilled water
PBS-BSA 2%	BSA
	PBS
Tris solubilization-buffer	Tris 0.01M
	Triton X-100
	NaCl
Tris-buffered saline (TBS)	Tris 0.01M
	Triton X-100
	Tween-20
	Calcium chloride 1M
	NaCL
Sulfuric acid-solution	Sulfuric acid 0.2M

Buffer / Solution	Components
	Distilled water
HIPA basic-buffer	Glucose 10%
	BSA 20%
	Distilled water
HIPA washing-buffer	Basic-buffer
	Apyrase 1 U/µl
	Hirudin 1 U/µl
	Hydrochloric acid 1M
HIPA resuspension-buffer	Basic-buffer
	MgCl2 0.2M
	CaCl2 0.2M
	Hydrochloric acid 1M

2.2 Methods

2.2.1 Patients and sera

The eligibility of ITP patients for the study was proofed by two, in the field of immune-hematology experienced physicians via retrospective review of medical records according to state-of-the-art ITP diagnostic-guidelines (159). In case of discrepancies, a third physician was consulted.

Experiments were performed using leftover serum material from ITP as well as from patients with non-ITP thrombocytopenia (hypo-proliferative thrombocytopenia). Patient serum samples were stored at the Department for Transfusion medicine Tübingen at -80°. Control sera with the blood group AB

were collected from healthy blood donors at the blood donation-centre Tübingen, Tübingen, Baden-Württemberg, Germany, after written consensus was obtained. To exclude unspecific effects like the activation of PLTs via complement or other serum proteins, all sera were heat-inactivated at 56°C for 25 min which was followed by a centrifugation step at 5000xg for 30 min. Pellets were discarded and supernatants were handled as described in the following sections. When indicated, IgG-fractions where isolated from ITP-sera as well as from healthy control (HC) sera by the use of a commercially available IgG-purification-kit in line with the manufacturer's instructions.

2.2.2 Detection of glycoprotein-specific autoantibodies

Detection of free and PLT-bound AAbs was performed by the monoclonal antibody-specific immobilization of PLT antigens (MAIPA) assay as described by Kiefel et al. with minor modifications at the HLA laboratory department of Transfusion medicine, Tübingen (160). Briefly, for the detection of free PLT-AAbs, PLTs ($20x10^6$) from a panel of well characterized donors were isolated from EDTA-blood by centrifugation (160xg, 20 min, no brake) and three washing steps (900xg, 20 min, with brake). Afterwards PLTs were stored at 4°C in isotonic saline (NaCl) containing 0.1% sodium-azide for at least 12 hours. After centrifugation (1200xg, 2 min), PLTs were resuspended in 50µl of isotonic PBS-BSA 2% (PBS-BSA) in microplate-wells. Subsequently, 50µl of human test-serum were added to the cell-suspension. Sera from healthy AB-controls and patients with known antigen-positivity served as negative and positive controls, respectively. After incubation (30 min, 37°C), cells were washed with 200µl of NaCl and resuspended in 50µl of PBS-BSA. The latter was followed by the addition of the GP-specific monoclonal Abs clones P2 and SZ1 (final concentration 7.5µg/ml), respectively and incubation at 37°C for 30 min. Subsequently, PLTs were washed three times with 200µl NaCl, resuspended in 100µl of solubilization-buffer and transferred to an Eppendorf-cup. After cell lysis (30 min, 4°C), the cell suspension was centrifuged (20 min, 15000xg) and 50µl of the supernatants, containing the soluble GP-moAB complexes, were mixed with 200µl TBS. Each 100µl portion of this solution was transferred into with goat anti-mouse IgG coated microplates in duplicate. Following incubation (90 min,

4°C) the cell-suspension was removed and the microplate washed four times with TBS. After washing, each well was incubated for 120 min at 4°C with 100µl of diluted peroxidase-labelled goat-anti human IgG (1:5000, v,v in TBS). In the last step, the microplate was washed five times with TBS and incubated with 100µl of TMB. The reaction was stopped after incubation (5 min, RT, dark) via the addition of 50µl 0.2M sulfuric-acid.

For the detection of PLT-bound AAbs, patient PLTs ($20x10^6$) were directly incubated with the corresponding moAbs and MAIPA was performed as described above. For both variants of MAIPA, test results were read using a photometer at a wavelength of 450 nm. An optical density of greater than 0.15 was considered as positive (161). MAIPA was mainly performed by the staff of the PLT laboratory at the centre of Transfusion Medicine. Results were analyzed by the doctoral candidate.

2.2.3 Preparation of washed platelets

Fresh PLTs were isolated as described previously from venous blood of healthy donors with the blood-group O after written consensus was obtained (162). In detail, whole blood was withdrawn by cubital venipuncture into anticoagulant citrate dextrose solution (ACD-A) containing vacutainers and allowed to rest for 30 min at 37°C. For the avoidance of PLT preactivation via tissue factor and other activating components, the first flow-through was discarded. After centrifugation (120xg, 20 min, RT, no brake) the supernatant PLT-rich-plasma (PRP) was gently separated by the use of a pasteur-pipet which was followed by the immediate addition of apyrase and pre-warmed ACD-A (5µl/ml PRP and 333µl/ml PRP, respectively). Afterwards, PLTs were isolated from PRP via centrifugation (650xg, 7 min, RT, no brake), resuspended in 5ml of wash-solution and allowed to rest for 15 min at 37°C. Following a final centrifugation step (650xg, 7 min, RT, no brake) PLTs were resuspended in 2ml of resuspension-buffer and adjusted to a PLT-count of $300x10^3$/µl after the measurement at a Cell-Dyn Ruby hematological analyzer was performed.

2.2.4 Assessment of desialylation via a lectin-binding assay

25µl of wPLTs ($7.5X10^6$) were incubated with 25µl of serum or IgG-fractions (1:1, v/v) for 120 min at RT under rotating conditions. Subsequently, samples were fixed with 2% paraformaldehyde [(PFA), 20 min, at RT] and washed twice with PBS (650xg, 7 min, RT, with brake). Alterations in the sialic pattern of PLTs were analyzed via flow-cytometry (FC) using FITC-labeled ricinus communis agglutinin (1 µg/mL, RCA) and erythrina crista galli lectin (0.5 µg/mL, ECL) that binds specifically to ß-Galactose and N-Acetylglucosamine residues, respectively. Afterwards, samples were washed twice, resuspended in 500µl PBS and analyzed via FC. Each serum sample was tested on wPLTs of three healthy donors. wPLTs that were treated with exogenous neuraminidase (sialidase, 1µl stock concentration) 15 min prior to fixation served as positive control in each experiment (**Figure 3**).

2.2.5 Determination of cutoff-value for desialylation

To determine valid cutoff-values for the detection of statistically significant desialylation, 20 sera from healthy donors with the blood-group AB were analyzed in the LBA. Cuffoff-values were determined as fold increase (FI) compared to mean of control plus two standard deviations.

2.2.6 Inhibition of platelet FcγRIIA

A monoclonal anti-CD32a-Ab (moAb IV.3) was used as previously described with minor modifications for the inhibition of PLT FcγRIIA (163). In detail, 75µl of wPLTs ($22.5x10^6$) were preincubated with 5µl of moAb IV.3 (31.25µg/ml) for 30 min at 37° and further handled as described in the LBA.

2.2.7 Inhibition of sialidase

The inhibition of sialidase was performed via preincubation of wPLTs with the sialidase inhibitor oseltamivir-acid. Briefly, after resuspension of the lyophilized sialidase-inhibitor with distilled water to a stock-concentration of 50µmol, 25µl of wPLTs ($7.5x10^6$) were preincubated with 1µl sialidase inhibitor (30 min, RT). Afterwards, wPLTs were incubated (1:1, v/v) with IgG-fractions as described in the sections above. To confirm the functionality of the sialidase inhibitor in each

experiment, exogenous sialidase was added to oseltamivir-acid preincubated wPLTs in a control sample.

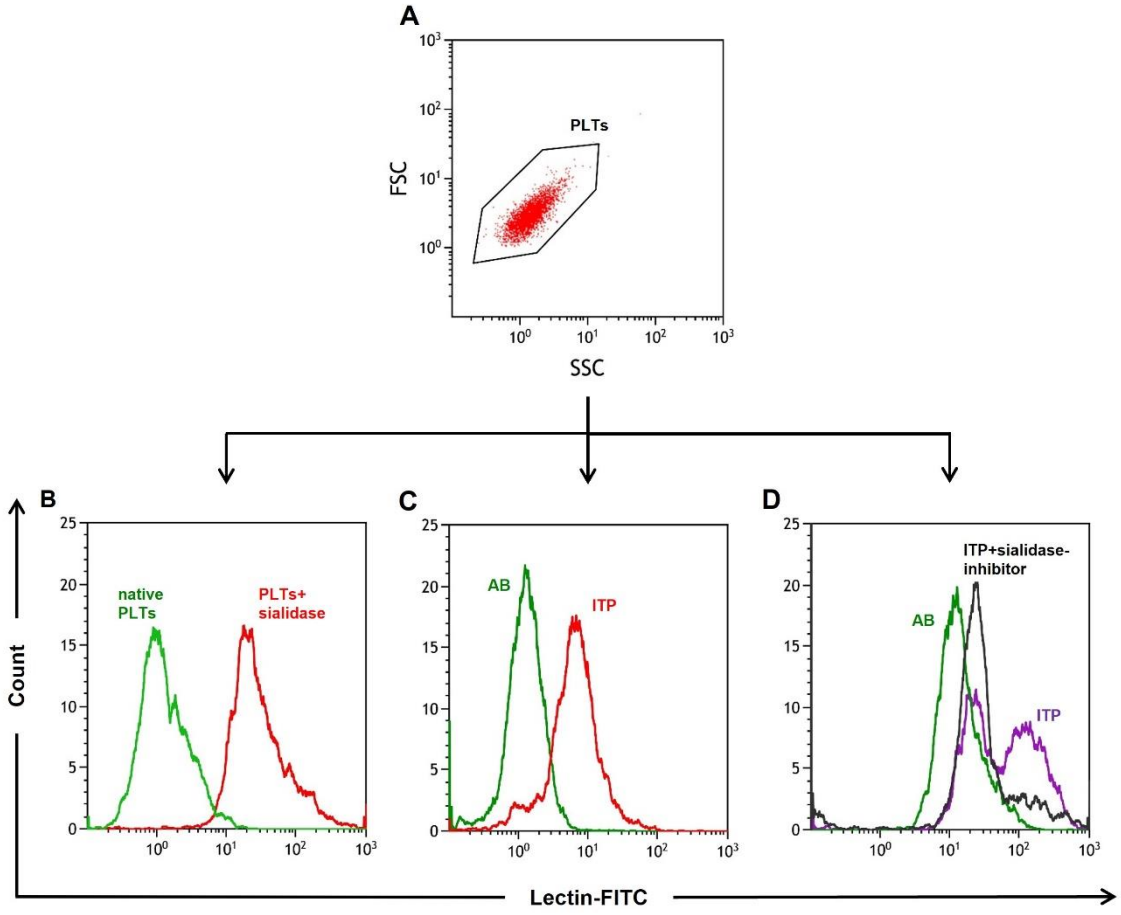

Figure 3: Flow-cytometry data showing histograms of lectin binding in different LBA approaches

A) wPLTs were gated based on their FSC vs. SSC properties as well as on the expression of the PLT-marker CD41. B) wPLTs that were incubated with exogenous sialidase to induce maximum levels of desialylation served as positive control in each experiment. C) To screen whether ITP patient sera have the capability to induce desialylation, wPLTs were incubated with ITP patient sera and compared with wPLTs that were incubated with sera from healthy controls. D) For the inhibition of desialylation, wPLTs were preincubated with the sialidase inhibitor oseltamivir-acid prior to ITP IgG incubation. FSC, forward scatter; FITC, fluorescein isothiocyanate; ITP, immune thrombocytopenia; SSC, sideward scatter; AB, blood group AB serum; PLTs, platelets.

2.2.8 Assessment of ITP antibody-mediated apoptosis

To investigate whether desialylation might induce PLT apoptosis, changes in the inner mitochondrial transmembrane potential (ΔΨ) were analyzed via FC. The binding of tetramethylrhodamine ethyl ester (TMRE) has been reported to be a reliable marker for the detection of changes in the inner mitochondrial transmembrane potential that are able to induce the intrinsic pathway of apoptosis via caspase activation (164, 165). Briefly, 25µl of wPLTs ($7.5x10^6$) were incubated with ITP or control IgGs (1:1, v,v, 120 min, RT) and stained with 10µM TMRE (30 min, RT). Carbonyl cyanide 4-(trifluoromethoxy) Phenylhydrazone (FCCP), an uncoupler of mitochondrial oxidative phosphorylation served as positive control in each experiment. After staining, the reaction was stopped via the addition of PBS to a final volume of 500µl that was followed by immediate FC analysis. Changes in ΔΨ of ITP IgG incubated wPLTs were compared with wPLTs that were incubated with control IgGs.

2.2.9 Analysis of antibody-mediated platelet destruction

2.2.9.1 Description of NSG mice

For the assessment of the biological relevance of desialylation on PLT survival, a NSG mouse model was used. In detail, sex- and age-matched NSG mice (NOD-scid IL2Rg^{null}; Stock No. 005557 purchased from The Jackson Laboratory Bar Harbor, ME, USA via Charles River, Research Models and Services Sulzfeld, Germany) at an age of 8-16 weeks, were utilized in this study. All procedures including animal-material were approved by the local Ethics Committee of the Medical Faculty, Eberhard Karls Universität, University Hospital of Tübingen, Germany and were performed in accordance with the declaration of Helsinki.

2.2.9.2 Platelet-preparation for in vivo assay

Whole blood from healthy donors with the blood group O was collected into ACD-A containing Vacutainers and further handled as previously described (166). To avoid preactivation of PLTs during sample withdrawal, the first flow-through was discarded. Additionally, Prostaglandin E1 (PGE1, 50ng/ml) was added periodically during the sample procedure. Whole blood samples were allowed to rest (10 min, RT) which was followed by the addition of PGE1 and subsequent

centrifugation (200xg, 10 min, RT, no brake). Afterwards, the supernatant PRP was gently separated with a Pasteur-pipet under the avoidance of shear as well as red blood cell contamination. After the transfer into PGE1 containing centrifugation tubes, the cell suspension was allowed to rest (10 min, RT). After final centrifugation (700xg, 10 min, RT, no brake), the remaining PLT-pellet was resuspended in 0.5 ml PPP (PLT poor plasma) and supplemented with PGE1.

2.2.9.3 Platelet injection and blood sample collection

10 min before PLT-injection, mice were placed under an infrared warm-lamp to improve vascular conditions. For subsequent injection purposes, mice were located into a small animal restrainer. Using a 1ml syringe with a 27 gauge needle, 200µl of the human PLT-suspension (~400x10^6 PLTs) were slowly infused into the mouse lateral tail-vein. For selected experiments, PLTs were preincubated with exogenous-sialidase**.** To determine a baseline value (100%) for the human PLT-count in the mouse circulation, a blood sample of each mouse was collected via tail vein puncture 30 min after the injection of PLTs was performed. Immediately afterwards, isolated IgG-fractions from ITP-sera or HCs were injected into the opposite lateral tail vein. For the assessment of the human PLT survival in the mouse circulation, periodical blood-sample collections at determined time-points (60,120 and 300 min) were performed. Collected blood samples were anticoagulated in 30µl of ACD-A containing PBS and further processed according to a commercially available fixation kit (PerFix-non centrifugation assay Kit). In detail, after anticoagulation, samples were immediately treated with a fixation buffer for 15 min at RT. Afterwards, red-blood-cell (RBC) depletion using 100µL of the kit specific lysis-buffer was performed (30 min, RT, in the dark). The reaction was stopped via the addition of 400µl of the kit specific stopping buffer and samples stored at 4°C (**Figure 4 A**).

2.2.9.4 Determination of human platelets in peripheral mouse blood

For species-specific PLT-identification, anti-human CD41-PE-Cy5 (CD41-PC5) and anti-mouse CD41-FITC Ab staining was performed (30 min, RT) and

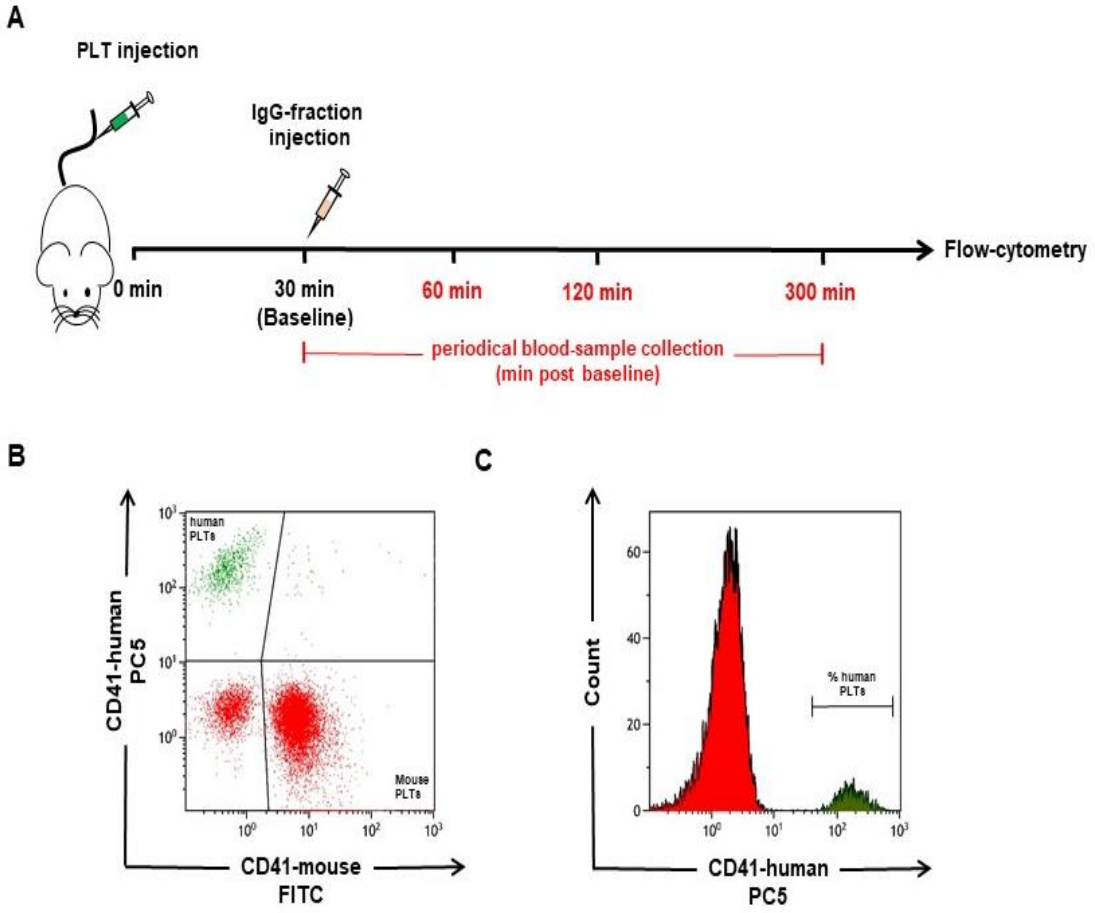

Figure 4: Survival of human platelets in the NSG-mouse model

A) After the injection of human PLTs into the mouse lateral tail vain, a blood-sample for the determination of the baseline (100% of human PLT-count in the mouse-circulation) was withdrawn 30 min after PLT injection. Thereafter, an immediate injection with human control or ITP IgGs was performed. For the longitudinal assessment of the human PLT survival, periodical blood-sample collections were performed at different time-points after baseline (60; 120 and 300 min) and analyzed via FC. B) and C) Representative dot-plot and histogram showing the FC gating strategy for the determination of human PLTs in mouse blood samples. The amount of human PLTs in the mouse circulation was assessed via the binding of a anti-human CD41-PC5 labelled monoclonal antibody and normalized to the total amount of human CD41-PC5 and mouse CD41-FITC positive PLT events. CD41, glycoprotein IIb; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; min, minutes; NSG, NOD scid gamma; PC5, phycoerythrin cyanin 5; PLTs, platelets.

followed by FC analysis. To determine the survival of human PLTs in the mouse circulation, a gating strategy that focuses on the FSC vs. SSC properties and species-specific surface marker expression of PLTs, was utilized. In detail, only cells with PLT-characteristics and single positive events for the expression of human-CD41 and mouse-CD41, respectively, were gated and considered as intact PLTs. The percentage of human-PLTs was normalized to the total amount of human-CD41 and mouse-CD41 positive events (**Figure 4 B+C**).

2.2.10 Platelet adhesion assay

To assess the impact of desialylation on PLT functionality, 8 well chamber- slides were coated with different extracellular-matrix-proteins (ECM) and adherent PLTs quantified via bright-field microscopy. Recombinant vWF was delivered as a lyophilized powder and resuspended with distilled water to a final concentration of 5U/ml. After coating with 150µl of fibrinogen (100 µg/mL) or vWF, culture slides were put on a shaker at 4°C overnight. The the next day, the coated slides were washed three times with PBS and blocked with human serum-albumin [HSA (250µg/ml)] for one hour at RT. In parallel, wPLTs ($7.5x10^6$/well) were incubated with ITP or control IgGs for 120 min at RT and subsequently filled up with PBS to a final volume of 600µl. The latter was followed by seeding the cells in aliquots of 150µl per surface coating and a resting time of 1 h at RT. In the last step, cells were fixed with 2% PFA for 20 min at RT and washed three times with PBS. For the quantification of adherent cells, 7 randomly chosen microscopic fields at 100X magnification were selected via bright-field microscopy and normalized to control.

2.2.11 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (La Jolla, USA). T-test was used to analyze normally distributed results. Nonparametric test (Mann-Whitney test) was used when data failed to follow a normal distribution as assessed by D'Agostino and Pearson omnibus normality test. Group comparison was performed using the Wilcoxon rank-sum test and the Fisher exact test with categorical variables. A p-value <0.05 was assumed to represent statistical significance.

2.2.12 Ethics

Studies involving human material were approved by the Ethics Committee of the Medical Faculty (025/2018BO2), Eberhard-Karls University, Tübingen, Germany, and were conducted in accordance with the declaration of Helsinki. All animal studies were conducted in Tübingen, Germany, and were approved by the responsible authorities of the State of Baden-Württemberg (M2/17).

3 Results

3.1 ITP-patient cohort

100 ITP patients were included in this study. The diagnosis of ITP was verified independently by two physicians, expert in the field of immune-haematology, according to current ITP guidelines (159). Patient material was collected for laboratory investigations of ITP between 2009-2019. Leftover samples were used in the current study. Overall, 100 patients were enrolled in the laboratory study. 40/100 (40%) patients were female and the median age at the time of laboratory testing was 54 years (interquartile range [IQR]: 0-86 years). The median PLT-count was 32x10^9/L (IQR: 7-74x10^9/L). Manifest bleeding events, reaching from petechiae to intracranial bleeding, were reported in 48/100 (48%) patients (**Table 6**). In the whole study cohort, ITP anti-PLT AAbs against different PLT GPs that were detected either in direct or indirect MAIPA-assay, were present in 51/100 (51%) patients (**Figure 5**).

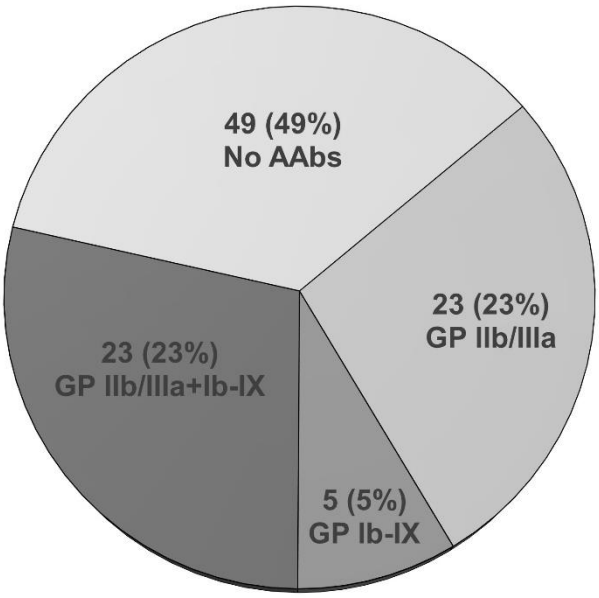

Figure 5: Glycoprotein specificities detected in ITP patient sera

GP specificities of ITP anti-PLT AAbs that were detected either in direct or indirect MAIPA were distributed as the following: 23% of patients presented with detectable anti-GP IIb/IIIa, 5% with anti-GP Ib-IX, 23% with anti-GP IIb/IIIa as well as anti-GP Ib-IX and 49% with no detectable anti-PLT AAbs. AAbs, autoantibodies; GP, glycoprotein; GP IIb/IIIa, fibrinogen-receptor GP Ib-IX, von Willebrand factor receptor.

3.2 Screening of the ITP patient cohort for desialylation

To investigate whether ITP patient sera have the capability to induce desialylation of PLTs, a screening for serum-induced changes in the sialic pattern of healthy wPLTs was performed. Via the utilization of a FC LBA assay, FIs of desialylation were calculated by comparing mean-fluorescence-intensities (MFIs) of wPLTs that were incubated with ITP patient serum with wPLTs that were incubated with HC serum.

Based on these analyses, a subgroup of 35/100 ITP patients was identified to induce increased desialylation of healthy wPLTs. Specifically, 28/100 sera led to a significant elevation in the exposure of ß-Galactose as well as 21/100 sera induced an increased exposure of N-Acetylglucosamine (mean FI: 3.50, range: 1.79-13.61, p value 0.0001; FI: 2.41, range: 1.54-5.47, p value 0.0001, respectively). Interestingly, serum-induced PLT desialylation was detected more often in patients with secondary ITP when compared with primary ITP patients (60% vs. 31%, p value 0.0395). For the validation of significant PLT desialylation in the serum screening, cutoff-values were calculated via the utilization of control sera (see method section), namely 1.75 and 1.42 for the expression of ß-Galactose and N-Acetylglucosamine, respectively (**Figure 6**).

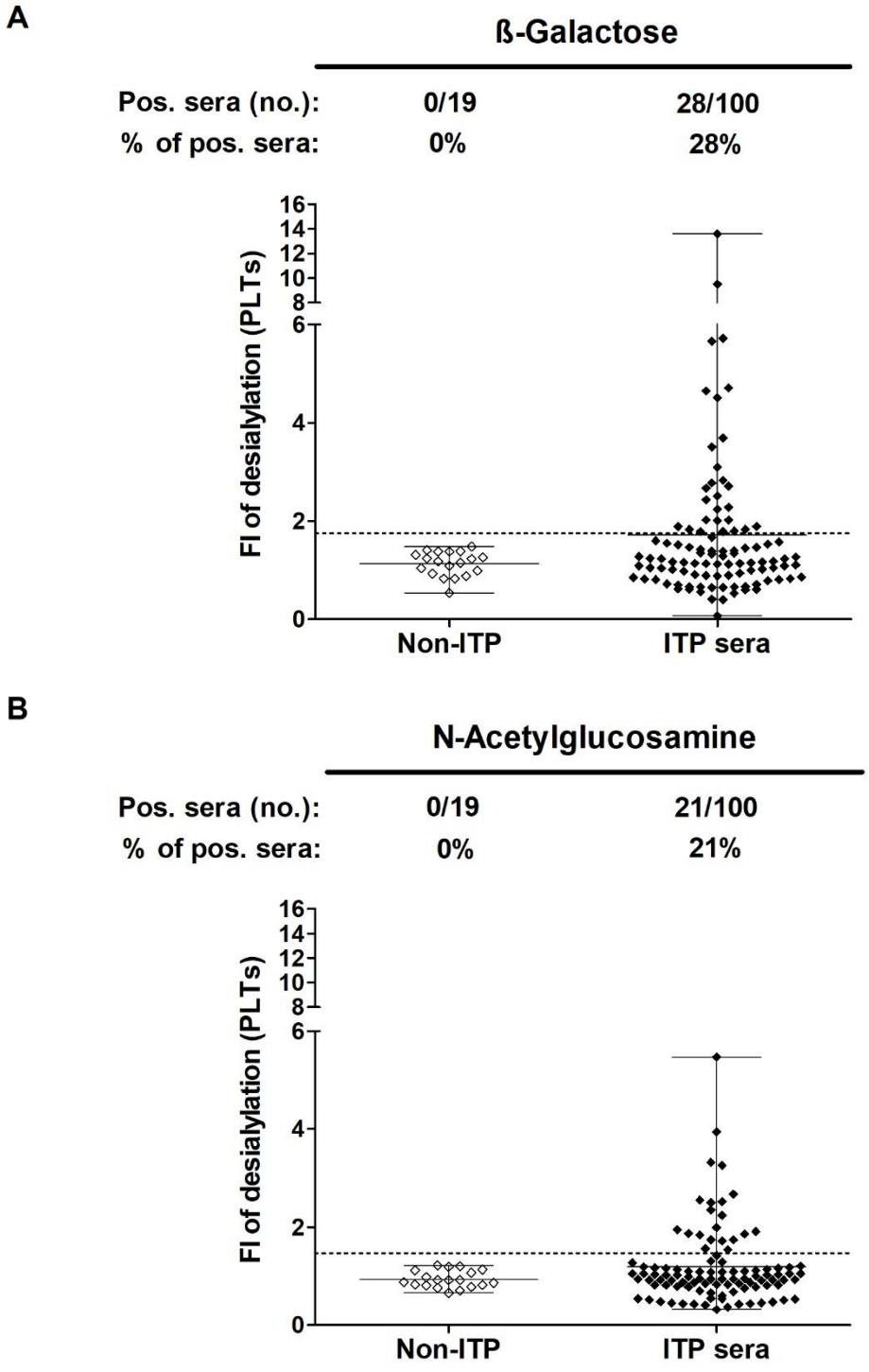

Figure 6: Screening of ITP sera for platelet desialylation

28/100 (A) and 21/100 (B) ITP patient sera (black diamonds) were able to induce an increased exposure of the sugar residues ß-Galactose and N-Acetylglucosamine on the surface of wPLTs. FI, mean fold-increase; ITP, immune thrombocytopenia; no, number; Dotted line, cutoff-values for the exposure of ß-Galactose and N-Acetylglucosamine, FI 1.75 and 1.42, respectively.

3.3 Correlation of desialylation and patient characteristics

To verify whether the biological effect of ITP serum induced changes in the sialic pattern of PLTs is of any clinical significance, desialylating and non-desialylating ITP patients were compared with their corresponding medical records. No correlation regarding gender as well as age distribution with serum induced PLT desialylation was revealed at the time of MAIPA testing. Of note and of high clinical interest, there was a significant correlation between the subgroup of patients that were found to be positive for desialylation and clinical overt thrombocytopenia. In particular, the subgroup of ITP patients with desialylating serum AAbs showed a marked decrease in the median PLT-count compared to patients with non-desialylating AAbs (PLT-count at MAIPA testing, median [IQR]: $13x10^9$/L, range 5-$42x10^9$/L vs. $46x10^9$/L, range 11-$81x10^9$/L, p value 0.0286, respectively**, Figure 7**).

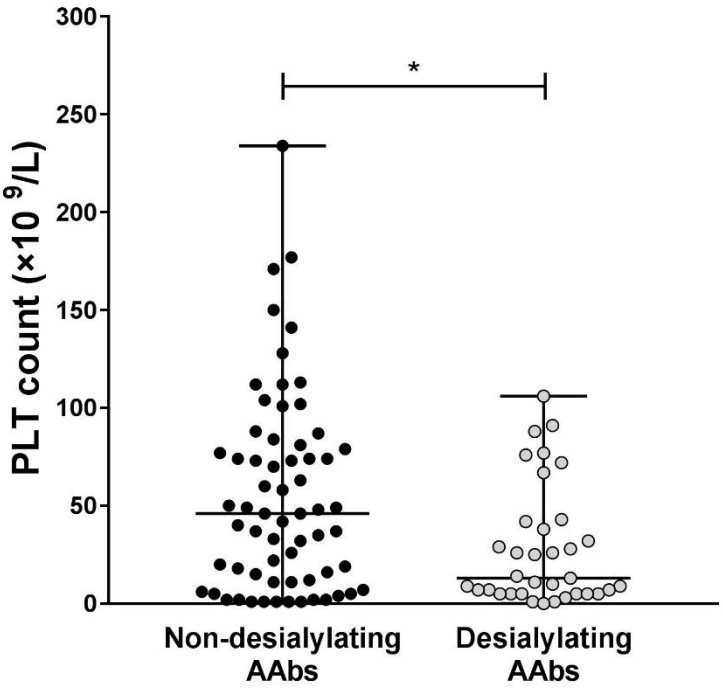

Figure 7: Desialylation is associated with lower platelet-counts

Patients with desialylating ITP-AAbs presented with significantly decreased PLT-counts compared to patients with non-desialylating ITP-AAbs. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. AAbs, autoantibodies; PLT, platelet.

Additionally, the effect of AAb-induced desialylation not only seems to be associated with low PLT-counts but also with an increased rate of bleeding manifestations including petechiae, hematoma as well as epistaxis (% of patients with bleeding at testing: 72% vs. 28%, p value 0.0008, respectively,**Table 6**).

Table 6: Desialylation is associated with higher bleeding prevalence

	All ITP patients included in the study (n=100)	Patients with desialylating AAbs (n=35)	Patients with non-desialylating AAbs (n=65)	p-value
PLT-count ($x10^9$/L) [median (IQR)]	32 (7-74)	13 (5-42)	46 (11-81)	0.0286
Any bleeding	48/10 (48%)	25/35 (72%)	23/65 (28%)	0.0008
Bleeding symptoms				
Petechiae	28/100 (28%)	16/35 (46%)	12/65 (19%9	0.0044
Hematoma	28/100 (28%)	17/35 (49%)	11/65 (17%)	0.0009
Mucosal	15/100 (15%)	7/35 (20%)	8/65 (12%)	0.3763
Epistaxis	12/100 (12%)	8/35 (23%)	4/65 (6%)	0.0208
Gastrointestinal	2/100 (2%)	1/35 (3%)	1/65 (2%)	0.9999
Central nervous system	1/100 (1%)	1/35 (3%)	0/65 (0%)	0.3434

3.4 Autoantibody specificity and its impact on desialylation

Following the identification of an ITP patient subgroup with desialylating AAbs, the relevance of human ITP AAb GP-specificities on PLT desialylation was investigated. Based on the LBA screening results, all patient sera were grouped and analyzed according to their GP-specificity as determined in the MAIPA-assay. Interestingly and in line with previous studies, a subgroup of human ITP sera of the patient cohort with detectable AAbs against the GP Ib-IX were identified to induce a significant cleavage of sialic acid on the PLT-surface. Of note, this human ITP AAb induced effect seems not to be restricted to AAbs against GP Ib-IX alone as almost 50% of ITP patients with detectable AAbs against GP IIb/IIIa were able to induce an increased expression of ß-Galactose and N-Acetylglucosamine (mean FI: 4.43, range: 1.83-13.61, p value 0.0001 for ß-Galactose and 2.58, range: 1.54-5.47, p value 0.0001 for N-Acetylglucosamine, respectively). Additionally, a subgroup of ITP sera with neither in direct nor indirect MAIPA detectable anti-PLT AAbs induced significant desialylation of healthy PLTs (mean FI: 2.46, range: 1.79-4.65, p value 0.0001 for ß-Galactose and mean FI: 2.23, range: 1.74-2.55, p value 0.0003 for N-Acetylglucosamine, respectively). This finding indicates that desialylating ITP AAbs may be present in this patient-group. However, due to the low-avidity to target epitopes and or to the lack of sensitivity in the MAIPA-assay, these AAbs seem to be missed in the standard detection assay (**Figure 8**).

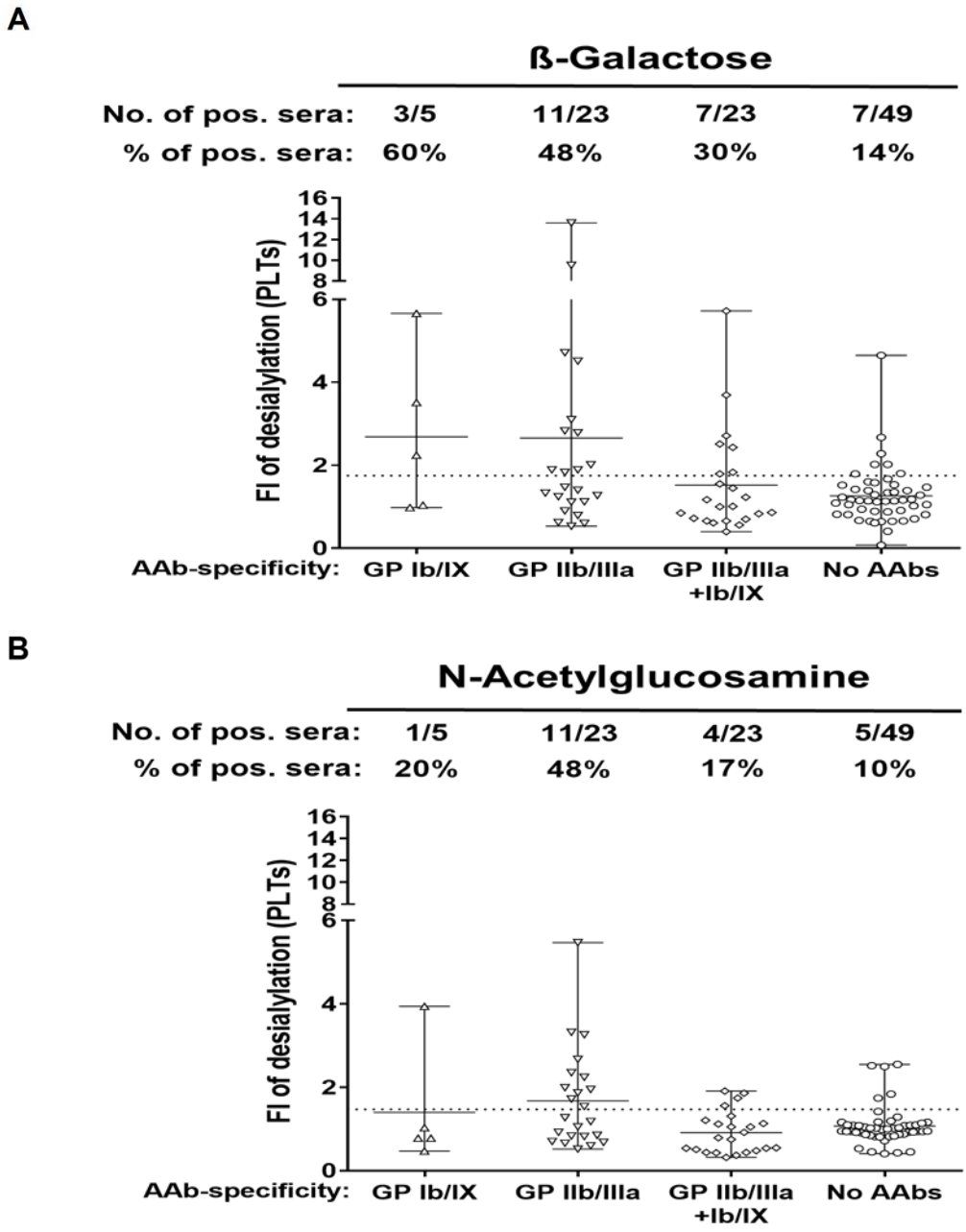

Figure 8: Human ITP autoantibody-induced platelet desialylation is not restricted to a single GP specificity

In addition to anti-GP Ib/IX AAbs, also sera with detectable anti-GP IIb/IIIa AAbs induced an increased exposure of (A) ß-Galactose as well as (B) N-Acetylglucosamine, respectively. Additionally, a subgroup of ITP patient sera without detectable AAbs induced increased desialylation. AAb, autoantibody; FI, fold increase; GP Ib/IX; von Willebrand factor receptor; GP IIb/IIIa, fibrinogen receptor; no., number; pos., positive; Dotted line, cutoff-value for significant desialylation 1.75 and 1.42 for ß-Galactose and N-Acetylglucosamine, respectively.

3.5 IgG fraction is responsible for desialylation in ITP sera

To further verify that the observed ITP serum-induced desialylation is due to the presence of specific anti GP IIb/IIIa ITP AAbs and not induced via other serum components, a proof of principle attempt using purified ITP IgG was performed. For this, IgG fractions were prepared from three corresponding ITP sera that were positive for desialylation and presented with detectable anti-GP IIb/IIIa AAbs in the first screening vice versa. Similar as previously observed with sera, also ITP IgG fractions that were prepared from corresponding desialylating anti-GP IIb/IIIa ITP AAb harbouring patient sera induced desialylation. This was confirmed by an anti-GP IIb/IIIa ITP IgG induced increased exposure of ß-Galactose and N-Acetylglucosamine on the PLT-surface (FI mean± SEM: 5.96±1.31 vs. 5.69±0.99, p value 0.8714 and FI mean± SEM: 3.69±0.76 vs. 5.20±0.21, p value 0.1028, respectively, **Figure 9**). Considering that these experiments were performed using wPLTs and isolated IgG fractions, this finding indicates that anti-GP IIb/IIIa ITP AAbs are able to induce the translocation of PLT associated sialidase that subsequently results in the cleavage of terminal sialic acid. Additionally, these results further contradict the hypothesis that desialylation might be induced by other serum-factors than ITP AAbs.

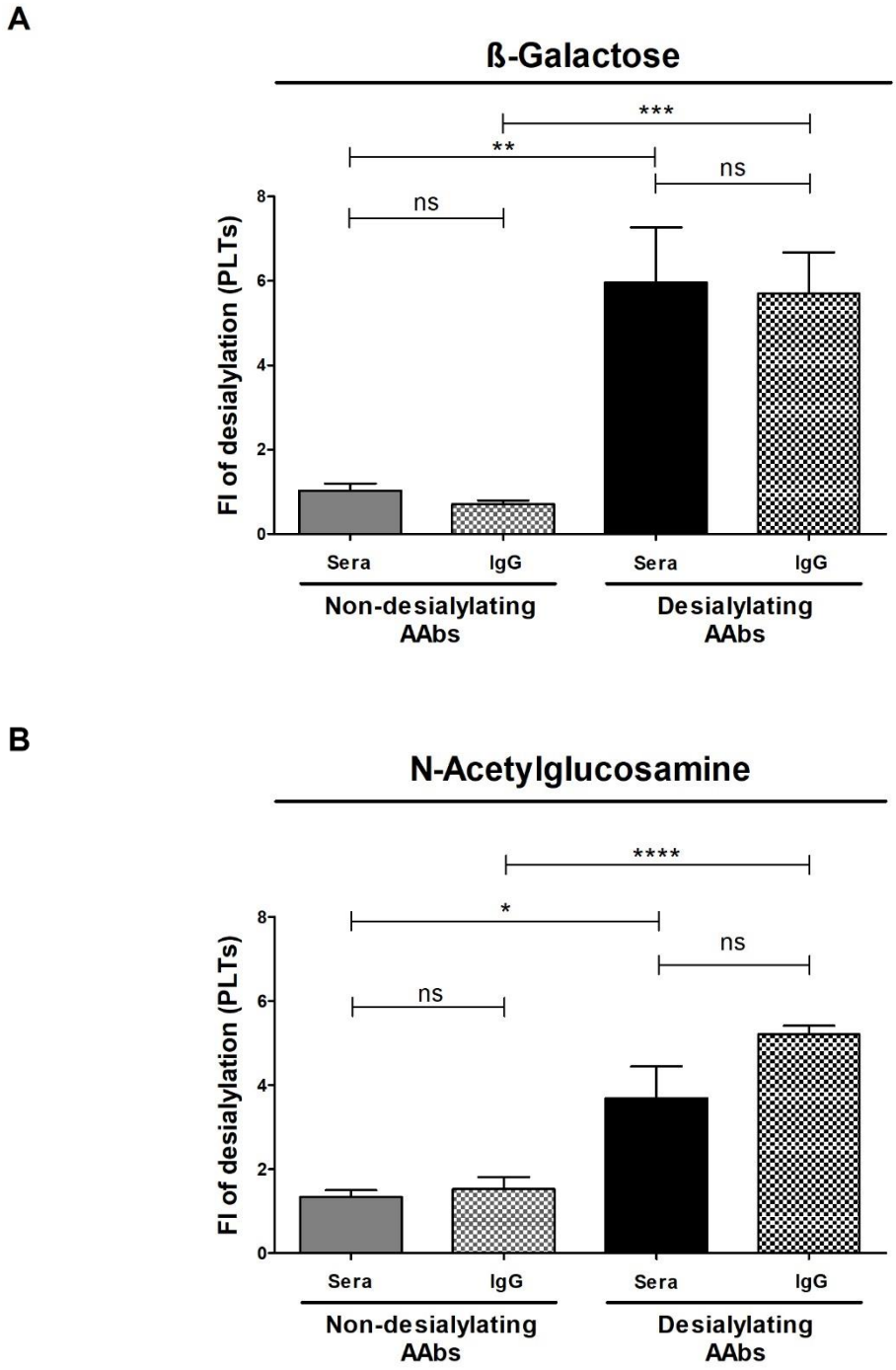

Figure 9: The impact of ITP autoantibodies on platelets' sialic pattern

IgG fractions were prepared from desialylating and non-desialylating ITP sera with detectable anti-GPIIb/IIIa AAbs. No significant changes in the amount of desialylation within these two groups were observed between IgG-fractions and the corresponding sera for both, (A) ß-Galactose and (B) N-Acetylglucosamine, respectively. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. AAbs, autoantibodies; FI, fold increase; IgG, immunoglobulin G, ns, not significant; PLTs, platelets.

3.6 Anti-GP IIb/IIIa autoantibodies induce desialylation via FcγRIIA

Since additional Fc-independent PLT clearance mechanisms in ITP were first suggested in the early 2000s, changes in the sialic pattern of PLTs became a target of various research approaches (135). Consequently, desialylation mediated by anti-GP Ib-IX AAbs is nowadays an adopted alternative PLT-clearance mechanism. However, in the present study, a subgroup of nearly 50% of patients with detectable ITP AAbs against PLT GP IIb/IIIa was identified to induce a significant desialylation of healthy PLTs. Taking into account, that PLTs carry the largest amount of the innate immune receptor FcγRIIA on their surface, an interaction with the Fc-portion of anti-GP IIb/IIIa AAbs causing subsequent PLT desialylation might be conceivable. For this and to gain further mechanistic insights, PLTs were preincubated with a monoclonal inhibitor of FcγRIIA (anti-CD32a moAb), namely IV.3, prior to IgG incubation. Of note, FcγRIIA blockade via IV.3 resulted in a significant reduction of anti-GP IIb/IIIa IgG-mediated desialylation of wPLTs. This was confirmed as IV.3 preincubation led to a significant inhibition of ß-Galactose as well as N-Acetylglucosamine exposure compared to wPLTs that were preincubated with the vehicle control (% desialylation mean±SEM: 100±0% vs.52±11%, p value 0.0110 and 100±0% vs. 60±9%, p value 0.0190, for ß-Galactose and N-Acetylglucosamine, respectively, **Figure 10**).

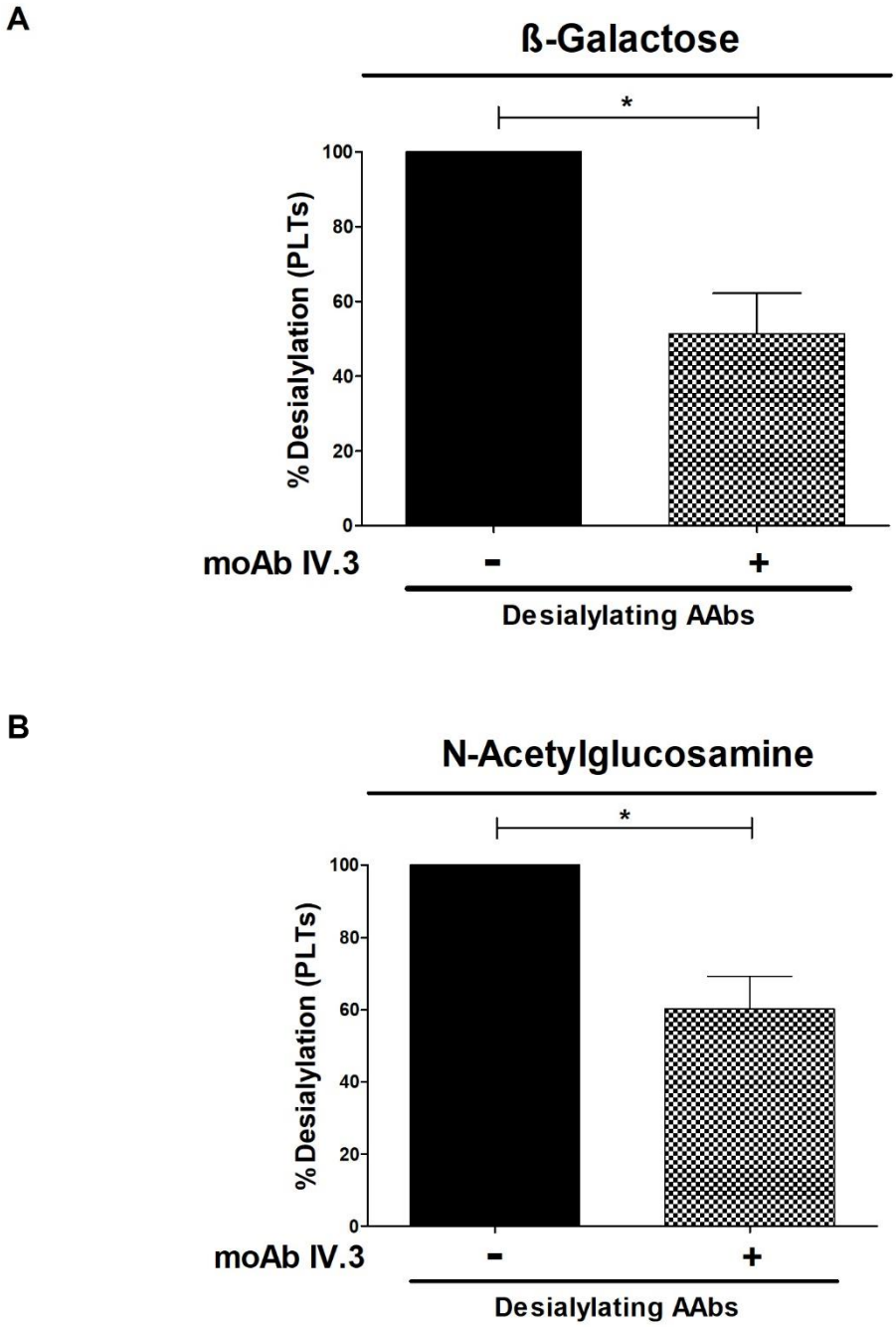

Figure 10: Anti-GP IIb/IIIa autoantibody-induced desialylation is significantly reduced by the blockade of FcγRIIA

Preincubation of human wPLTs with anti-CD32a monoclonal antibody (moAb IV.3) resulted in a significant decrease in the exposure of (A) ß-Galactose and (B) N-Acetylglucosamine, induced via desialylating anti-GP IIb/IIIa ITP AAbs. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. AAbs, autoantibodies; moAb IV.3, anti-human CD32a monoclonal antibody; PLTs, platelets.

3.7 Treatment with a sialidase inhibitor reduces desialylation

It is well accepted, that the translocation of both, intracellular and membrane-bound sialidase results in the cleavage of terminal sialic acid on the PLT surface (167). Additionally, different pathogens, like streptococcus pneumoniae or the influenza virus, utilize sialidase as a pathogenic factor and the inhibition via sialidase inhibitors is a safe and a well-established treatment option (149, 168, 169). Based on this evidence, the next approach of the study was to verify whether desialylation on the PLT surface can be efficiently blocked via the use of the sialidase inhibitor oseltamivir-acid in vitro. According to literature data and a prior performed dose-effect titration curve (data not shown), an optimum concentration of 50µmol oseltamivir-acid was efficient to prevent desialylation via exogenous sialidase on the PLT surface. In particular, the preincubation of wPLTs with oseltamivir-acid significantly decreased the expression of both ß-Galactose and N-Acetylglucosamine compared to the vehicle control (% desialylation mean±SEM: 100±0% vs. 23.17±6%, p value 0.0058 and 100±0% vs. 21.62±2%, p value 0.0005, respectively, **Figure 11**).

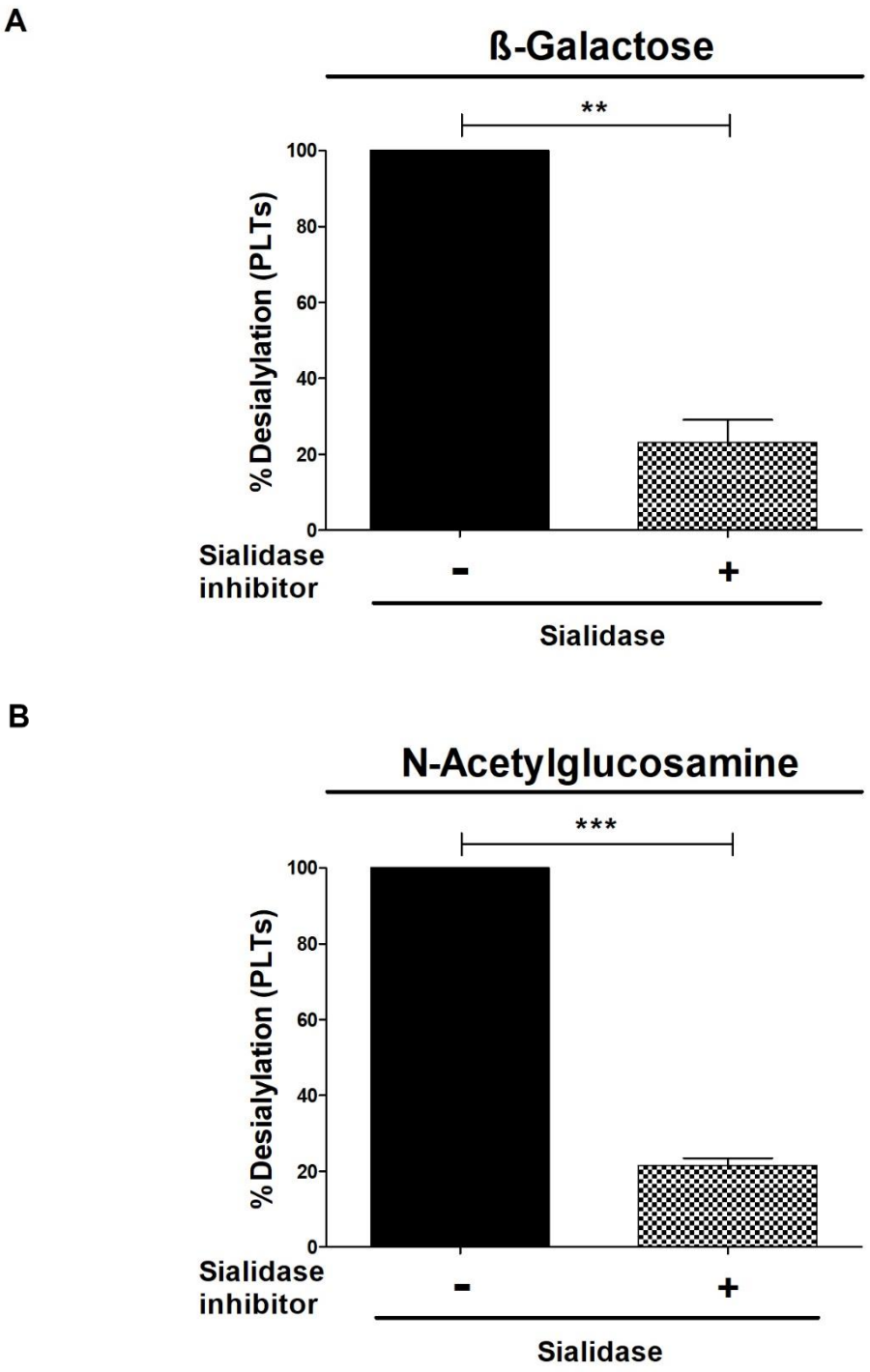

Figure 11: The use of a sialidase inhibitor prevents platelet desialylation induced via exogenous-sialidase

Preincubation of wPLTs with a sialidase inhibitor efficiently blocked an increased exposure of (A) ß-Galactose and (B) N-Acetylglucosamine that was induced via exogenous-sialidase. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. PLTs, platelets.

3.8 Blockade of ITP autoantibody-induced desialylation via sialidase inhibition

Following these data, the next question was whether the preincubation of PLTs with oseltamivir-acid shows the potential to prevent ITP AAb-induced PLT desialylation. Of note, sialidase-inhibition resulted in a marked reduction of ITP AAb-induced desialylation. In detail, preincubation with oseltamivir-acid led to a significant reduction of anti-GP IIb/IIIa AAb-induced expression of ß-Galactose as well as N-Acetylglucosamine compared to the vehicle control (% mean±SEM: 100±0% vs.63±2%, p value 0.0001 and 100±0% vs. 57±6%, p value 0.0002, respectively, **Figure 12**).

Via the use of wPLTs and isolated IgG-fractions, these findings point towards the ability of ITP AAbs to induce the release of sialidase from the inner-compartment of PLTs. Interesting is also the observation that sialidase-inhibition resulted in a marked reduction of desialylation despite the presence of ITP AAbs. This finding indicates with high probability that potential quantitative changes in the total sugar amount, due to the presence of PLT binding AAbs from the IgG subclass that are known to harbour a specific glycosylation pattern, can be ruled out as an artefact (170).

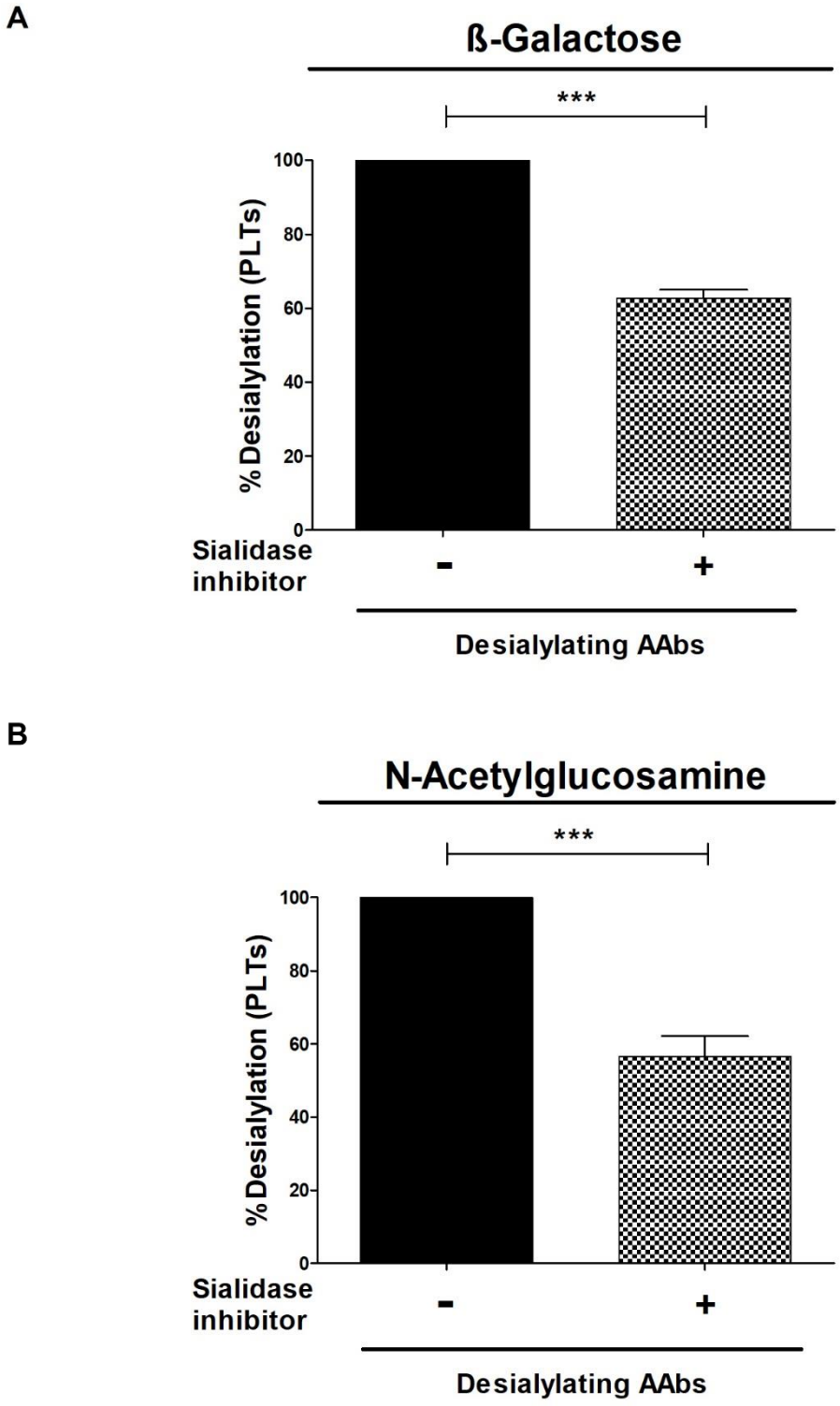

Figure 12: Inhibition of ITP autoantibody-induced desialylation via a sialidase inhibitor
Preincubation with oseltamivir-acid resulted in a significant reduction of anti-GP IIb/IIIa ITP AAb-induced increased exposure of (A) ß-Galactose and (B) N-Acetylglucosamine, respectively. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. AAbs, autoantibodies; PLTs, platelets.

3.9 Desialylation impairs platelet survival

To investigate whether desialylation affects the survival of human PLTs in vivo, an NSG-mouse model was utilized.

3.9.1 Desialylation via exogenous-sialidase reduces platelet survival

In a first step, the question whether desialylation of PLTs via exogenous sialidase affects PLT survival was addressed. Remarkably, while native PLTs showed a stable overall survival, desialylation via exogenous-sialidase resulted in a nearly complete depletion of human PLTs in the mouse circulation 300 min after PLT administration (% human PLT survival after 300 min, mean±SEM: 62.5±9% vs. 3.5±0.5%, p value 0.0054). This finding suggests that changes in the sialic pattern of PLTs seem to have a drastic effect on PLT survival, namely the destruction of human PLTs independent of Fc-mediated PLT-clearance mechanisms (**Figure 13**)

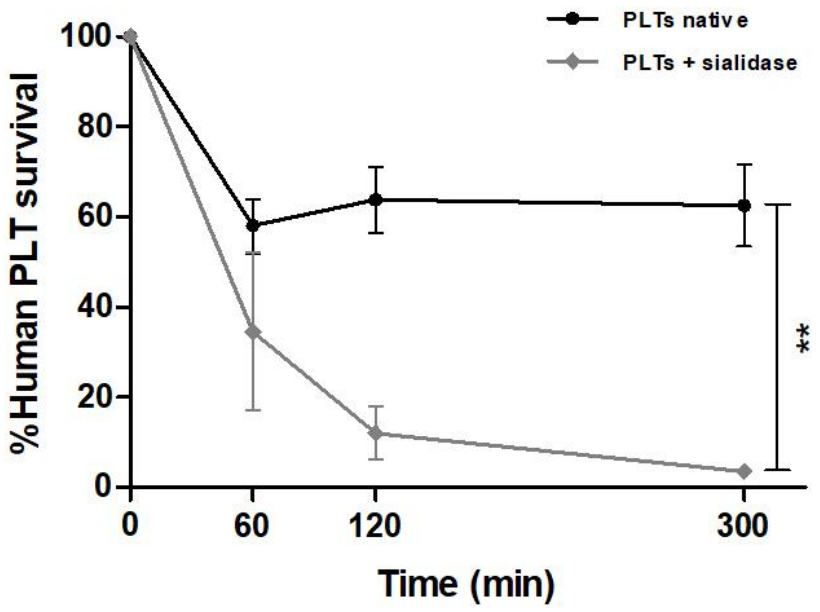

Figure 13: Desialylation of human platelets via exogenous-sialidase induces increased platelet clearance in vivo

Changes in the sialic pattern of human PLTs induced via exogenous-sialidase resulted in a marked decrease in the number of circulating human PLTs in mice (grey diamonds), whereas native, vehicle preincubated PLTs (black diamonds) showed stable PLT-counts during the observation period. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. min, minutes; PLT, platelet.

3.9.2 ITP autoantibody-mediated desialylation induces increased platelet clearance

Considering the findings of the previous experiment comparing the in vivo survival of native and via exogenous sialidase desialylated PLTs, the next step of the study was as to determine the effect of ITP AAb induced PLT desialylation on PLT survival.

The performance of these experiments showed that the administration of desialylating ITP IgGs via tail vein injection induced a dramatic decrease of the human PLT-count in the mice circulation when compared with control (mean % human PLT survival after 300 min±SEM: 28±5% vs. 79±5%, respectively, p value 0.0005). Most important, the preincubation of human PLTs with a sialidase inhibitor resulted in a significant rescue of the human PLT-count compared with PLTs treated with the vehicle control (mean % human PLT survival after 300 min±SEM: 28±5% vs. 45±3%, p value 0.019, **Figure 14**). This positive outcome of human PLT-counts after sialidase-inhibition indicates that ITP AAb-induced PLT desialylation might be one of the factors that contribute to the increased destruction of PLTs in ITP.

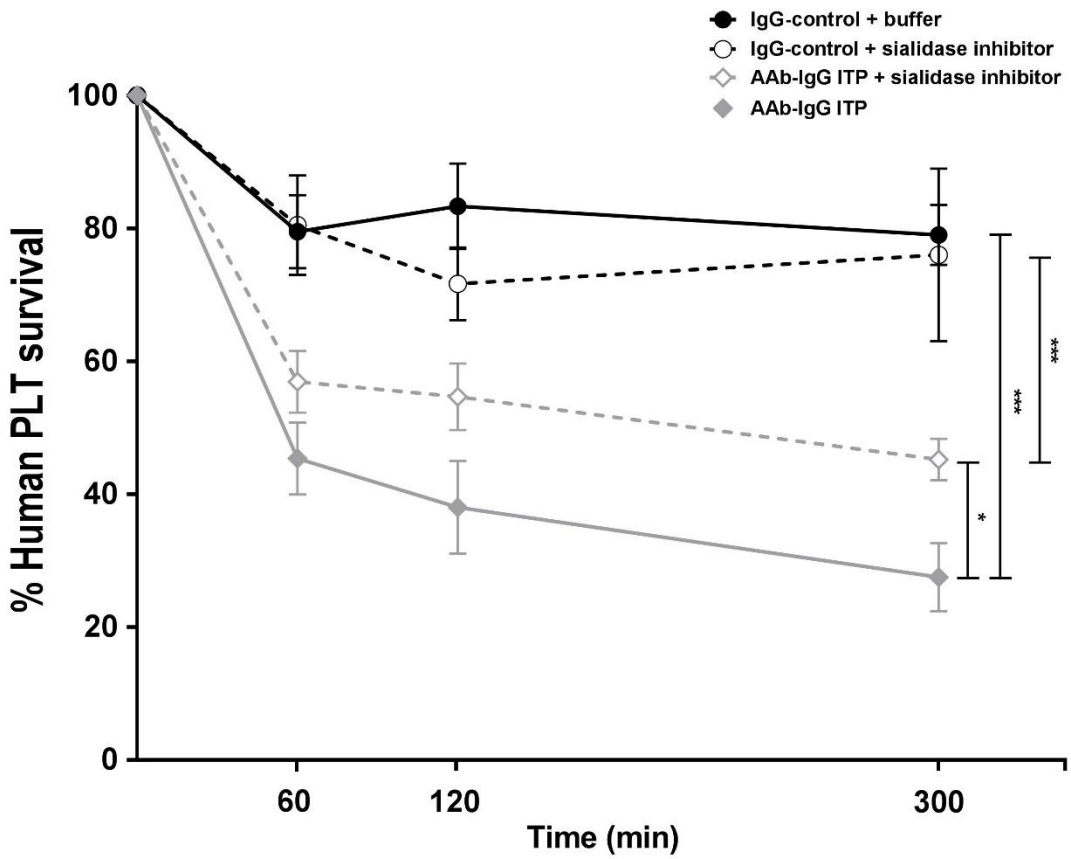

Figure 14: The impact of desialylation on platelet life span

The injection of desialylating ITP-AAbs into NSG mice resulted in an accelerated clearance of human PLTs from the mouse circulation (grey diamonds). The destruction of human PLTs by ITP AAbs was reduced, but not completely blocked, by a sialidase inhibitor (oseltamivir-acid, empty diamonds) that prevents the desialylation of PLTs. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. AAb, autoantibody; IgG, immunoglobulin G; ITP, immune thrombocytopenia; min, minutes; PLT, platelet.

3.10 Desialylation affects platelet functionality

3.10.1 Desialylation via exogenous-sialidase impairs platelet function

Considering that ITP-patients with desialylating AAbs presented with increased bleeding tendency, the impact of desialylation on PLT function was investigated. Therefore, a PLT adhesion assay that utilizes two extracellular matrix-protein coatings, namely fibrinogen and vWF, was performed under different conditions.

In a first step, wPLTs were preincubated with exogenous-sialidase prior to the spreading on fibrinogen and vWF, respectively, to verify whether desialylation affects PLTs' adhesion ability. Interestingly, desialylation via exogenous-sialidase resulted in a significant reduction of PLT adhesion on fibrinogen as well as vWF when compared with the vehicle control (% adherent cells/field mean±SEM: 100±0% vs. 49±4%, p value 0.0001 and 100±0% vs. 48±6%, p value 0.0001, respectively). The impact of desialylation on the adhesion function of PLTs was further shown, as the inhibition of exogenous-sialidase via a sialidase inhibitor led to a significant rescue of the deteriorated PLT function on both fibrinogen as well as vWF (% adherent cells/field mean±SEM: 49±4% vs. 76±4%, p value 0.0072 and 48±6% vs. 90±3%, p value 0.0031, respectively, **Figure 15**).

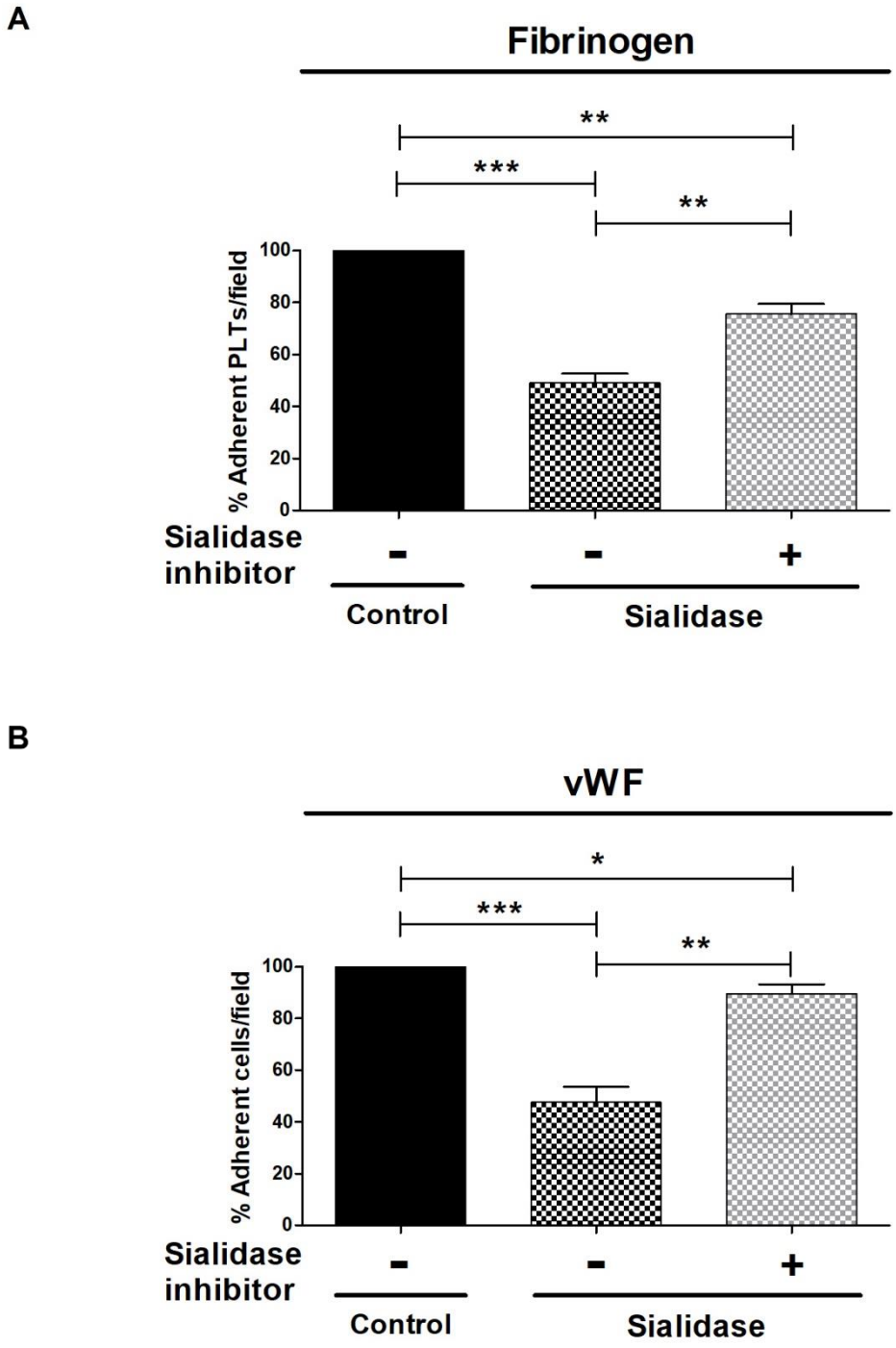

Figure 15: Desialylation impairs platelets' function to adhere

Changes in the sialic pattern reduce PLTs' ability to adhere on (A) fibrinogen as well as (B) vWF. Interestingly, the reduction of PLT functionality on both ECM-proteins was ameliorated via the use of a sialidase inhibitor. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. vWF, von Willebrand factor.

3.10.2 Desialylating ITP autoantibodies deteriorate platelets' functionality

Based on the findings that PLTs' adhesion ability seems to be dramatically impaired by exogenous-sialidase induced desialylation, the impact of ITP AAb-induced desialylation on PLT function was investigated. Notably, the incubation of wPLTs with desialylating ITP AAbs led to a dramatic decrease of the adhesion function on fibrinogen and vWF. In contrast wPLTs that were incubated with non-desialylating ITP IgGs only showed a moderate functional impairment (% adherent cells/field mean±SEM: 74±8% vs. 34±7%, p value 0.0021 and 67±5% vs. 26±2%, p value 0.0027, for fibrinogen and vWF, respectively, **Figure 16**).

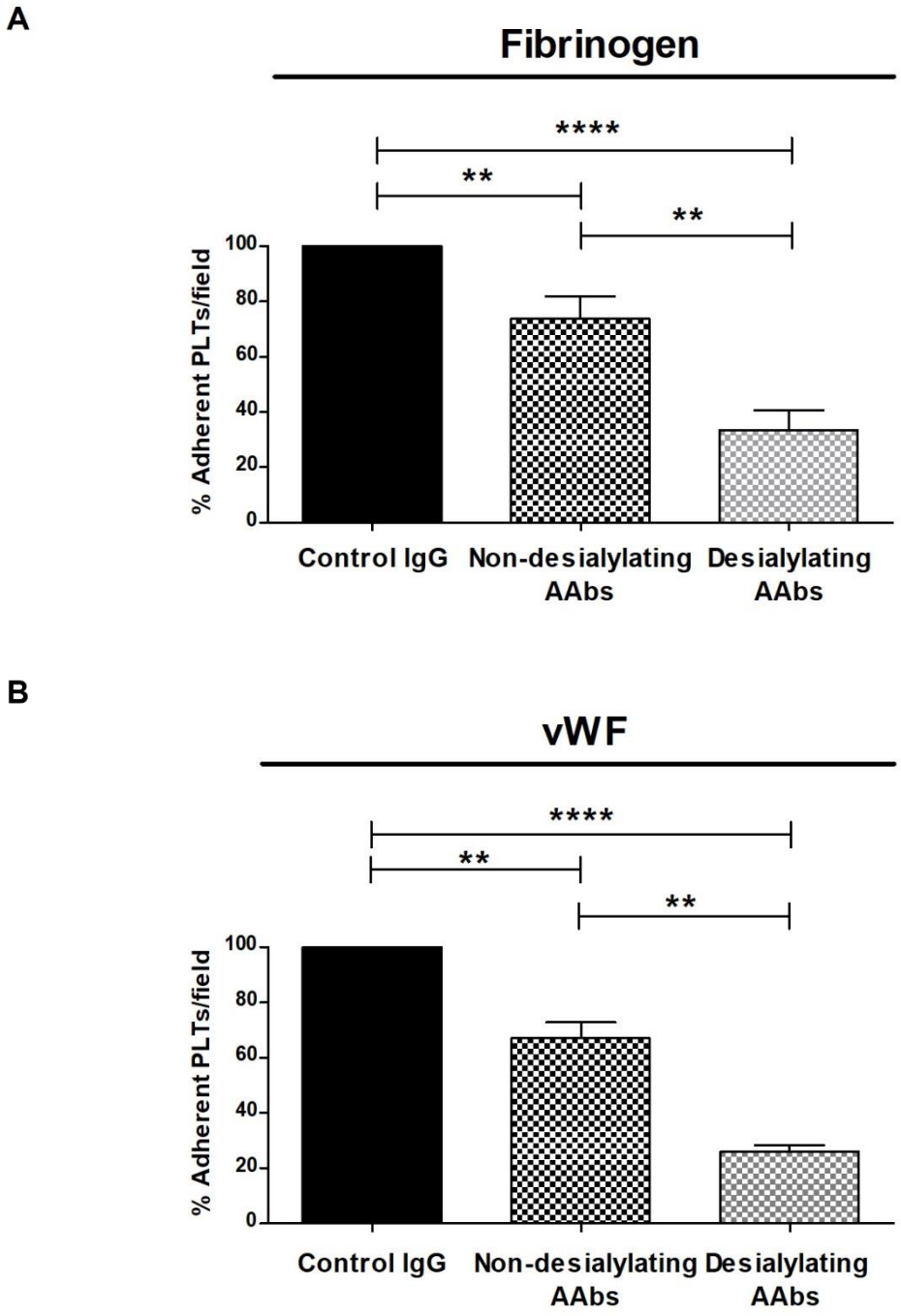

Figure 16: ITP autoantibody-induced desialylation affects platelet function

Desialylating ITP AAbs led to an inhibition of PLT adhesion on (A) fibrinogen as well as (B) vWF compared with non-desialylating AAbs. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. AAbs, autoantibodies; IgG, immunoglobulin G; vWF, von Willebrand factor.

3.10.3 Pretreatment with a sialidase inhibitor protects platelet adhesion ability

Considering the findings of the previous experiments, the next approach focused on confirming whether ITP AAb-induced reduction of PLT adhesion on fibrinogen as well as vWF is primarily related due to desialylation or to other factors like the interference of target receptors via ITP AAbs. Therefore, PLTs were preincubated with a sialidase inhibitor. Intriguingly, the preincubation with oseltamivir-acid led to a significant increase of PLT function. In detail, compared to the vehicle control, the function of sialidase inhibitor treated PLTs was significantly enhanced as there was a higher number of adherent PLTs on both ECMs (% adherent cells/field mean±SEM: 36±5% vs. 86±6%, p value 0.0001 and 26±2% vs. 67±11%, p value 0.0203, for fibrinogen and vWF, respectively, **Figure 17**).

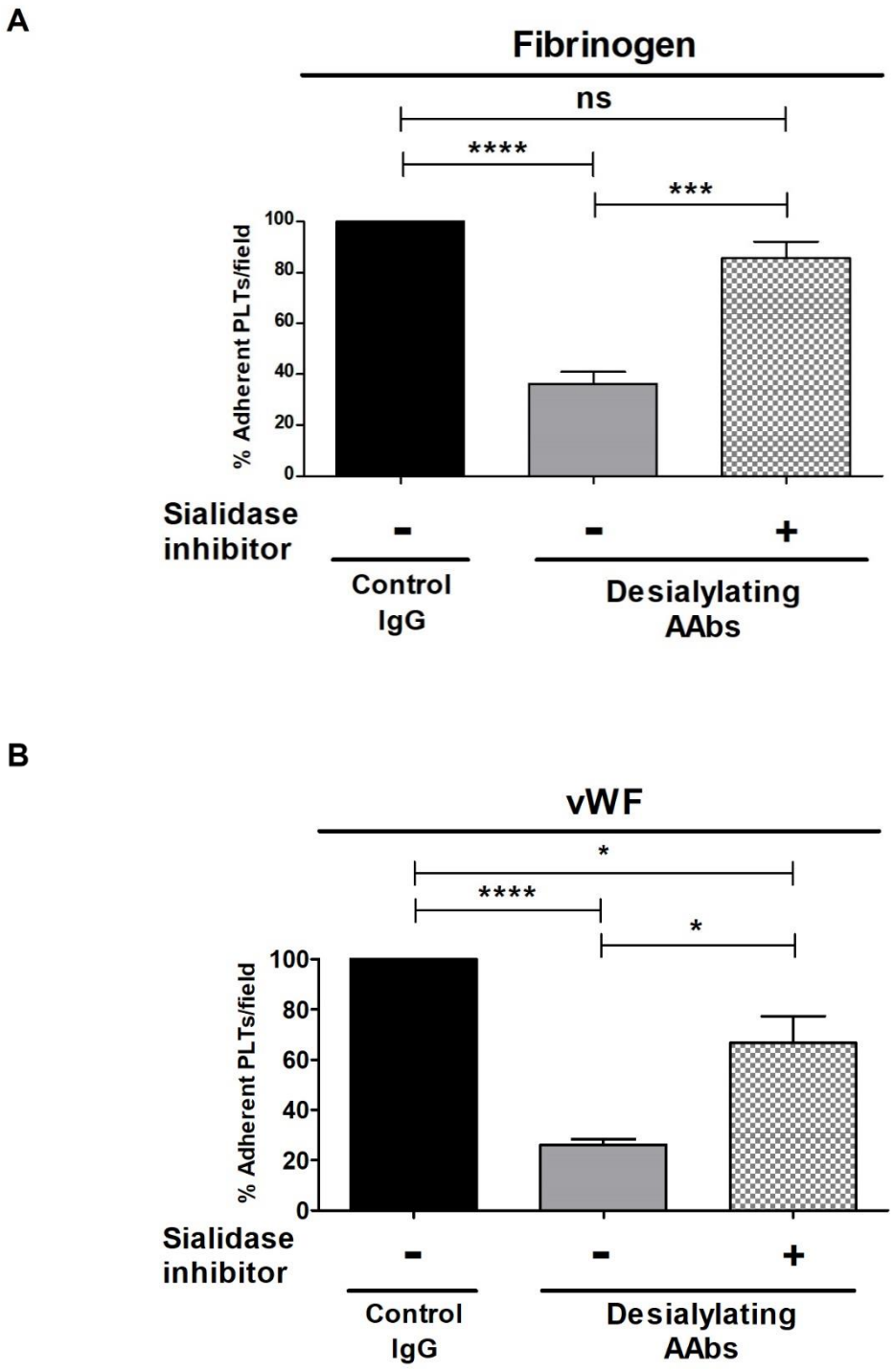

Figure 17: The use of a sialidase inhibitor rescues platelet functionality

ITP AAb-induced reduced PLT adhesion on (A) fibrinogen as well as (B) vWF was significantly improved by the pretreatment of PLTs with the sialidase inhibitor oseltamivir-acid. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. AAbs, autoantibodies; IgG, immunoglobulin G; ns, not significant; vWF, von Willebrand factor.

3.11 Platelet apoptosis does not correlate with ITP autoantibody-induced desialylation

The last step of this study investigated the impact of desialylation on the induction of PLT apoptosis as an alternative PLT clearance mechanism. Therefore, wPLTs were incubated with non-desialylating and desialylating human ITP AAbs. Subsequently, changes in the inner mitochondrial transmembrane potential were measured by FC. Interestingly, no correlation between desialylating AAbs that mediate increased exposure of ß-Galactose as well as of N-Acetylglucosamine and the induction of apoptosis was observed (r=-0.33, p value 0.351 and r=-0.344, p value 0.331 for ß-Galactose and N-Acetylglucosamine vs. TMRE, respectively, **Figure 18**).

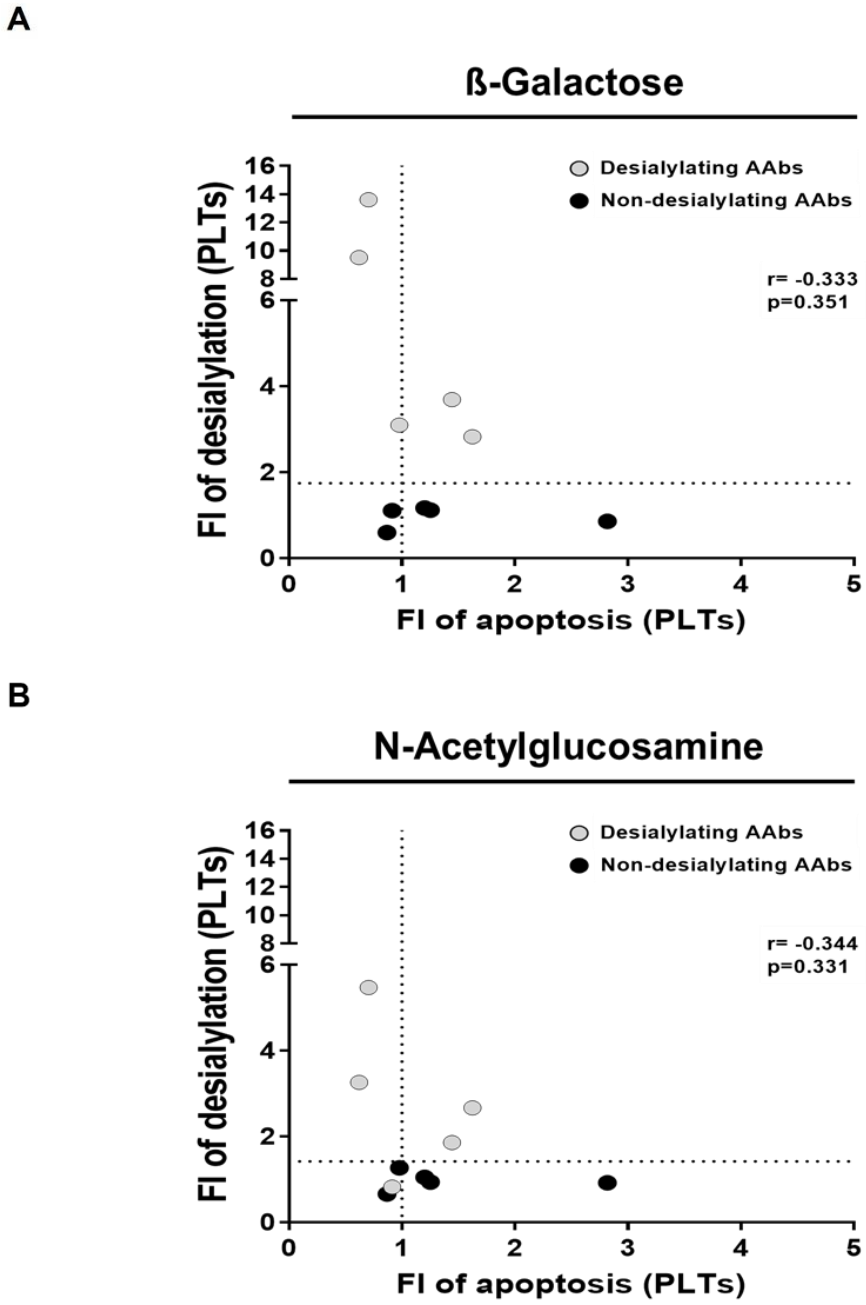

Figure 18: Platelet apoptosis is not associated with ITP autoantibody-induced platelet desialylation

No correlation between the increased exposure of (A) ß-Galactose as well as (B) N-Acetylglucosamine and the induction of PLT apoptosis, measured by changes in the inner mitochondrial trans- membrane potential (TMRE) was observed. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. AAbs, autoantibodies; FI, fold increase, PLTs, platelets.

4 Discussion

ITP is a bleeding disorder that is characterized by a low PLT-count and increased bleeding tendency (171). The aetiology of ITP is multifactorial, including alterations in the central as well as the peripheral immune-tolerance which result in modifications of T- and B-cell immunity (16). Altogether, these preconditions lead to an autoimmune state that results in an increased PLT destruction and/or an impaired production of PLTs in the bone marrow niche (172). While Fc-dependent PLT depletion mechanisms have been characterized since the early beginnings of ITP research, greater evidence regarding the suppression of PLT production via ITP AAbs in the bone marrow has been gained more recently (172-174). Hence, most of the current therapeutic approaches, like the administration of IVIG, anti-D or thrombopoietin receptor agonists target these Fc-dependent pathways or the impaired PLT-production (17).

In consideration of the well-established Fc-dependent pathomechanisms, the identification of an additional, Fc-independent PLT clearance pathway, namely Ab-induced desialylation, was a striking discovery (157). However, a systematic analysis focusing on the prevalence as well as the potential of different human ITP AAb-subsets to induce PLT desialylation has not been performed in a bigger ITP patient cohort. Moreover, most other studies performed did not investigate the role of ITP AAb-induced desialylation on PLT function and the consequences on the prevalence and tendency of bleeding in ITP patients.

In the first step of this study, a screening of 100 well characterized ITP patients regarding the potential to induce desialylation of healthy donor PLTs was performed. Interestingly, subsequent analysis of the sialylation status of PLTs confirmed that 35 out of 100 patient sera were able to induce an increased expression of different sugar-residues, namely N-Acetylglucosamine and ß-Galactose, that are physiologically capped with terminal sialic acid. To validate that the observed desialylation is induced via a specific subset of desialylating AAbs and not due to the binding of unspecific PLT-bound IgGs or immune complexes that might be present in the sera of the normal population, pooled AB sera from healthy individuals served as control (175). Additionally, every

experiment was performed using blood-group O PLTs. This allows the exclusion of unspecific activating PLT reactions induced via ABO-antibodies that might interact with their corresponding antigen on the PLT surface and potentially lead to sialidase release (176, 177). Moreover, it has to be considered that blood group A and B antigens represent different sugar residues of the galactose family that reside on the PLT-surface, namely N-Acetylgalactosamine and D-galactose, respectively (178, 179). Via the utilization of blood-group O PLTs that lack these carbohydrate structures on their surface, an unspecific binding of the used lectins in the screening assay can be excluded with high probability. Additionally, for the assessment of PLT desialylation, a predictive cutoff-value was calculated to validate a significant ITP AAb-induced increase in the expression of ß-Galactose and N-Acetylglucosamine.

The fact that 60% of desialylating AAbs were detected in the sera of patients with secondary ITP suggests that there might be a higher prevalence of desialylating AAbs in patients with certain underlying malignancies (e.g., chronic lymphocytic leukemia or myelodysplastic syndrome). As skewed T- and B-cell subsets have been described as capable of promoting the generation of PLT-reactive AAbs this hypothesis might be feasible (16, 180). However, as the main cause for thrombocytopenia in this patient collective is still assumed to be myelosuppression, it is necessary to prove whether the observed PLT desialylation and thrombocytopenia is due to the presence of PLT-specific AAbs or not (117). Therefore, a test cohort of 19 sera from thrombocytopenic non-ITP patients without in MAIPA detectable PLT-targeting AAbs and different underlying haematopoietic malignancies were investigated. Despite clinical overt thrombocytopenia, none of these patient sera induced increased desialylation on the surface of healthy human PLTs. This result suggests that the presence of ITP AAbs that are inducing the removal of terminal sialic acid on PLTs could next to myelosuppression represent an additional thrombocytopenic factor in a subgroup of secondary ITP patients.

A key finding of this study is, that 48% of human ITP sera harbouring anti-GP IIb/IIIa AAbs were able to induce a significant desialylation on the PLT-surface.

This finding supplements the data of a prior study where desialylation was reported to be Fc-independent and only induced via moAb and human ITP-AAbs that target PLT GP Ib-IX (157). Although Fc-independent sialidase release through anti-GPIbα AAbs is well accepted, this study demonstrates that human ITP-AAbs against the PLT GP IIb/IIIa also show the potential to induce PLT desialylation. This discrepancy might be explained by the fact, that most of the previous studies were performed using murine PLTs (155, 157). It is well known that these PLTs lack the expression of FcγRIIA (CD32a) whereas this receptor is highly expressed on the surface of human PLTs (99). Based on this consideration, it is reasonable to hypothesize that human anti-GP IIb/IIIa AAb-induced desialylation might be mediated via the engagement of FcγRIIA by the AAb's Fc-domain similar to that observed in other diseases like heparin-induced thrombocytopenia (181). This hypothesis was reinforced as the inhibition of FcγRIIA via moAb IV.3 led to a significant reduction of anti-GP IIb/IIIa AAb-induced desialylation. The reduction of desialylation via the inhibition of FcγRIIA suggests that a subgroup of human anti-GP IIb/IIIa ITP AAbs have the potential to induce changes in the sialic pattern of PLTs via a Fc-dependent pathway. This finding was further confirmed by another study published quite recently by another research group (182). However, the question whether anti-GP IIb/IIIa ITP AAbs induce Fc-dependent desialylation via intra- or inter-PLT crosslinking still remains unsolved. Nonetheless, the identification that a subset of human anti-GP IIb/IIIa ITP AAbs has the capability to engage PLT FcγRIIA and induce subsequent PLT desialylation might have potential implications for future FcγRIIA targeting therapeutic approaches.

Notably, desialylation was also observed in PLTs that were incubated with patient sera tested negative for the presence of ITP AAbs. This finding could most likely be explained by the lack of test accuracy of MAIPA. Although this detection assay is commonly used in the routine testing for the detection of anti-PLT Abs, its sensitivity was described to be limited by several groups (183). Based on this limitation, the presence of desialylating ITP anti-PLT AAbs from a low-avidity type with the ability to dissociate from target epitopes after binding and subsequent induction of desialylation might be conceivable (57, 113). Additionally, the

presence of alternative PLT epitopes, that are targeted by different ITP anti-PLT AAbs and not part of common MAIPA test panels have to be considered.

In addition, it was demonstrated that modifications of the PLT glycan pattern are mostly due to the presence of ITP AAbs and not due to other serum components. In fact, PLT desialylation was induced to the same extent via isolated IgG fractions as observed in corresponding patient sera. Considering that isolated ITP IgG fractions were incubated with high purified wPLTs, these data indicate that PLTs might serve as the main source of sialidase which is released upon AAb-binding either from the PLT's intracellular compartment or membrane (142). Nevertheless, AAbs are harbouring sialic acids on their surface that have been described to alter disease activity in different autoimmune states (184). Consequently, a potential contribution of sialic acid harbouring ITP anti-PLT AAbs that are bound to the PLT surface and might artificially alter the sialylation status has to be considered. These concerns were partially excluded, as the pre-treatment of PLTs with the sialidase inhibitor oseltamivir-acid noticeably reduced the level of desialylation on the PLT surface despite the presence of isolated desialylating ITP IgG fractions. This finding is of great significance as it might identify human PLTs as an important source for sialidase in ITP disease.

In this study, the clinical relevance of PLT desialylation was further confirmed as AAb-induced sialic acid removal was found to correlate with low PLT-counts in ITP patients. In contrast to patients where serum-induced changes in the PLT glycan pattern were absent, patients tested positive for desialylation presented with marked thrombocytopenia. This finding might point towards an additional, amplifying PLT clearance mechanism that occurs in parallel to well characterized Fc-dependent PLT depletion, namely phagocytosis, complement activation or cytotoxic cell-lysis (49, 59, 115). Hence, it is feasible that in ITP patients with non-desialylating ITP AAbs the effect of this alternative Fc-independent PLT clearance mechanism is absent. This could also explain the higher detected PLT-counts in this patient subgroup. Also, and in line with clinical overt thrombocytopenia, bleeding tendency was detected to correlate with the desialylation ability of ITP sera in this study cohort. In fact, thrombocytopenic ITP-

patients with desialylating AAbs presented a higher rate of bleeding events reaching from petechiae, hematoma as well as epistaxis. As low PLT-counts have been reported to harbour only limited predictive value for the prediction of bleeding events, these data are not clearly dissecting whether the decrease in PLT-count or an impaired PLT function is leading to higher bleeding tendency (22). In this work, the incubation of wPLTs with exogenous sialidase resulted in a drastic reduction of PLTs' ability to adhere to the two ECMs fibrinogen and vWF. These data point towards a functional role of sialic acid loss and are reinforced due to the fact that especially the GP Ibα-subunit of GP Ib-IX as well as GP IIb/IIIa are covered with a significant amount of surface sialic acid (185, 186). The observation that the loss of sialic acid content induced via exogenous-sialidase resulted in a reduced adhesion ability of PLTs suggests that sialylation seems to be relevant for the function of GP IIb/IIIa as well as of GP Ib-IX. This was further underlined by the inhibition of sialidase via a sialidase inhibitor which significantly rescued PLTs' adhesion function.

Most importantly, the observation of a sialidase-induced functional impaired PLT phenotype was reinforced as PLTs that were incubated with ITP IgG fractions showed similar functional defects. Incubation with desialylating human ITP IgGs resulted in a significant impaired ability of PLTs to adhere to fibrinogen as well as vWF. This finding indicates that human ITP IgG induced PLT desialylation, similar to that observed with PLT desialylation via exogenous sialidase, might interfere with the function of GP IIb/IIIa as well as GP Ib-IX. Strikingly, the hypothesis of a potential functional role of GP sialylation was confirmed as the inhibition of sialidase via oseltamivir-acid significantly rescued the impaired PLT function that was induced via desialylating ITP IgGs. Nonetheless, as also non-desialylating ITP IgG fractions induced a reduced binding of PLTs to both ECMs to some extent, the direct binding of GP targeting ITP AAbs with subsequent functional defects of PLT surface GPs has to be considered (187).

Following the detection of a functional impaired PLT phenotype, the next step of the study addressed the question whether ITP AAb-induced desialylation affects PLT survival Remarkably, desialylation of healthy human PLTs via exogenous

sialidase resulted in a significantly faster removal of PLTs from the mouse circulation compared with vehicle control incubated PLTs. This finding indicates that the loss of terminal sialic acid might have the potential to induce an increased depletion of human PLTs in the mouse circulation. As these experiments were performed in the absence of human ITP AAbs, the involvement of Fc-dependent PLT clearance mechanisms, despite the potential prevalence of physiologically PLT-bound Abs, are not assumed to be the main cause for the rapid PLT-depletion (49, 59, 135). A more likely explanation in line with previous findings is, that the increased uptake of exogenous-sialidase treated PLTs might be induced via alternative, Fc-independent PLT clearance mechanisms due to the loss of terminal sialic acid. As the expression of the AMR is highly conserved in mammals, an increased uptake of desialylated human PLTs via the AMR expressed on the hepatocytes of the liver is a potential explanation for the observed increased depletion of human PLTs (137, 145).

Most importantly, the impact of human ITP AAb-induced desialylation on PLT survival is shown in this study. Desialylating human ITP patient IgGs induced a significant reduction of circulating human PLTs during the observation period. Contrary, these changes were nearly absent in the presence of control IgGs. These findings indicate that human ITP AAb-induced desialylation also has the capability to induce a significant increase in the depletion of human PLTs in vivo. However, as these experiments were performed in the presence of human AAbs, the involvement of Fc-dependent in parallel to Fc-independent PLT clearance mechanisms has to be considered. The contribution of Fc-independent PLT-clearance, namely desialylation, was further confirmed, as sialidase inhibition via oseltamivir-acid showed the potential to significantly reduce, but not completely block the increased ITP AAb-induced PLT-depletion. This finding indicates that Fc-independent clearance of ITP AAb-desialylated PLTs represents a distinct pathway that might amplify the consumption of PLTs in ITP patients. These data are reinforced by the detection of lower PLT-counts in ITP patients that were screened positive for the ability to induce serum induced PLT desialylation in this study.

Following the finding of an increased depletion of desialylated PLTs, the presence of alternative ITP AAb-induced PLT-depletion mechanisms was investigated. The finding that no correlation between the extent of ITP AAb-induced desialylation and the induction of apoptosis was detected might point towards the absence of apoptotic processes in ITP AAb induced PLT desialylation. However, these preliminary findings must be evaluated carefully due to the small sample size in these experiments. Furthermore, the involvement of PLT apoptosis due to ITP AAb-induced desialylation remains controversial, as previous studies that utilized small ITP patient cohorts show contrary data (188, 189). Additionally, and potentially a further limitation, in our study ITP AAb-induced apoptosis was detected via the measurement of ΔΨ depolarization. As changes of the ΔΨ were previously reported to not only be induced via the intrinsic pathway of apoptosis but also in procoagulant PLTs, the measurement of ΔΨ changes might be not specific enough to detect apoptotic changes that are induced via desialylating human ITP AAbs (3). As PLT apoptosis represents a complex process, additional apoptotic markers such as the expression of prosurvival protein BCL-XL, activation of the apoptotic proteins Bak or Bax and most importantly the detection of active caspases would further confirm whether PLT apoptosis is induced via desialylating human ITP AAbs or not (165). However, as this topic would exceed the range of this thesis, future research approaches are necessary to clarify whether PLT apoptosis is induced via desialylating human ITP AAbs.

5 Summary

5.1 English Summary

Introduction: Immune thrombocytopenia (ITP) is an autoimmune disease that is characterized by a low platelet (PLT)-count and increased bleeding tendency. Antibody-induced PLT desialylation, the loss of sialic acid on the surface of PLT glycoproteins (GPs) has been recently suggested to contribute to an increased PLT depletion in ITP. Based on this previous finding, the presence of desialylating autoantibodies (AAbs) in a larger human ITP cohort as well as the potential impact of ITP AAb-induced PLT desialylation on PLT functionality and PLT survival were investigated in this study.

Methods: In a first step, sera from well characterized ITP patients were tested using a lectin binding assay (LBA). After the incubation of PLTs from healthy individuals with ITP or control sera, glycan changes were analyzed via flow cytometry (FC). To investigate the impact of desialylation on PLT functionality, an adhesion assay on the extracellular matrix proteins fibrinogen as well as von Willebrand factor (vWF) was established. To prove whether ITP AAb-induced desialylation affects PLT survival, an NSG mouse model was utilized.

Results: 100 ITP sera were investigated in this study. 28/100 sera led to a significant elevation in the exposure of the sugar residues ß-Galactose as well as 21/100 sera to an increased exposure of N-Acetylglucosamine (mean fold FI: 3.50, range: 1.79-13.61, p value 0.0001; FI: 2.41, range: 1.54-5.47, p value 0.0001, respectively). Human ITP AAb-induced PLT desialylation was further identified being not only restricted to AAb targeting GP Ib-IX as also anti-GP IIb/IIIa ITP AAbs were identified to induce desialylation that was significantly reduced via PLT FcγRIIA blockade (% desialylation mean±SEM: 100±0% vs. 52±11%, p value 0.011; 100±0% vs. 60±9%, p value 0.0190, respectively). Moreover, ITP AAb-induced desialylation led to an impaired PLT adhesion function on fibrinogen and vWF, whereas these changes were only moderate in PLTs that were incubated with non-desialylating AAbs (% adherent cells/field mean±SEM: 74±8% vs. 34±7%, p value 0.0021 and 67±5% vs. 26±2%, p value 0.0027, respectively). Notably, the impaired PLT function was significantly

reduced in the presence of a sialidase inhibitor (% of adherent cells/field mean±SEM: 36±5% vs. 86±6%, p value 0.0001 and 26±2% vs. 67±11%, p value 0.0203, for fibrinogen and vWF, respectively). Interestingly, the presence of desialylating ITP AAbs was identified to be also associated with low PLT-counts and an increased bleeding tendency in this ITP patient cohort. The biological relevance of PLT desialylation was demonstrated as the injection of desialylating human ITP AAbs resulted in an accelerated clearance of human PLTs in a murine model of ITP. Importantly, the increased PLT depletion was significantly reduced by the pretreatment of PLTs with a sialidase inhibitor that prevents desialylation on the PLT surface (survival of human PLTs after 300 min: 28±5% vs. 45±3%, p value 0.0190, respectively).

Conclusion: Our findings indicate that desialylating AAbs, that have the potential to induce significant changes in the glycan pattern of PLTs, are present in a subgroup of ITP patients. Furthermore, this study demonstrates that ITP AAb-induced desialylation seems not be a solely Fc-independent mechanism as also human anti-GP IIb/IIIa ITP AAbs showed the potential to induce significant PLT desialylation in a Fc-dependent pathway. Moreover, the presence of desialylating ITP AAbs was also associated with clinical overt thrombocytopenia, increased bleeding tendency and impaired PLT function. These data suggest that desialylation might result in an increased bleeding phenotype in ITP patients. Most importantly, human ITP AAb-induced changes in the glycan pattern of PLTs were identified to contribute to an increased PLT destruction in an apoptosis independent pathway in vivo, which was significantly reduced via sialidase inhibition. Taken together, findings of this study suggest that a routinely performed screening for the presence of desialylating AAbs and the use of sialidase inhibitors might further dissect the complex pathomechanisms present in ITP patients and be promising for novel ITP treatment approaches.

5.2 Deutsche Zusammenfassung

Einleitung: Die idiopathische Immunthrombozytopenie (ITP) ist eine Autoimmunerkrankung die durch einen isolierten Abfall der Thrombozytenzahl unter 100x10^9/L und eine erhöhte Blutungsneigung charakterisiert wird. Erst kürzlich wurde ein neuer Abbauweg, die sogenannte Plättchen Desialinisierung mithilfe der Verwendung von monoklonalen Mausantikörpern, identifiziert. Diese Antikörper zeigten das Potential das Enzym Sialidase aus den Plättchen freizusetzen und folglich eine Desialinisierung der Plättchen zu induzieren. Diese Modifikation der Plättchenoberfläche führt zu einer Erkennung durch den Ashwell-Morell Rezeptor auf den Hepatozyten in der Leber und zu einem vermehrten Abbau von desialinisierten Plättchen.
Gegenstand dieser Studie ist die Frage, ob desialinisierende ITP Autoantikörper in einer größeren humanen ITP Kohorte vorkommen und ob die Autoantikörper vermittelte Desialinisierung die Plättchen Funktion und deren Überleben beeinträchtigt.

Methode: In einem ersten Screening wurden Plättchen von Gesundspendern mit Seren von gut charakterisierten ITP Patienten inkubiert und mittels Durchflusszytometrie auf das Vorkommen von Desialinisierung untersucht. Die Konsequenzen der Desialinisierung auf die Plättchenfunktion wurden mittels Adhäsionsversuchen auf den extrazellulären Matrixproteinen Fibrinogen und von-Willebrand Faktor (vWF) untersucht. Um die biologische Relevanz der Desialinisierung auf das Plättchenüberleben zu überprüfen, wurde ein murines ITP Model verwendet.

Ergebnis: 100 ITP Seren wurden in dieser Studie untersucht. 28/100 Seren induzierten eine erhöhte Expression des Oberflächenzuckers ß-Galactose sowie 21/100 ITP Seren eine erhöhte Expression des Oberflächenzuckers N-Acetylglucosamin, welche unter physiologischen Bedingungen von Sialinsäure überdeckt sind. Interessanterweise, und entgegen dem bisherigen Kenntnisstand induzierten auch Seren mit detektierbaren Antikörpern gegen Glykoprotein IIb/IIIa eine erhöhte Expression von ß-Galactose und N-Acetylglucosamin welche durch die Inhibition von Fc-gamma-Rezeptor IIA auf der Plättchenoberfläche signifikant

gehemmt werden konnte (% Desialinisierung mean±SEM: 100±0% vs. 52±11%, p=0.011 für ß-Galactose und 100±0% vs. 60±9%, p=0.0190, für N-Acetylglucosamin). Die Folgen der Desialinisierung auf die Plättchenfunktion werden dahingehend gezeigt, als dass eine verminderte Adhäsionsfähigkeit von mit desialinisierenden ITP Autoantikörpern inkubierten Plättchen auf den extrazellulären Matrixprotein Fibrinogen und vWF beobachtet wurde. Dieser Befund korreliert mit einer erhöhten Blutungsneigung in jener Untergruppe von ITP Patienten in der eine signifikante Desialinisierung im Screening detektiert wurde. Bemerkenswert ist, dass dieser Funktionsverlust in vitro durch eine Vorbehandlung der Plättchen mit einem Sialidase Inhibitor erheblich gehemmt werden konnte (% adhärierende Plättchen/Feld mean±SEM: 36±5% vs. 86±6%, p=0.0001 für Fibrinogen und 26±2% vs. 67±11%, p=0.0203, für vWF). Letztlich konnte ebenfalls gezeigt werden, dass die durch humane ITP Autoantikörper vermittelte Desialinisierung ein reduziertes Überleben von humanen Plättchen im Mausmodel induzieren. Interessanterweise konnte die Hemmung der Sialidase mittels spezifischen Sialidase Hemmers eine deutliche Verbesserung des durch desialinisierende ITP Autoantikörper reduzierten Plättchenüberlebens in vivo erzielen (% mittleres Plättchenüberleben nach 300 min±SEM: 28±5% vs. 45±3%, p =0.0190)

Fazit: Zusammenfassend wurde in dieser Studie das Vorkommen der Plättchendesialinisierung anhand einer gut charakterisierten ITP Patientenkohorte untersucht. Hierbei wurde deutlich, dass eine Untergruppe von ITP Patienten desialinisierende Autoantikörper aufweist die unterschiedliche Oberflächenglykoproteine auf der Plättchenoberfläche angreifen. Die Folgen auf das Schicksal von Plättchen wurden dahingehend gezeigt, als dass die durch humane ITP Autoantikörper vermittelte Desialinisierung zu einer verminderten Funktionsfähigkeit als auch zu einem reduzierten Überleben der Plättchen im Mausmodel geführt haben. Ein routinemäßig durchgeführtes Screening auf das Vorkommen der Plättchen Desialinisierung als auch der therapeutische Einsatz des Sialidase Inhibitors Oseltamivir könnten daher in Zukunft insbesondere bei therapierefraktären ITP Patienten eine therapeutische Option darstellen und weiters die komplexe Pathophysiologie in der ITP dechiffrieren.

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7 Erklärung zum Eigenanteil

Die vorliegende wissenschaftliche Arbeit wurde am Institut für Klinische und Experimentelle Transfusionsmedizin durch Univ. Prof. Dr. Tamam Bakchoul betreut.

Die Konzeption der Studie und Versuche erfolgte unter Anleitung von Herrn Univ. Prof. Dr. Tamam Bakchoul.

Versuche wurden nach Einarbeitung durch die Labormitglieder Dr. rer. nat. Irene Marini und Dr. rer. nat. Rabie Jouni (Thrombozytenisolation, Durchflusszytometrie) von mir eigenständig durchgeführt.

In Zusammenarbeit mit Frau Flaviana Rigoni erfolgte die Etablierung und Durchführung der Thromzytenfunktionstests (Plättchenadhäsion). Die Tierversuche erfolgten in Zusammenarbeit und unter direkter Aufsicht von Dr. rer. nat. Rabie Jouni und Dr. rer. nat. Lisann Pelzl, als wissenschaftliche Leitung des zugrunde liegenden Tierversuchsantrages. Frau Dr. Karina Althaus hat die Patientendaten zur Verfügung gestellt. Die statistische Auswertung erfolgte nach initialer Einweisung von Dr. rer. nat. Irene Marini eigenständig durch mich.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den 12.12.2021

8 Publications and Acknowledgements

8.1 Publications

Parts of this thesis were published in the following publication:

Autoantibody-mediated desialylation impairs human thrombopoiesis and platelet lifespan. Irene Marini, Jan Zlamal, Christoph Faul, Ursula Holzer, Stefanie Hammer, Lisann Pelzl, Wolfgang Bethge, Karina Althaus, and Tamam Bakchoul. Haematologica.2021 Jan 1;106(1):196-207.

Parts of this dissertation were presented at the following scientific meetings by the MD doctoral candidate:

51. Jahrestagung der Deutschen Gesellschaft für Transfusionsmedizin und Immunhämatologie e.V. (DGTI), 18.09.-21.09.2018 in Lübeck, Germany (oral talk).

63. Jahrestagung der Gesellschaft für Thrombose- und Hämostaseforschung e.V. (GTH), 27.02.-02.03.2019 in Berlin, Germany (oral talk).

27th Congress of the International Society on Thrombosis and Haemostasis (ISTH), 06.07.-11.07.2019 in Melbourne, Australia (oral talk).

52. Jahrestagung der Deutschen Gesellschaft für Transfusionsmedizin und Immunhämatologie e.V. (DGTI), 18.09.-20.09.2018 in Mannheim, Germany (oral talk).

64. Jahrestagung der Gesellschaft für Thrombose- und Hämostaseforschung e.V (GTH), 18.02.-21.02.2020 in Bremen, Germany (poster presentation).

8.2 Acknowledgements

First, I would like to thank my primary supervisor Prof. Dr. med. Tamam Bakchoul for opening the wonderful world of research to me and teaching me to be ambitious and critical about my research results. Thank you for giving me the opportunity to work on this project and to present my data at various conferences in front of experts in the field. Your approaches supported me in reaching more advanced levels of research and your enthusiasm will always inspire me.

I also want to thank the other members of the lab, especially Dr. rer. nat. Rabi Jouni and Dr. rer. nat. Irene Marini who introduced me to the methods, taught me how to work scientifically and to be curious about the data of each experiment.

Next, I would like to show my gratitude to Flavianna Rigoni who's kind nature and technical skills as TA supported my work from the first day onwards.

Not only important for scientific discussions and her clinical skills, but also for her supportive nature, I would especially like to thank Dr. med. Karina Althaus.

I am also grateful that I was surrounded by my great student colleagues Oleg Hidiatov and Miriam Wagner. Together we had various funny moments at the lab and without you as comrades it would not have been the same.

Furthermore, I want to thank the TA team of the HLA-laboratory, Transfusion medicine Tübingen, especially Franziska Lissy and Simone Weit. Both kindly offered their help and support whenever it was needed.

Finally, I want to thank my family and especially the most important and inspiring person in my life, my wife Susanne. You always supported and motivated me during several phases of this thesis. You always had an open ear and were patient, especially during stressful times. Thank you also for encouraging me to proceed. My love, my soul mate, without you and your endless support this work would not have been done.