Department for Biomedical Sciences
University of Veterinary Medicine Vienna

Institute of Animal Breeding and Genetics
(Head: O.Univ.-Prof. Dr.med.vet. Mathias Müller)

# Altered Hemostatic Potential and Autoantibody-Induced Platelet Desialylation in Adults with Primary Immune Thrombocytopenia

Master thesis submitted for the fulfillment of the requirements for the degree of
**Master of Science (MSc)**
of the University of Veterinary Medicine Vienna

Submitted by
Theresa Schramm, BSc.

Vienna, July 2021

# Supervision

This Master Thesis was supervised by:

Ap. Prof Priv.-Doz. Dr. Johanna Gebhart, PhD (External Supervisor)
Medical University of Vienna
Department of Internal Medicine I
Division of Haematology and Haemostaseology
Vienna General Hospital
Währinger Gürtel 18-20, 1090 Vienna, Austria

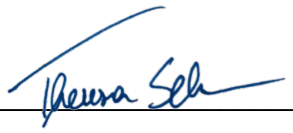
Univ.-Prof. Dipl.-Ing. Dr.rer.nat. Richard Moriggl (Internal Supervisor)
University of Veterinary Medicine, Vienna, Austria
Institute of Animal Breeding and Genetics
Functional Cancer Genomics
Veterinärplatz 1, 1210 Vienna, Austria

Reviewer:
Ass.-Prof. Dr.med.vet. Martina Patzl
University of Veterinary Medicine, Vienna, Austria
Institute of Immunology
Department for Pathobiology
Veterinärplatz 1, 1210 Vienna, Austria

# Statutory Declaration

Theresa Schramm, BSc.
Raggendorferstraße 27,
2221 Gr. Schweinbarth, Austria

“I assure that this thesis is a result of my personal work and that no other than the indicated aids have been used for its completion. Furthermore, I assure that all quotations and statements that have been inferred literally or in general manner from published or unpublished writings are marked as such. Beyond this I assure that the work has not been used, neither completely nor in parts, to pass any previous examination.”

Vienna, 30.06.2021 

(Place, Date) (Signature Author)

# Acknowledgements

Firstly, I want to thank Univ.-Prof. Dr. Ingrid Pabinger-Fasching and Assoc.-Prof. Priv. Doz. Dr. Cihan Ay for giving me the opportunity to join their outstanding research group at the Medical University of Vienna. Furthermore, I really appreciated Ap. Prof. Priv.-Doz. Dr. Johanna Gebharts excellent guidance through this internship and believing in me and my work as part of her study. Thanks for answering all my questions and the feedback, I will gladly look back at my internship at the Anna Spiegel Institute.

Special thanks to Dr. Dino Mehic and Pia Glaser for their help with practical laboratory tasks and especially statistics, I wish them all the best for their future careers. Thank you also to the rest of the lab group, who gave me a warm welcome and thereby created a great work environment!

Thank you to my internal supervisor Univ.-Prof. Dr. Richard Moriggl for his insightful comments and supervision. Additionally I want to thank Ass.-Prof. Dr.med.vet. Martina Patzl for her in-depth comments and corrections, which have greatly improved this Master Thesis.

Furthermore, I would like to thank my family for their support during my master studies at the University of Veterinary Medicine Vienna. A big thank you to my dear friends Linda, Meli, Ines, Günter and Berni for always being keen to know how my work progresses, for cheering me up when I was stressed out and for always believing in me.

# Abstract

Immune thrombocytopenia (ITP) is an acquired thrombocytopenia caused by an autoimmune reaction against thrombocytes and megakaryocytes, which results in decreased platelet counts (<100 x$10^9$/l). Primary ITP patients show heterogeneous bleeding phenotypes and partially thromboembolic risk. Further characterization of cell physiological and immunological processes causing the abnormal platelet numbers and connected symptoms can help improve treatment options and improve life quality of patients enormously.

Within this Master thesis we have studied the global hemostatic capacity in ITP patients compared to non-immunological thrombocytopenia patients and age- and sex- matched healthy controls. We further hypothesized that an autoantibody-related reaction causes platelet desialylation, which induces Fc-independent platelet clearance via Ashwell-Morell receptors. The global hemostatic capacity was assessed by Thrombin generation and plasma clot formation and lysis according to the recommendations of the Scientific Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH). Thrombosis risk was investigated via Tissue factor (TF) bearing microparticles, according to a protocol established by Hisada and Mackman 2019. A Lectin binding assay was established to measure the platelet desialylation status by autoantibodies in primary ITP patients.

We found impaired capacity of Thrombin generation in primary ITP patients, compared to non-immunological thrombocytopenia patients and healthy controls. Furthermore, clot formation was slightly altered and clot lysis was significantly delayed in primary ITP patients. No difference in the extravesicular-associated Tissue factor activity was found. Two primary ITP patients tested positive for platelet desialylation in our newly established assay.

To sum up, there is altered hemostatic potential between the ITP patients and the control groups. Mildly reduced Thrombin generation and lower clot formation might impact the individual bleeding severity. The delayed clot lysis might counteract the clinical bleeding tendency to favor a prothrombotic state. As TF-levels were not increased, no strong association to thrombosis risk can be drawn based on solely this parameter. The established Lectin-binding assay did not show increased Lectin binding in most measured primary ITP patients. These findings indicated the need for further investigation of this disease and the identification of a biomarker unique to primary ITP.

# Table of Contents

# 1 Introduction

## 1.1 Scope of Work

This Master thesis addressed the hemostatic potential of patients with primary immune thrombocytopenia (ITP) and aims to link primary immune thrombocytopenia clinical patient data of coagulation markers to antibody glycoprotein specificity and the antibody-related ability for platelet desialylation.

This thesis hypothesized that,

*"ITP patients have an altered hemostatic potential and show autoantibody-related ability for platelet desialylation."*

and findings were examined and discussed as detailed in the following:

## 1.2 Methodical Approach

To proof the hypothesis, three cohorts (primary ITP patients, non-immunological thrombocytopenic patients and healthy individuals) were examined and compared. The cohort of interest consisted of 88 adult patients with primary ITP (>18 years, platelet counts ≤150 x$10^9$/l) from the Vienna ITP Biobank. Citrated plasma and serum samples of these patients from the biobank were examined. For comparison, samples of 19 patients with non-immunological thrombocytopenia and of up to 150 age- and sex-matched healthy individuals were investigated.

The global hemostatic capacity was assessed by Thrombin generation (Thrombin generation assay, Technoclone, Vienna, Austria) and plasma clot formation and lysis using a previously published method (Wolberg et al. 2005) based on the recommendations of the Scientific Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) (Pieters et al. 2018).

Furthermore, Tissue factor bearing microparticles were assessed using a method established by Hisada and Mackman (Hisada and Mackman 2019a).

Platelet desialylation of the majority of primary ITP patients was previously assessed in cooperation with the research group of Prof. Backoul, Eberhart-Karls University, Tübingen by Ap. Prof. Priv. Doz. Dr. Johanna Gebhart using a Lectin binding assay (LBA) (Marini et al. 2019). Platelet desialylation by autoantibodies, was confirmed and tested for reproducibility using the same LBA. For that, washed platelets were extracted out of fresh whole blood samples.

## 1.3 Structure of the Thesis

First, a theoretical background on platelets, hemostasis and primary ITP was given, followed by a detailed description of the used materials and performed methods. Afterwards the scientific findings were summarized in the results-section. Under the fifth bullet point, the data were concisely discussed, and conclusions were drawn. Additional Tables are found in the Annex at the very end of this Master thesis.

# 2 Theoretical Background

Symptoms of ITP were first noticed even before the discovery of platelets as a blood component. From the 11th to the 17th century various forms of reddish-purple skin lesions, named “purpure”, resulting from bleeding in the dermis were described. The first detailed clinical description of ITP was in 1735 by the German physician Paul Gottlieb Werlhof, based on the case of a 16-year-old girl. In some older publications this disease is found by its eponym *Werlhof Disease* (Stasi and Newland 2011, Remiker and Neunert 2020). After microscopy technology was more advanced, blood characterization improved, and platelets were finally discovered in 1841. Within the following years a greater insight into the pathophysiology of thrombocytopenia was gained, also revealing the immunological aspect of platelet destruction in this specific disorder (Remiker and Neunert 2020).

The following chapter will discuss hemostasis, a physiological core process in mammalian blood, and give a broad introduction on primary ITP. Within the hemostasis subchapter, primary and secondary hemostasis are explained. Primary hemostasis deals with platelet formation, structure, and physiology while secondary hemostasis mainly encompasses the plasmatic coagulation. The second big subchapter gives a broad overview on primary ITP, including clinical and epidemiological features as well as information on ITP diagnosis and therapy. Furthermore, cellular and physiological aspects behind primary ITP are described in great detail.

## 2.1 Hemostasis

Hemostasis is a core physiological process in mammalian blood, which regulates blood clotting, platelet activation and vascular repair (Engelmann and Massberg 2013). The three main tasks are (1) closure of damaged blood vessels, (2) maintaining the fluid state of blood and (3) eliminating blood clots after vascular integrity is restored, making hemostasis to an important biological self-defense mechanism within the body. Irregularities within this system might cause bleeding diathesis or the other extreme, thrombosis (Smith et al. 2015).

Hemostasis divides into primary and secondary hemostasis. Primary hemostasis includes platelet activation and aggregation, whereas secondary hemostasis includes the activation of the plasmatic blood coagulation and Fibrin formation (Engelmann and Massberg 2013).

## 2.1.1 Primary Hemostasis

### 2.1.1.1 Platelets

Platelets are small, anucleated cells derived from megakaryocytes (MK) and they are produced in the bone marrow and lung (Holinstat 2017, Lefrancais et al. 2017). A healthy person has a normal platelet count of approximately 150-350 x $10^9$/l blood (Michelson 2003) and their average lifespan is five to seven days, depending on environmental conditions within the vessel (p.ex. high shear forces). Platelet clearance from the vessel or after assembly into a Fibrin clot, is executed by macrophages and neutrophils, which transport their cargo to the spleen for complete destruction and elimination from the organism (Holinstat 2017).

The platelet surface structure is highly complex carrying various types of glycoproteins (GP). The most important ones are the GPIb-IX-V complex, GPVI, as well as integrin alpha IIb/beta 3 (GPIIb/IIIa). These glycoproteins mediate adhesion, activation and aggregation processes. (Gremmel et al. 2016). GPIIb/IIIa is the most abundant platelet surface glycoprotein (Niiya et al. 1987) and also found on other cells like MKs, mast cell basophils and even tumor cells (Uzan et al. 1991, Huang et al. 2019, Gremmel et al. 2016).

Platelets play a crucial role in many physiological processes within the body, reaching from fighting infections and inducing inflammation to tumor angiogenesis and metastasis. Not to forget their most important function: prevent hemorrhage after vascular injury. Under normal conditions platelets flow within the vessel, without interacting with the vessel wall. In case of damage, they however will bind to the extracellular matrix in a highly regulated process, resulting in a tight adhesion and subsequent platelet activation – also known as primary hemostasis (Broos et al. 2011).

### 2.1.1.2 Platelet Activation

Primary hemostasis describes the process from initial platelet adhesion to thrombus formation after tissue trauma. Upon vessel injury platelets initially get into contact with the extracellular matrix which exposes a lot of adhesive macromolecules (p.ex. Laminin, Fibronectin, Collagen, von Willebrand Factor (VWF)). Platelet adhesion is greatly influenced by shear forces in the vessel, as high shear forces will enforce platelet tethering. In short, after binding of VWF to GPIb-IX-V, platelet velocity is slowed down, and platelet activation is initiated (De Marco et al.

1985). This platelet activation stabilizes surface integrins, which support the adhesion mechanism (Varga-Szabo et al. 2008). While platelets are in close proximity to the endothelial surface, GPVI forms a bond with Collagen, which further stabilizes platelet adhesion. Upon collagen binding, platelet degranulation and subsequent release of platelet agonists (like ADP) is triggered, which reinforces the platelet recruitment process and in turn leads to rapid growth of the thrombus (Varga-Szabo et al. 2008). Besides the two mentioned agonists (VWF and collagen) in platelet activation, Thrombin and ADP are other main activators of platelets (Figure 1).

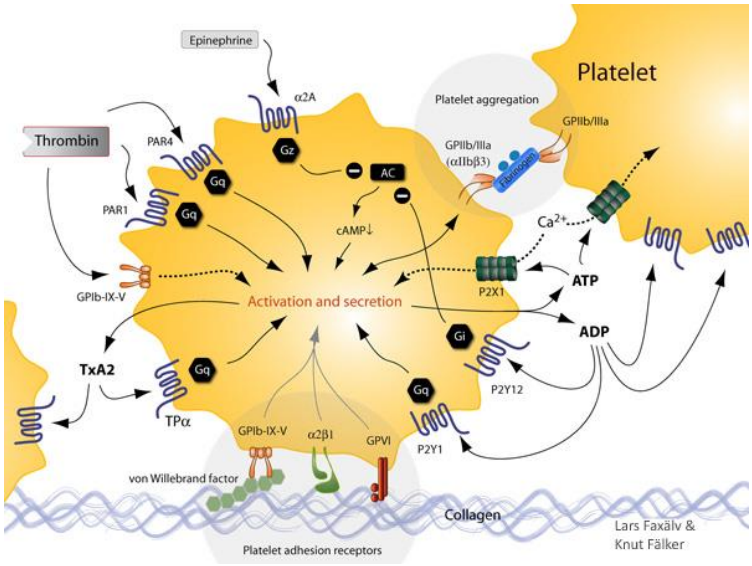



***Figure 1: Platelet Activation and Interaction.*** *Binding to damaged vessels is facilitated by the three major platelet adhesion receptors (1) GPVI and (2) GPIIb/IIIa, which directly bind to Collagen, while (3) GPIb-IX-V binds to von Willebrand factor (VWF). The GPIb-IX-V receptor complex further interacts with other platelet ligands and procoagulant factors like Thrombin. Thrombin is a potent platelet activator which binds to the protease-activated surface receptors 1 and 4 (PAR1, PAR4). P2Y12 and P2Y1, ADP stimulated surface receptors and the thromboxane proteinoid alpha (TP alpha) receptor, stimulated by thromboxane A2 ($TXA_2$) activate strong positive feedback signaling cascades for platelet activation. Under high shear flow, VWF will bind and activate GPIIb/IIIa, and platelet-to-platelet aggregation is facilitated through Fibrin binding and therefore connecting to this complex. $Ca^{2+}$ is involved in most proteolytic coagulation steps, it enters the platelet via the P2X1 ion channel. Epinephrine is another component to enforce stable platelet aggregation (Gremmel et al. 2016, Broos et al. 2011). Source: http://www.diapensia.se/free-figures/ [22.04.2021]*

## 2.1.1.3 Tissue Factor-Bearing Microvesicles

Microparticles are secreted by various cells like platelets, monocytes, endothelial cells and even tumor cells into the system. They are released via exocytosis and form microvesicles (MV) or ectosomes from the cell plasma membrane. Ectosomes (40-100 nm) are not only smaller than MVs (100 nm-1 µm), their formation, lipid composition as well as content differs. Notably, integrin alpha IIb/ beta 3/1, GPIb alpha and P-selectin were detected on the

membrane of MVs, which might facilitate interactions with other cells or Fibrin (Heijnen et al. 1999). MVs can survive enormous environmental conditions like high shear stress, and are believed to have pro- as well as anticoagulant activity (Heijnen et al. 1999). The procoagulant activity is mainly facilitated by the membrane proteins Phosphatidylserine (PS) and Tissue factor (TF), which is a highly pro-coagulant protein (Owens and Mackman 2011). The procoagulant activity of platelet-derived MVs is further promoted by membrane phospholipids (Thiagarajan and Tait 1991), which are ligands for gamma-delta T cells (Handgretinger and Schilbach 2018).

TF plays central roles in hemostasis, inflammation, angiogenesis and embryogenesis (Butenas 2012). Furthermore, it promotes thrombosis in arteries and veins, sepsis, participates in disseminated intravascular coagulation (DIC) (Østerud and Bjørklid 2001, Mork et al. 2018) as well as antiphospholipid antibody syndrome (Grover and Mackman 2018) and is involved in cancer progression (Hisada and Mackman 2019b).

TF itself is primarily expressed by cells associated with the blood vessel wall, like vascular smooth muscle cells, fibroblasts and pericytes (subendothelial cells of the microvasculature) (Bouchard et al. 1997, Schecter et al. 2000). TF-bearing MVs are specifically secreted by monocytes and macrophages into the circulation (Mork et al. 2018, Del Conde et al. 2005). In a feed-forward fashion TF-bearing MVs bind with their surface PS the coagulation factors (F) Va, VIIa, and Xa. These coagulation factors then interact with the activated TF, located on the outer membrane and promote Thrombin generation (Rao et al. 2012) and therefore exhibit a procoagulant function. Increased TF-activity and elevated levels of TF-bearing MVs were detected in several thrombosis-associated diseases (Owens and Mackman 2011).

### 2.1.2 Secondary Hemostasis

Secondary hemostasis is divided into the intrinsic and extrinsic pathways of coagulation. Together they combine to one common pathway upon activation of FX (Figure 2) (Loof et al. 2014) and facilitate stable anchoring of the Fibrin network and thereby ensure clot stabilization.

TF initiates the cascade. After tissue trauma, TF is exposed to inactive enzymes (zymogens) in the blood. Afterwards it forms a complex with FVIIa, which initiates coagulation by activation of FX and FIX on a PS-rich membrane (Østerud and Bjørklid 2001, Mackman et al. 2007).

Activated FIX and its cofactor FVIIIa again form a complex (intrinsic tenase complex) and activate, together with the TF-FVIIa complex, FX to FXa. Activation of FX marks the common pathway of coagulation. FXa together with its cofactor FVa build the prothrombinase complex, capable of converting Pro-thrombin (FII) into Thrombin (FIIa). Thrombin is in a positive-feed-back-loop-fashion able to activate FIX and FXI, triggering even more Thrombin generation. Thrombin then cleaves Fibrinogen into Fibrin and activates platelets by binding to their protease activated receptors (PARs) (Kahn et al. 1999, Rauch and Nemerson 2000, Smith et al. 2015).

Summing up, hemostasis is tightly regulated, and deviations can have drastic outcomes in form of thrombosis or bleeding. Several anticoagulant proteins, like Antithrombin and Tissue factor pathway inhibitor (TFPI) regulate this system. Furthermore, Thrombomodulin and Protein C exhibit proteolytic activity against cofactors and therefore slow down Thrombin generation (Engelmann and Massberg 2013).

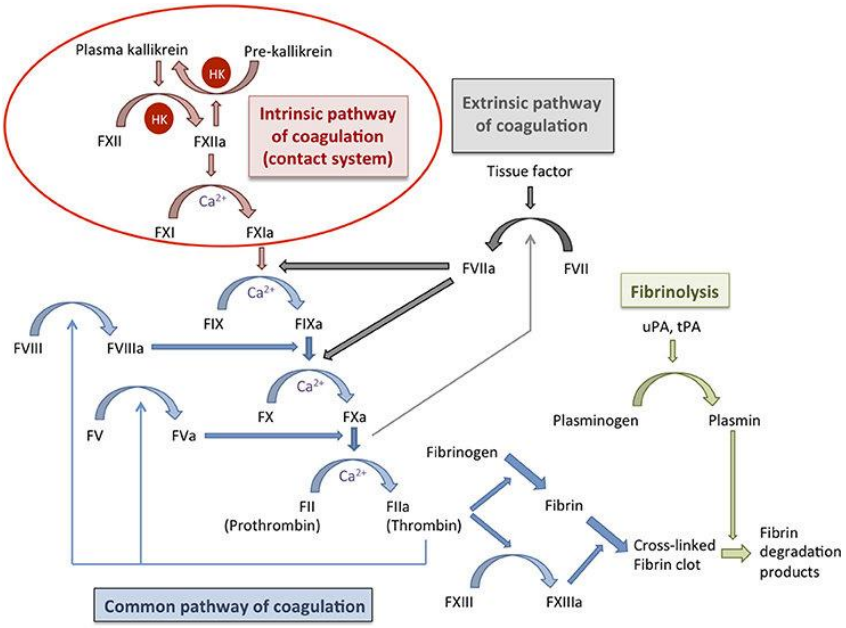



***Figure 2: Coagulation Cascade and Fibrinolysis.*** *(A) Intrinsic pathway of coagulation: Plasma kallikrein, supported by high molecular weight kininogen (HK), activates FXII which forms FXIIa. FXIIa and Calcium ($Ca^{2+}$) cleave FXI, which generates FXIa and more HK. HK in turn participates in the generation of more plasma kallikrein from pre-kallikrein. B) Extrinsic pathway of coagulation: Tissue factor (TF) has a high affinity for FVIIa. Upon TF:FVIIa complex formation, proteolytic activity of FIX, FX and FVIIa are enhanced. (C) Common pathway of coagulation: Activation of FIX and FX leads in the presence of $Ca^{2+}$ to the synthesis of Thrombin. Thrombin regulates in a feed-forward stimulation its own synthesis by binding and activating FVIII and FV. Besides that, it activates Fibrinogen, which synthesizes Fibrin. Fibrin cross-links platelets or other blood cells and leads to clot formation. (D) Fibrinolysis: urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) induce plasmin formation. Plasmin in turn leads to the destruction on Fibrin clots into small Fibrin degradation products (FDP). Source: (Loof et al. 2014)*

### 2.1.3 Fibrinolytic System

Coagulation and fibrinolysis are tightly regulated processes, which keep the blood in fluid state and prevent blood loss through injury. As discussed above, coagulation generates Thrombin, which ultimately leads to thrombus formation, followed by Fibrin synthesis. Fibrinolysis ensures the destruction of the formed thrombus and prevents excessive pro-coagulatory behavior (Loof et al. 2014). The key player in this process is the serine protease plasmin. Plasmin is activated via the extrinsic activators tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Hoffman et al. 2013).

Tissue-type PA and uPA convert plasminogen (PLG), a circulation plasma zymogen, into plasmin (Cesarman-Maus and Hajjar 2005). Both serine proteases are secreted by the endothelium, but show structurally different features and exhibit different affinities for PLG (Hoffman et al. 2013).

The fibrinolytic system is a feed-forward mechanism, starting by the cleavage of tPA and uPA from inactive single chain to active double-chain polypeptides, by plasmin. Tissue-type PA and PLG both bind to the C-terminal lysin residues of Fibrin, leading to subsequent destruction of whole blood clots (Rijken and Lijnen 2009) and simultaneously promote plasmin synthesis (Cesarman-Maus and Hajjar 2005). As more plasmin is generated, tPA and uPA cleavage is enhanced which in turn activates PLG and leads to Fibrin destruction. Without the presence of Fibrin, tPA has low affinity for PLG activation and subsequently less Fibrin destruction will take place (Cesarman-Maus and Hajjar 2005). Urokinase-type PA primarily triggers the activation of cell-bound plasminogen by binding to the cellular u-PA receptor (u-PAR) (Blasi et al. 1987). Urokinase PA regulates extracellular proteolysis in tissue construction and destruction, also it is involved in cell migration and invasion, macrophage function, ovulation, and embryo implantation (Figure 3) (Blasi et al. 1987, Rijken and Lijnen 2009, Qin et al. 2015).

Inhibition of fibrinolysis can occur at several points along the pathway. The key players are the Plasminogen activator inhibitor (PAI-1), Thrombin-activatable fibrinolysis inhibitor (TAFI) and $\alpha_2$-antiplasmin ($\alpha_2$-AP). They account to the serpin (serine protease inhibitor) superfamily and upon cleavage through their target enzyme they release a peptide which binds to the enzyme, thereby blocking further catalytic steps (Rijken and Lijnen 2009, Rau et al. 2007). In case of a dysbalanced fibrinolytic system, increased bleeding tendency is caused through overactivation

or thrombotic events and this occurs from deficient activation (Rijken and Lijnen 2009). Hemorrhagic disorders result from insufficient inhibition of fibrinolysis, caused through defective key inhibitors ($\alpha_2$-AP and PAI-1) or excessive amounts of tPA and uPA (Saes et al. 2018).

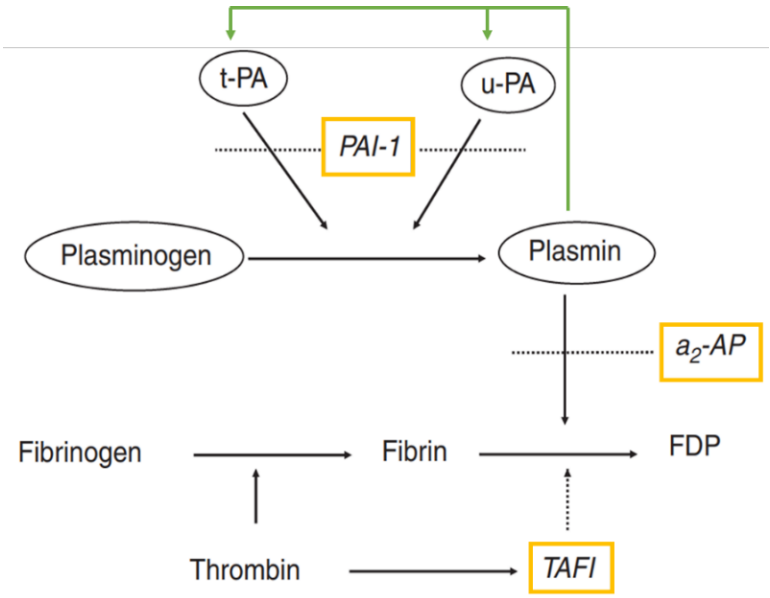



***Figure 3: Fibrinolysis Schema.*** *Plasminogen is activated through tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA) and generates plasmin. Tissue-type PA and u-PA can be blocked by Plasminogen activator inhibitor-1 (PAI-1). Plasmin leads to the breakdown of Fibrin into soluble Fibrin degradation products (FDP) and can be regulated through $\alpha_2$-antiplasmin ($\alpha_2$-AP). Thrombin not only promotes Fibrin generation, it also activates the Thrombin-activatable fibrinolysis inhibitor (TAFI), which blocks Fibrin destruction. Graphic slightly adapted from (Rijken and Lijnen 2009)*

## 2.1.4 Global Hemostatic Potential

Thrombin is a serine protease that binds to PARs and the GPIb-IX-V complex (Kahn et al. 1998, D rmann et al. 2000). This serine protease plays a key role in clot formation and lysis. As discussed above it is a main player in hemostasis and responsible for the stability, structure, and strength of the Fibrin clot. Low Thrombin generation will result in thick Fibrin strands and instable clots susceptible to fibrinolysis, while higher Thrombin production will result in thinner Fibrin strands and stronger clots (Wolberg 2007). The coagulation is a highly dynamic process and Thrombin levels will change according to the state of coagulation. According to Fitzgerald et al. Thrombin generation reaches its peak at 500 nM (Fitzgerald et al. 2020). As Thrombin generation capacity is a key component in coagulation and influenced by several other parameters within the system, it represents an excellent indicator of global hemostatic function (Brummel-Ziedins and Wolberg 2014). Its concentration might play a crucial role in

management of bleedings, which can occur during surgery (Fitzgerald et al. 2020) or are manifested in other coagulation disorders like von Willebrand disease (VWD) and hemophilia (Rugeri et al. 2007, Negrier et al. 2019). These patients specifically showed decreased Thrombin generation, whereas increased Thrombin generation increases the risk of thrombosis (Manco-Johnson et al. 2020). Especially development of clots in the coronary or cerebral blood vessels is dangerous, as they can result in ischemic stroke or acute myocardial infarction (Jackson 2011).

A reliable Thrombin generation test as part of routine coagulation testing in the clinics is currently lacking (Brummel-Ziedins and Wolberg 2014). Thrombin generation is measured via functional coagulation tests like activated partial thromboplastin time (aPTT), Prothrombin time (PT) and activated clotting time (ACT) (Fitzgerald et al. 2020, Hofer et al. 2019). These assays investigate the formation of the Fibrin clot, which already occurs when less than 5 % of Prothrombin is converted to Thrombin. Therefore, these assays do not give appropriate information on the Thrombin generation capacity (Fitzgerald et al. 2020).

Hemostatic capacity can additionally be investigated by analysis of Fibrin clot formation, its structure and capability to resist fibrinolysis (Pieters et al. 2018, Wolberg et al. 2005). Patients with bleeding disorders (p.ex. hemophilia A and B, bleeding of unknown cause (BUC)) exhibit delayed/reduced clot formation (Gray et al. 2011, Hofer et al. 2019). Furthermore, Fibrin clot and structure show altered characteristics in thrombotic disorders (Undas 2014). Fibrin clot characteristics measured in patients after idiopathic venous thromboembolism have lower clot permeability, are less compact and have an increased clot lysis time (Undas et al. 2009).

## 2.2 Thrombocytopenia

According to the National Heart, Lung and Blood Institute (NIH) thrombocytopenia is described as a condition with lower platelet numbers than normal, nevertheless there is no clear cut-off value for the diagnosis of clinically relevant thrombocytopenia (NIH 2021).

A healthy individual has a platelet count of 150-350 x $10^9$/l blood. If the platelet count drops below 100 x $10^9$/l, thrombocytopenia is diagnosed. Too few platelets can lead to bleeding of different severity. Bleedings can manifest as internal bleedings, in the mucosa or external, mostly as skin bleedings (NIH 2021).

Thrombocytopenia can be caused by a dysfunctional platelet production in the bone marrow and/or increased platelet destruction. The treatment of thrombocytopenia depends on the underlying cause and the severity of thrombocytopenia (NIH 2021).

Various causes and conditions can result in thrombocytopenia, including non-immunologic or immunologic reactions. Non-immunologic thrombocytopenia, amongst other causes, may result from sepsis, fever, splenomegaly, hematopoietic stem cell transplantation, increased platelet consumption in coagulation activation, hemorrhage, hematologic and other malignant diseases and/or chemotherapy (Ferreira et al. 2011). However, thrombocytopenia can also result from genetic alterations (= inherited form) (Kistangari and McCrae 2013). Furthermore, non-immunologic thrombocytopenia might be drug-induced (p.ex. by heparin, quinidine or vancomycin, etc.) (Vayne et al. 2020).

ITP is defined by isolated thrombocytopenia (platelet count <100 x $10^9$/l) and not associated with the typical causes for non-immunological thrombocytopenia. (Kistangari and McCrae 2013). Two subforms are classified in ITP, primary ITP and secondary ITP, according to the underlying condition.

## 2.3 Primary ITP

Primary ITP is a rare autoimmune disease, characterized through isolated thrombocytopenia. Its pathological mechanisms are not completely elucidated yet, but include immunological reactions resulting in both, decreased platelet production and increased platelet destruction (Cines et al. 2009). Hence, diagnosis remains one of exclusion as no reliable, robust, and uniformly used clinical and/or laboratory parameters exist (Rodeghiero et al. 2009). Secondary ITP is induced through underlying diseases like autoimmune or hematological disorders (p.ex. systemic lupus erythematosus, antiphospholipid syndrome, chronic lymphocytic leukemia, lymphoproliferative disorders) or infections. Furthermore, it can occur after transfusion or due to drugs (Cines et al. 2009). Primary ITP accounts for 80 % and secondary ITP for 20 % of immune thrombocytopenias (Cines et al. 2009).

### 2.3.1 Epidemiology and Clinical Features

According to the research group of Terrell et al., who reviewed many publications reporting incidence data on ITP from 1966 to 2009, an incidence rate of acute ITP in children of

1.9-6.4 per 100,000 children/year and 1.6-3.9 per 100,000 adults/year is estimated (Terrell et al. 2010). This categorized primary ITP as an orphan disease. In the nationwide population-based study by Moulis et al., it is reported that incidence rates peak among children (1-5 years) and people over 60 years, especially in men >75 years (Moulis et al. 2014). Furthermore, the authors state that the overall incidence of ITP was higher in females than in males. Eighteen percent of ITP cases in adults were categorized as secondary ITP, which was mostly associated with a hematological disease (Moulis et al. 2014).

Starting from ITP diagnosis its disease progression can be divided into three stages: (1) newly diagnosed ITP (<3 months within diagnosis), (2) persistent ITP (3-12 months from diagnosis) and (3) chronic ITP (<12 months) (Rodeghiero et al. 2009; Neunert et al. 2019). Rodeghiero et al. further described a fourth stage, severe ITP. In this phase, patients suffer from bleeding symptoms requiring treatment, or development of new bleedings which require treatment modification (Rodeghiero et al. 2009).

The most common clinical manifestation observed in primary ITP is bleeding. The broad term bleeding includes mucocutaneous bleedings within the skin, oral cavity as well as the gastrointestinal tract. *Purpura* (“dry purpura”) on extremities are observed and in severe forms oral *hemorrhagic bullae* (“wet purpura”) can occur. Intracranial bleeding, although rare, is the worst possible bleeding complication associated with ITP. However, bleeding in ITP hardly occurs with platelet levels >30 x $10^9$/l (Kistangari and McCrae 2013). Besides bleeding, fatigue is a common ITP related symptom which drastically impacts the patients quality of life (Provan et al. 2019, Neunert et al. 2019). Paradoxically, also an increased thrombosis risk was detected in several studies on ITP cohorts. The clear mechanisms behind this paradox clinical complication are yet not known (Kistangari and McCrae 2013).

### 2.3.2 Diagnosis of Primary ITP

The diagnosis of primary ITP is challenging and performed by exclusion of other possible causes for the low platelet count. The physician needs to consider the patient history, perform a physical examination, analyze peripheral blood counts, in some cases examine the bone marrow, test for infections (p.ex. *Helicobacter pylori*, *hepatitis B virus* (HBV), *human immunodeficiency virus* (HIV), *hepatitis C virus* (HCV)), perform quantitative Ig level testing and blood group Rh(D) typing (Provan et al. 2019, Neunert et al. 2019).

When examining the patient history, following points are taken into account: family and professional history, earlier/recent bleedings, medication, alcohol consumption, pregnancy, thrombosis history, symptoms pointing to other autoimmune diseases, preceding infections (p.ex. HIV, HCV, *Human alphaherpesvirus 3* (HHV3), *Rubella virus, Measles virus*), vaccinations, iron levels, fever and weight loss (Pabinger et al. 2012).

The overall physical appearance of ITP patients usually is normal, however, during the examination special attention should be paid to the skin, mucosa as well as joints and muscles as these are frequent sites of bleeding (Pabinger et al. 2012). Other conditions like fever, weight loss, hepato- and/or splenomegaly, or enlarged lymph nodes could indicate amongst others a HIV infection, systemic lupus erythematosus or a lymphoproliferative disease (Provan et al. 2019).

In ITP, the peripheral blood count usually does not show any abnormalities, except for isolated thrombocytopenia (Provan et al. 2019). The detection of small, hypochromic red blood cells is termed microcytic anemia and frequent in bleeding conditions. Microcytic anemia is commonly caused through iron deficiency and correlates to the time and amount of blood loss (Massey 1992). A low reticulocyte (immature red blood cells) count indicates that erythropoiesis in the bone marrow is disturbed, and it subsequently leads to anemia (Janus and Moerschel 2010). The evaluation of the iron status and reticulocyte count helps to identify the cause of anemia. For example, severe vitamin B12 and folate deficiency can be detected in thrombocytopenic patients (Provan et al. 2019). Investigation of the peripheral blood smear is important to avoid diagnosis of pseudo thrombocytopenia, which arises due to EDTA-dependent platelet agglutination and to exclude other causes of thrombocytopenia and/or hematological disorders (Braester 2003).

Examination of the bone marrow is not inevitable necessary, especially in children suffering from ITP (Neunert et al. 2019). It is advised to perform this procedure to differential diagnoses of thrombocytopenia in case of systemic symptoms or suspected underlying disorders. However, bone marrow examination should in any case be done if a splenectomy is considered (Provan et al. 2019). A thorough examination includes an aspirate, a biopsy, analysis via flow cytometry as well as cytogenetic analysis to make sure lymphoproliferative diseases, myelodysplastic syndrome or primary bone marrow disorders are not misdiagnosed as ITP (Sajjan et al. 2008).

In case ITP patients express digestive symptoms or in geographic areas of high prevalence (p.ex. developing countries), testing for *H. pylori* should be done (Stasi et al. 2009). Reliable test methods are the urea breath test and the stool antigen test, serological detection is neither as sensitive nor specific (Provan et al. 2019). After initial diagnosis with ITP, the American Society of Hematology highly recommends to test for HIV and HCV in all patients (Neunert et al. 2019). The viral testing needs to be done due to the fact that thrombocytopenia with underlying viral infections cannot be distinguished from primary ITP on a solely clinical examination (Liebman and Stasi 2007). Furthermore, quantitative detection of immunoglobulin (Ig) levels is advised, as reduced levels could indicate an immune deficiency syndrome (p.ex. common variable immunodeficiency, systemic lupus erythematosus, antiphospholipid syndrome) (Provan et al. 2019).

#### 2.3.2.1 Differential Diagnosis

The definite diagnosis of primary ITP can be challenging, as other causes of non-immunologic or immunologic thrombocytopenia have to be excluded. Especially secondary ITP must be ruled out. Thus, a broad panel of investigations to exclude other diagnoses must be performed. Table 1 lists relevant differential diagnoses.

Non-immunologic thrombocytopenia is caused by decreased platelet production due to abnormal physiological processes, genetic alterations or exposure to chemotherapeutic drugs or toxins (Kistangari and McCrae 2013). At first sight several immunologic conditions can be misdiagnosed as primary ITP, which is why a thorough laboratory investigation as well as evaluation of the patient medical history is necessary (Provan et al. 2019).

**Table 1: Differential Diagnosis of Primary ITP.**

| Non-immunologic | Immunologic / Secondary ITP |
|---|---|
| ➢ **Decreased platelet production**<br>– Acute/chronic leukemia<br>– Myelodysplasia<br>– Aplastic anemia<br>– Congenital/acquired amegakaryocytic thrombocytopenia<br>– Toxic exposure (radiation, chemotherapy, alcohol)<br>– Nutritional deficiency (B12, folate)<br>– Myelofibrosis<br>– Myelopthistic processes<br>– Viral infection of hematopoietic precursors<br><br>➢ **Enhanced platelet destruction**<br>– Splenic sequestration<br>– Disseminated intravascular coagulation<br>– Thrombotic thrombocytopenic purpura (no direct immune response to platelets)<br>– Cardiopulmonary bypass<br>– Infection/sepsis<br><br>➢ **Inherited thrombocytopenia** | ➢ **Autoimmune disorders**<br>• Systemic lupus erythematosus<br>• Evans syndrome<br>• Antiphospholipid antibodies<br><br>➢ **Drug induced thrombocytopenia**<br>• Antibiotics (Bactrim, Vancomycin, etc)<br>• Quinine<br>• Valproic acid<br>• Heparin, …<br><br>➢ **Infection** (H. pylori, HIV, HCV)<br><br>➢ **Lymphoproliferative disorders**<br><br>➢ **Immunodeficiency**<br>• Autoimmune lymphoproliferative syndrome<br>• Common variable immunodeficiency<br><br>➢ **Post transfusion purpura**<br><br>➢ **Vaccinations** |

*Table was slightly adapted from (Kistangari and McCrae 2013)*

## 2.3.3 Risk Factors for ITP

An infection with *H. pylori* is a possible risk factor for the disease outbreak, some cases even evolved after vaccination (p.ex. for *measles-mumps-rubella*). As the incidence numbers are very low, it could be an accidental association (Matzdorff et al. 2018). Epidemiological studies on CD16 and CD32 gene polymorphisms in ITP, showed that individuals expressing CD16 polymorphism have a significantly higher risk on developing ITP than individuals with CD32 polymorphism (Xu et al. 2016).

Still, in most of the patients the key event/mechanism leading to ITP is not identified and there are probably many more risk factors than the mentioned ones.

## 2.3.4 Bleeding Phenotype

Bleeding is the most characteristic symptom in ITP. However, platelet counts are highly individual and do not correlate with the bleeding risk. This phenomenon is one reason why therapeutic choices are not made solely based on the platelet count but rather due to the bleeding potential (Pabinger et al. 2012). Still, researchers confirmed that bleeding risk increases if platelet counts drop below 30 x $10^9$/l with a mortality rate of 0.02-0.04 cases per patient-year (Cohen et al. 2000, Portielje et al. 2001). Studies showed that ITP patients

>60 years have an increased risk of non-fatal and fatal bleedings (Cohen et al. 2000) even though the median platelet count did not significantly differ compared to ITP patients <40 years (Michel et al. 2011). Cohen et al. performed a study on mortality rates and incidences of major bleeding events in ITP. Regarding a 5-year-mortality-rate, they predicted a 2.2 % chance of a fatal outcome in <40-year-old patients and a 47.8 % chance for patients >60 years (Cohen et al. 2000). However, most age-related deaths are not solely due to the disease, but to other conditions or side effects of ITP therapy (Pabinger et al. 2012; Kistangari and McCrae 2013). According to a model created by Cohen et al., a young female patient (25 years) will only lose 5.4 quality-adjusted-life-years when receiving the correct treatment in comparison to 14.9 quality-adjusted-life-years if no treatment is administered (Cohen et al. 2000).

Piel-Julian et al. performed a study assessing the bleeding risks factors in 302 patients. They found that the bleeding incidence was increased in women (odds ratio [OR] 2.6, 95 % confidence interval [CI]) (Piel-Julian et al. 2018). However, men more frequently tend to have severe bleedings. Furthermore, exposure to non-steroidal anti-inflammatory drugs (NSAIDs) showed increased bleeding risk (OR 4.8, 95 % CI) and low platelet counts were specifically noted in patients with mucosal bleedings. Lastly anticoagulant drugs correlated with cases of severe bleeding (OR 4.3, 95 % CI) (Piel-Julian et al. 2018).

### 2.3.5 Molecular and Cellular Pathophysiology of Primary ITP

The following chapter describes platelet clearance mechanisms and the processes resulting in impaired platelet production, which together lead to the low platelet count in primary ITP.

#### 2.3.5.1 Platelet Clearance Mechanisms

Under physiological conditions, platelets circulate for about 7-10 days within the system, during which they lose their terminal sialic acid and undergo cellular aging (senescence) (Swinkels et al. 2018).

A sialidase/neuraminidase (NEU) is a glycohydrolytic enzyme capable of removing sialic acid residues form glycoproteins and glycolipids. Sialic acid is an acidic monosaccharide placed at the terminal end of the glycoprotein. Loss of sialic acid disturbs direct cell-cell interactions as well as interaction with other players of their environment (Miyagi and Yamaguchi 2012). It is therefore believed to be a biomarker for platelet clearance.

The human genome encodes for at least four sialidases, NEU1- NEU4 (Jansen et al. 2012). Upon upregulation of NEU1 and NEU3, increased loss of sialic acid was detected. Jansen et al. confirmed that desialylation induces platelet clearance. Cold stored platelets express significantly higher NEU1 levels, leading to VWF receptor desialylation. Targeted treatment with sialidase inhibitors hinders the capping of sialic acid from the glycoproteins and therefore prolong the platelet lifespan (Jansen et al. 2012). The sialidase inhibitors DANA and oseltamivir phosphate build a promising therapy for ITP patients, still more studies remain to be conducted (Li et al. 2016).

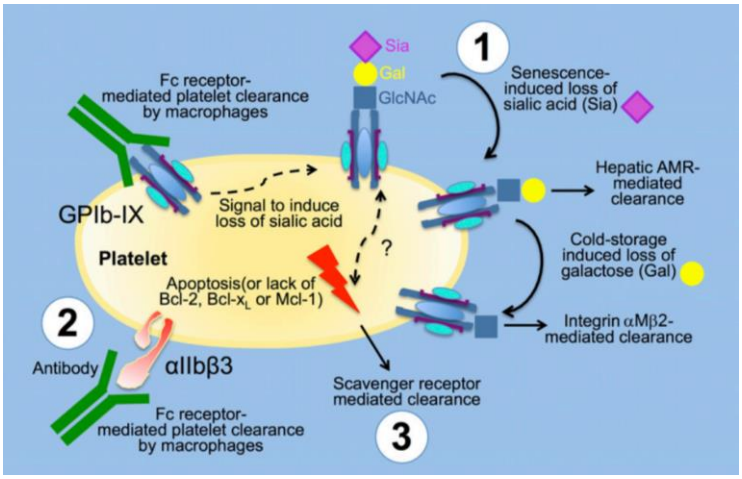



***Figure 4: Graphic Summary of Platelet Clearance Mechanisms.*** *(1) Glycan-Lectin mediated platelet destruction (2) Autoantibody Fc-receptor mediated clearance (3) Apoptosis (programmed cell death); AMR, Ashwell-Morrell receptor Source:(Grozovsky et al. 2015)*

**Glycan Mediated Clearance**

As platelets undergo senescence, they lose sialic acid from their surface and instead expose surface galactose. This is recognized by the Ashwell-Morell Receptor (AMR) on hepatocytes, which upon binding will initiate the JAK2-STAT3 or STAT5 pathway, facilitating thrombopoietin (TPO) production (Grozovsky et al. 2014, Li et al. 2016).

*In vitro* experiments conducted with Chinese Ovary Hamster cells and THP-1 cells (= human monocytic cell line) showed, that platelets can lose their galactose upon cold storage, which leads to the exposure of N-acetylglucosamine (GlcNAc), which attracts hepatic macrophages. Hepatic macrophages (Kupffer cells) expose alpha-M beta-2 integrin on their surface, which initiates this process (Josefsson et al. 2005). The alpha-M-subunit is not only involved in phagocytosis, but also cell mediated cytotoxicity, chemotaxis and cellular activation

(Grozovsky et al. 2015). Additionally, researchers detected that it binds the C3b component of the complement cascade (Springer 1994, Petty and Todd III 1993, Xia et al. 2002).

**Autoantibody and Lectin Mediated Platelet Clearance**

In ITP autoantibodies primarily target the platelet glycoproteins (GPIIb/IIIa, GPIb-IX complex). The Fc-portion of the platelet-associated immunoglobulin G (IgG) antibodies in turn bind the Fc-gamma-receptors (Fc-gamma-R) on splenic macrophages or $CD8^+$ cytotoxic T cells. Upon receptor-antibody binding, phagocytosis is initiated (Grozovsky et al. 2015). Fc-gamma-R-dependent elimination of platelets is part of the adaptive immunity. Targeting the Fc-gamma-R-mediated pathway via intravenous immunoglobulin G (IVIg), which binds to the Fc-portion of Fc-gamaRs, competing with splenic macrophages (Nagelkerke and Kuijpers 2014), proofed effective in inhibition of macrophages and therefore a quick restorage of platelet numbers in ITP patients can follow (Audia et al. 2017).

Antibodies against GPIb alpha as part of the GPIb-IX (von Willebrand receptor) complex facilitate desialylation and clearance through AMR in the liver. This GP is especially attractive because it carries terminal beta-galactose and beta-GlcNAc (Josefsson et al. 2005).

**Programmed Cell Death: Apoptosis**

Members of the Bcl-2 family regulate the intrinsic apoptotic pathway. The balance between pro- and anti-apoptotic signals regulates the programmed cell death. The most prominent pro-apoptotic proteins are Bak and Bax, whereas Bcl-2, Bcl-$x_L$ and Mcl-1 are pro-survival proteins. It is uncertain if specifically, members of the Bcl-2 family alter the platelet surface (alter the sialic acid content) or if this happens at some unknown time point along the process. By inhibition of Bcl-2 and Bcl-$x_L$, platelet clearance was induced in the liver, but not in the spleen (Kile 2014). Other studies showed that expression of NEU3, relates to resistance to apoptosis. This phenomenon was specifically detected in tumor cells (Tringali et al. 2009, Miyagi et al. 2012, Miyagi and Yamaguchi 2012).

**Immunological Platelet Clearance**

Sixty percent of the low platelet counts detected in ITP patients are caused by autoantibodies. Most antibodies target the GPIIb/IIIa (approx. 70 %) and the GPIb-IX-V (approx. 25 %) complex (Swinkels et al. 2018).

Production of antibodies is part of the adaptive immune system and facilitated by B-cells. Splenic macrophages or dendritic cells (DCs) will present platelet antigens to B-cells. Together with $CD4^+$ follicular helper cells a geminal center is formed and B-cells differentiate into platelet-reactive plasma cells (PC) (Catani et al. 2006, Kuwana et al. 2009). Plasma cells then secrete specific antibodies targeting the initially by macrophages and DCs presented antigens. Besides the PCs in blood and bone marrow, memory B-cells are released into the blood circulation, to support platelet elimination (Figure 5) (Kuwana et al. 2002). Summing up, the thereby generated autoantibodies hinder thrombopoiesis (McMillan et al. 2004, Chang et al. 2003), promote apoptosis (Goette et al. 2016) and platelet desialylation as well as deposition via the complement cascade system (Peerschke et al. 2010). During autoantibody classification studies, IgG class autoantibodies appeared to play a major role in antibody-driven autoimmunity. Besides IgG, also IgM and some IgA class antibodies were found (Nishioka et al. 2005). However, solely the IgG (subtype) expression is no sufficient biomarker for ITP (Swinkels et al. 2018).

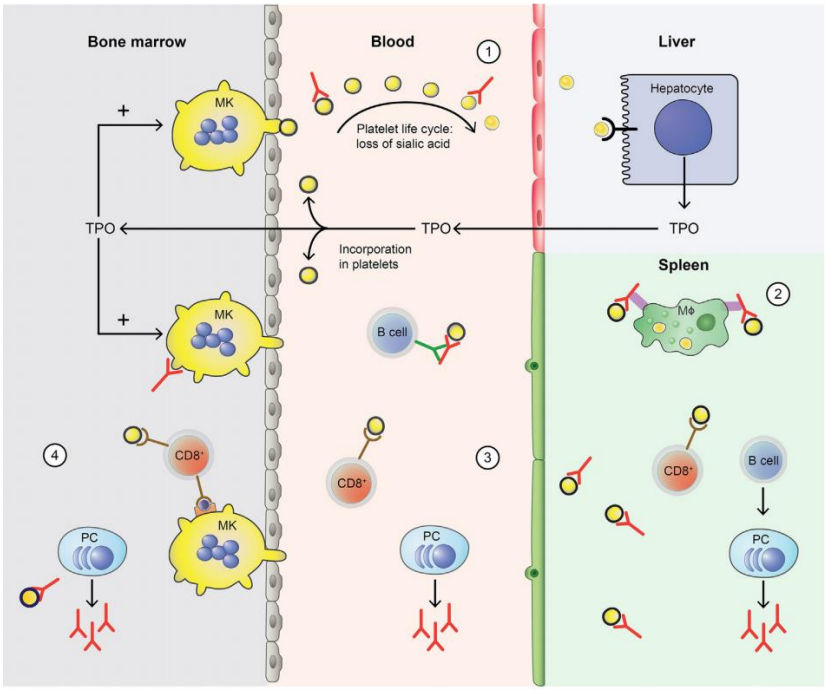



***Figure 5: Immune Cell Mediated Platelet Clearance in ITP**. (1) During aging platelets (depicted in yellow) undergo apoptosis and respectively lose sialic acid residues (depicted as black circle around the platelets). Under normal circumstances platelets are cleared via hepatocytes in the liver which triggers Thrombopoietin (TPO) formation. In ITP it is suggested that autoantibodies enhance the desialylation process and therefore platelet clearance. (2) Spleen: Macrophages directly phagocytose platelets. $CD4^+$ T helper (Th) cells are primed, which again help B-cells to differentiate into platelet-reactive plasma cells (PC). PC then secrete autoantibodies (red). $CD8^+$ cytotoxic T-cells (Tc) can directly bind to platelets and lyse them. (3) Peripheral Blood: PC and Tc further drive the autoimmune reaction against platelets, supported by memory B-cells. Tc might support the desialylation process. (4) Tc and PC both target MK in the bone marrow, inhibiting platelet production. Source: (Swinkels et al. 2018)*

### 2.3.5.2 Impaired Platelet Production

Megakaryopoiesis describes the development of MKs and ultimately platelets. It takes place in the bone marrow niche, which provides the perfect microenvironment (Zufferey et al. 2017). The most important hormone in this process is thrombopoietin. TPO is produced by hepatocytes and sinusoidal endothelial cells in the liver, as well as in the kidney by proximal convoluted tubule cells, and it stimulates platelet production by MKs via the TPO receptor (cMpl) (Grozovsky et al. 2014, Swinkels et al. 2018). Additionally, TPO binds to cMpls on platelets which leads to its destruction (Audia et al. 2021). Interestingly, ITP patients show relatively normal TPO levels, even though they have reduced platelet counts. For example, patients with reduced bone marrow MKs and platelet counts had significantly elevated TPO levels in comparison to healthy controls (Hou et al. 1998). Plasma TPO levels are counter regulated by the amount of circulating platelets and MKs. TPO binds to receptors on their cell surface and will be subsequently destroyed due to platelet clearance. This phenomenon indicates, that in ITP patients TPO binds to the circulating platelets and MKs before they undergo phagocytosis (Audia et al. 2021). Different TPO levels were observed across several other conditions like chronic liver disease, myelodysplastic syndrome, or aplastic anemia, leaving many questions around TPO and a high potential for further investigation (Hou et al. 1998, Zufferey et al. 2017). TPO action promotes also cycling of hematopoietic stem cells and is critical for their maintenance and replenishment, being a central cytokine in hematopoiesis (Gao et al. 2021).

In ITP megakaryopoiesis is dysregulated, which results in impaired MK-maturation and platelet release. Megakaryocytes show decreased ploidy (decreased sets of chromosomes), granularity and platelet release (Zufferey et al. 2017). *In vitro* plasma autoantibodies tended to detain MK-maturation and promote apoptosis (Chang et al. 2003; McMillan et al. 2004). So far, these autoantibodies are not classified in regard of their epitope recognition, and several Ig-classes of autoantibodies could be involved (Zufferey et al. 2017). However, researchers confirmed that especially GPIb and GPIIbIIIa cause this process (Chang et al. 2003, Iraqi et al. 2015). MKs exhibit a regulatory role within the bone marrow niche. They, act on plasma cells for example which produce antibodies (Winter et al. 2010) and impair T-cell activation as well as IL-10 production (Zufferey et al. 2017). Besides MK-clearance through autoantibodies, clearance by neutrophils and macrophages was reported.

### 2.3.5.3 Treatment of Adult ITP Patients

Therapy is administered after individual patient assessment. Besides a thorough examination, patient age (children or adult), specific life circumstances (p.ex. pregnancy) and disease stage require different therapeutical strategies. The main goals of treatment are the prevention of severe bleeding and achievement of platelet counts of >20-30 x $10^9$/l in symptomatic ITP patients. Furthermore, the administered drugs should cause as little toxic side effects as possible and health-related quality of life should significantly improve (Provan et al. 2019, Neunert et al. 2019).

If the bleeding tendency is very low and platelet counts are <30 x $10^9$/l, pros and cons about therapy and side effects have to be taken into account. Nevertheless, usually treatment is initiated (Matzdorff et al. 2018). In case the platelet count is >20-30 x $10^9$/l and bleeding is not detected, treatment is evaluated on an individual basis and mostly not required (Provan et al. 2019). A close eye has to be kept on patients having a platelet count above that threshold, so therapy can be given if the condition worsens. If severe bleeding is detected in newly diagnosed ITP, hospitalization is advised (Matzdorff et al. 2018).

**First-Line Treatment in ITP Patients**

First line treatment in adults after initial diagnosis are (1) corticosteroids (p.ex. dexamethasone, methylprednisolone, prednis(ol)one) (2) IVIg or (3) IV anti-D (licensed for ITP only in some countries; not available in Europe) (Neunert et al. 2019, Provan et al. 2019).

Corticosteroids are steroid hormones produced and released by the adrenal cortex and they are under control of the hypothalamus pituitary adrenal gland axis. They are part of main regulatory functions in cell development, homeostasis, metabolism, cognition, and inflammation. Glucocorticoids represent one of the worlds most prescribed drugs for a variety of inflammatory and autoimmune diseases (p.ex. asthma, allergy, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease or COVID-19 infections) (Ramamoorthy and Cidlowski 2016, Alexaki and Henneicke 2021). However, only short-term treatment is advised, as severe side effects (p.ex. immunosuppression, anxiety, memory loss, lipid depot rearrangement due to lipolysis, osteoporosis, diabetes, obesity, hypertension, etc.) and resistance like frequently observed in acute leukemia long term treatment can evolve (Ramamoorthy and Cidlowski 2016, Pravosudov 2019, Neunert et al. 2019). In regard to ITP, approximately 80 % of patients

respond to corticosteroids, but their symptoms often relapse upon a stop of therapy. Thus, corticosteroid treatment is effective, but contains also many dangerous and unwanted systemic side effects. Cell type specific targeting of steroid compounds was so far not successfully achieved (Kistangari and McCrae 2013).

Intravenous immunoglobulin is commonly used in combination with corticosteroids, especially if rapid rise of platelet count is required p.ex. in case of severe bleeding symptoms. Also, this treatment can be administered if corticosteroids fail, and a more definitive therapy is to be determined (Kistangari and McCrae 2013). Successful increases in platelet counts were detected in 60-80 % of patients post treatment. However, responses are temporary (Stasi 2012). The mode of action is blockage of T cell activation, inhibition of macrophage Fc-receptors, cytokine modulation, as well as complement neutralization (Kistangari and McCrae 2013) among many other effects eluted briefly above.

**Second-Line Treatment in ITP Patients**

Subsequent medical therapies (second line therapies) recommended by the American Society of Hematology for persistent or chronic ITP forms are thrombopoietin receptor agonists (TPO-RAs) (p.ex. eltrombopag, avatrombopag, romiplostim) and fostamatinib (Provan et al. 2019, Neunert et al. 2019).

Currently three TPO-RAs (romiplostim, eltrombopag and avatrombopag) are approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of adults with chronic ITP. TPOs are usually administered after first line treatment failure or failure of splenectomy (Kapur et al. 2020) as they show good response rates (>60 %) in patients with and without splenectomy and durable response rates of 6-8 years (Lete et al. 2008). Due to the overall good response, other therapies could be reduced. However, upon stop of TPO-RAs ITP will relapse in most patients (Provan et al. 2019). Their mode of action is promotion of platelet differentiation by binding to cMpl on MKs (Michel 2013). Also, immunomodulatory functions have been reported. Romiplostim supports regulatory T cell (Treg)-activity and TGF-beta 1 levels in the circulation while eltrombopag can downregulate monocyte/macrophage-mediated phagocytosis and an increase in plasma levels of TGF-beta 1 was reported. All together these drugs reduce anti-platelet antibody levels and subsequently rescue platelet counts in ITP patients (Kapur et al. 2020).

Fostamatinib is a SYK-(spleen tyrosine kinase) inhibitor and used for treatment of chronic ITP. Via inhibiting SYK, the signal transduction cascade, responsible for phagocytosis of erythrocytes and thrombocytes within the spleen, is disturbed (Matzdorff et al. 2021).

**Third-Line Therapy**

Rituximab is a B cell depleting anti-CD20 agent commonly used in lymphoma therapy, but it is also used for ITP patients trying to deplete autoantibodies in patients who do not reach remission after first- and second-line therapy. The drug generally shows good response and durable platelet restorage. Possible side effects are infusion reactions, cardiac arrhythmias, and serum sickness. Special care with administering rituximab needs to be taken in case of a preceding HBV infection or other infections (Kistangari and McCrae 2013).

Immunosuppressive agents, like mycophenolate mofetil might be an option in case common therapies fail (Taylor et al. 2015). In patients in whom splenectomy is not advised or other medications are not available, treatment with danazol and dapsone ("corticosteroid-sparing agents") showed good outcomes (Liu et al. 2016, Colella et al. 2018). Nevertheless, nowadays these treatments are rarely applied and restricted to chronic, multi-refractory and clinically symptomatic ITP patients. Therapy involving vinca alkaloids (p.ex. vincristine) is not advised, as neurotoxicity was observed as severe side effect in chronic ITP (Park et al. 2016).

**Surgical Treatment in ITP Patients**

If medical treatments fail, surgical therapy in form of splenectomy can be administered. This surgical intervention is only suggested, if after 12-24 months from diagnosis, no platelet stabilization is achieved, and chances of remission are poor (Provan et al. 2019). Nevertheless, response rates are quite high and persistent after splenectomy. However, it should be noted that long-term risks go along with spleen removal, possible candidates should be aware, that thrombosis, severe sepsis, infection (Thai et al. 2016) and cancer risk are increased (Kristinsson et al. 2014).

# 3 Materials and Methods

The following chapter gives a detailed description of Materials and Methods used in this study.

## 3.1 Patient Samples and Healthy Controls

Between 2016 and 2019, adult patients diagnosed with primary ITP were included in the Vienna ITP Biobank at the Clinical Division of Hematology and Hemostaseology, Department of Medicine I, Medical University Vienna or at the third Medical Department for Hematology and Oncology, Hanusch Hospital Vienna. Patients with primary ITP had to be over 18 years and had to have platelet counts of ≤150 x$10^9$/l in case of no ITP-specific treatment to be included in the ITP Biobank. Active neoplastic disease, secondary/hereditary thrombocytopenia, secondary ITP or a platelet count >150 x $10^9$/l without treatment were exclusion criteria. Written consent was obtained from all patients before inclusion into the study (EC 1843/2016). Trained personal recorded the individual medical and bleeding history of each patient. Several coagulation- and other routine parameters were determined at the Department of Laboratory Medicine, Medical University Vienna, Vienna, Austria. Furthermore, the ISTH-BAT bleeding score was recorded.

In the conducted experiments, 88 plasma samples and 32 serum samples from primary ITP patients included in the Vienna ITP biobank were investigated.

For comparison, a group of patients with non-immunological thrombocytopenia was included. Plasma samples from 19 non-immunological thrombocytopenic patients were investigated. These patients were recruited at the patients ward of the Clinical Division of Hematology and Hemostaseology, Department of Medicine I, Medical University of Vienna, and the study included patients with thrombocytopenia after chemotherapy.

Healthy control (HC) samples were obtained from the Vienna Bleeding Study (VIBS) (EC 039/2006) and included healthy individuals with normal platelet counts (range 150-350 x $10^9$/l). The healthy controls were age and sex-matched for each experiment. In total 159 plasma samples were analyzed in the TGA, 157 in the CLA, and 106 in the microparticle Tissue factor test. For the LBA 20 serum samples of healthy controls were tested.

### 3.1.1 Standardized Bleeding Assessment – SMO Grading Index

In 2008 the International Working Group (IWG) on ITP decided to define a new ITP-specific Bleeding Assessment Tool (ITP-BAT) as previously used scales were not uniform and thus not comparable. (Rodeghiero et al. 2013).

The ITP-BAT concentrates on three main areas: skin (S), visible *mucosae* (M) and organ (O) (also including internal *mucosae*). Each area is divided in sub-areas which are graded individually. Zero is the lowest and five the highest grade, corresponding to fatal bleeding. To evaluate the final SMO Grade (SMOG) index, the highest grades of each area are summed up (Rodeghiero et al. 2013).

### 3.1.2 Blood Sampling and Sample Preparation.

Blood was obtained by antecubital venipuncture with a 21-gauge butterfly needle (Greiner Bio-One International GmbH, Kremsmünster, Austria) into vacutainers containing 3.8 % trisodium citrate (Greiner Bio-One International GmbH, Kremsmünster Austria) and it was placed into primary tubes containing separation gel and z-clot activators (Greiner Bio-One International GmbH, Kremsmünster Austria). Further processing and storage were performed at the MedUni Vienna Biobank (www.biobank.at) according to standard operating procedures in an ISO 9001:2015-certified environment (Haslacher et al. 2018).

Platelet poor plasma (PPP) was obtained by an initial centrifugation at 2,000 x g for 15 min at 15 °C, followed by a second centrifugation at 18,000 x g for 2 min. Aliquots were stored at <-70 °C. After a 30 min incubation of the primary tubes, followed by a centrifugation at 1,884 x g for 10 min at room temperature (RT), serum aliquots were prepared and stored at <-70 °C (Haslacher et al. 2018).

Routine laboratory parameters used within this thesis were generated in the central laboratory of the General Hospital Vienna and Hanusch-Krankenhaus Vienna according to standard routine clinical practice (http://www.kimcl.at/).

## 3.2 Thrombin Generation Assay (TGA)

Thrombin generation was measured with the TECHNOTHROMBIN® TGA kit (Technoclone GmbH, Vienna, Austria) in PPP. In this assay, the clotting cascade is initiated by micelles of negatively charged phospholipids, which comprise human TF and $CaCl_2$. The TECHNOTHROMBIN® TGA kit is a fluorescence-based method, whereby a fluorogenic substrate is cleaved by Thrombin. Negatively charged phospholipids and TF activate the coagulation cascade, respectively changing the Thrombin levels over time, causing the absorbance curve shown in Figure 6.

For the measurement PPP citrated plasma samples are thawed at 37 °C for 15 min in a water bath. Meanwhile TGA substrate (Fluorogenic substrate, 1 mM Z-G-R-AMC, 15 mM $CaCl_2$), TGA reagent C (RC) Low (low concentration of phospholipid micelles containing rhTF in Tris-Hepes-NaCl buffer), TGA control high (Human plasma with increased Thrombin generation, lyophilized) and TGA control low (Human plasma with decreased Thrombin generation, lyophilized) are dissolved in distilled water and incubated for at least 20 min at RT. Afterwards a 96-well plate frame containing well-insets for six columns is prepared and 40 µL of PPP/well and 40 µL/control high or low/well were pipetted in duplicates onto the plate. Next, one part TGA RC Low and five parts TGA substrate are mixed. After adding 60 µL of the reagent/substrate mixture to each well, the photometric reading (FLx800 Fluorescence Reader, BioTek Instruments Inc., Winooski, VT, USA) is immediately started at 37 °C for 120 min in one minute measurement intervals. Data evaluation is done via an excel evaluation sheet (downloaded from www.technoclone.com), considering the current standard curve according to the substrate lot number. The evaluation sheet transforms the measured RFU (relative fluorescence units) data from the different Thrombin standard concentrations into a calibration curve, which is further used to assess nM Thrombin present in each individual sample at a given time point. Five parameters (lag phase, peak height of Thrombin, time-to-peak (TTP), velocity index (VI), area under the curve (AUC)) are generated as readouts. Additionally, a graph depicting the individual Thrombin generation curve of each patient or control sample per point of time during the coagulation is generated.

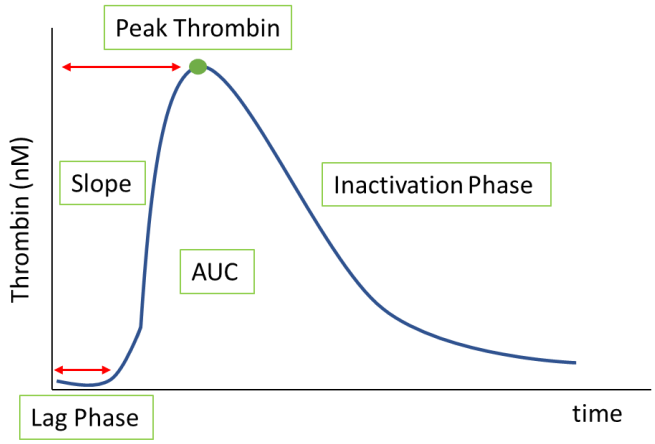



***Figure 6: Thrombin Generation Curve.*** *Lag phase, start to time point when $CaCl_2$ and phospholipid/Tissue factor mixture lead to the first burst of Thrombin formation; slope or velocity index, steepest rate of Thrombin formation; peak Thrombin, maximal concentration of Thrombin formed; AUC, area under the curve; ©Theresa Schramm*

## 3.3 Turbidimetric Clot Formation and Lysis Assay (CLA)

The CLA is an *ex vivo* kinetic turbidimetric assay, used to determine real-time clot formation and lysis in human plasma. During the 2-hour (20 sec interval) long optical density measurement, a specimen-specific turbidity curve is generated. This curve mirrors the clot formation in which the plasma turns progressively turbid, followed by the clot lysis in which the turbidity decreases over time. Figure 7 mimics the plasma clot formation and lysis curve. Several clot parameters are extracted for each well. The baseline absorbance represents the optical density (OD) of the first measurement, the lag phase defines the time (in minutes) to the first reading with an OD ≤10 % higher than the baseline OD during the first 100 points of measurement. The maximum clot formation rate ($V_{max}$, OD/min) shows the highest positive change in absorbance within three sequenced measurements, while the amplitude (ΔAbs) states the difference between peak absorbance and baseline absorbance. Time-to-peak absorbance (TTP) describes the timespan from the starting point to the measurement with the highest absorbance, whereas the clot lysis time (CLT) defines the time from the midpoint of the clot formation section of the curve to the midpoint of the clot lysis section (Posch et al. 2020).

First, Tissue factor (Dade® Innovin®, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) is diluted with HEPES buffer saline (HBS) (2 mM HEPES, 150 mM NaCl; pH 7.4) to a working concentration of 24 pmol/l and HTPA-TC (LOXO GmbH, Dossenheim, Germany) is diluted with HBS with bovine serum albumin (HBSA) (137 nM NaCl, 5.38 nM KCl,

5.55 nM glucose, 10 nM HEPES, 0.1 % bovine serum albumin (BSA); pH 7.4) to 12 µg/ml. Both reagents can be aliquoted and stored at -80 °C until usage.

Next, the plasma samples are thawed at RT and in the meantime pre-dilutions for the cascade induction mix are prepared. Therefore, 1 M $CaCl_2$ solution is diluted with HBSA buffer to 0.12 M and a Phospholipid (Rossix AB, Mölndal, Sweden) working solution at 0.024 mM is prepared. HTPA-TC (12 µg/mL) is diluted with HBSA buffer to 4 µg/mL. Afterwards, 60 µL of the patient or healthy plasma are transferred, in duplicates, onto the 96-well plate. Afterwards the cascade induction mix (40 mM $CaCl_2$, 8 µM phospholipids, 4 pM TF, 666 ng/mL HTPA-TC) is prepared and thoroughly vortexed. Next, 60 µL of the cascade induction mix were added to each well containing plasma and the photometer protocol was immediately executed. It is important to pre-heat the photometer (Varioskan LUX Multimode Microplate Reader, Thermo Fischer Scientific, Vienna, Austria) to 37 °C and take measurements in 20 sec intervals for 2 h at 405 nm.

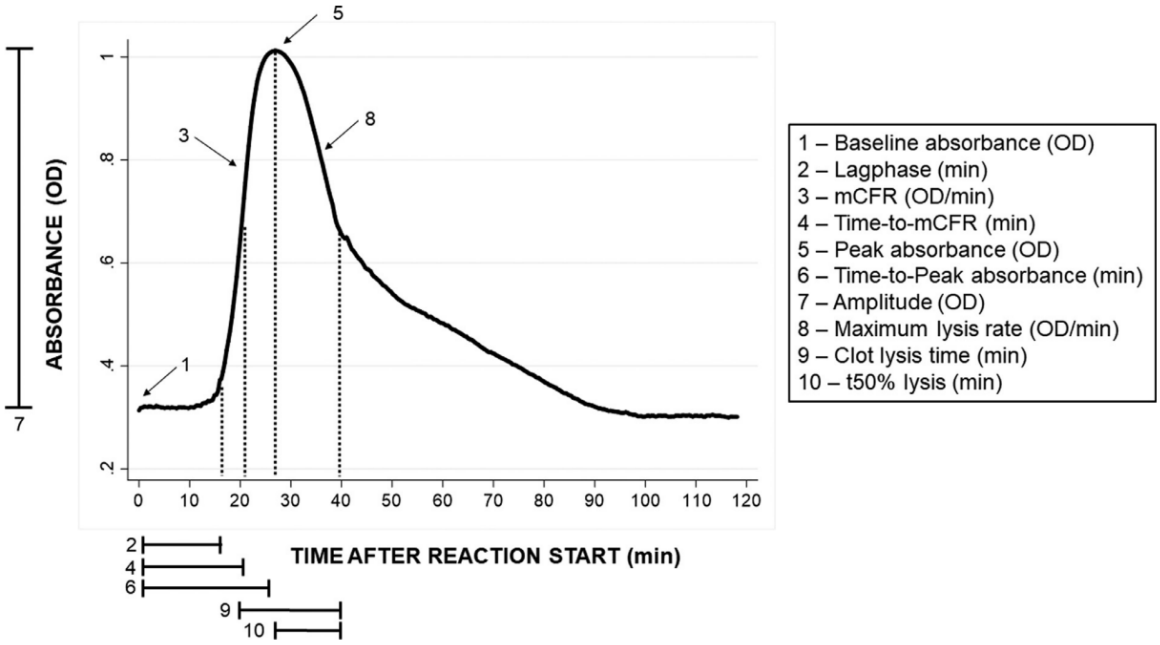



***Figure 7: Plasma Clot Formation and Lysis Density Curve**. The box represents parameters which can be generated and calculated from the curve. OD, Optical density. Graph taken from: (Posch et al. 2020)*

## 3.4 Microparticle Tissue Factor Test (Mackman-Assay)

This experiment is based on the protocol for measurement of TF-activity in extracellular vesicles (EV) derived from human plasma samples and was developed by Yohei Hisada and Nigel Mackman (Hisada and Mackman 2019a). Within this Master thesis this experiment will also be referred to as “Mackman-Assay”. TF is a membrane bound receptor for FV/FVIIa and the initiator of the coagulation cascade. EVs are submicron membrane vesicles that are amongst other cells secreted by activated monocytes and tumor cells (Hisada and Mackman 2019a). The Mackman assay is a highly sensitive test to investigate levels of EV TF-activity, as elevated EV TF-activity is thought to be a biomarker for increased thrombotic risk (Hisada and Mackman 2019a).

### 3.4.1 Preparation Positive and Negative Controls

Venous blood from a healthy donor (male, 38 years) is collected in 9 mL 3.8 % sodium citrate vacutainers (Greiner Bio-One International GmbH, Kremsmünster, Austria). The first 2-3 mL of blood are discarded to avoid contamination of TF from the vessel wall. Furthermore, it is important to avoid agitation of the vacutainers and process the blood within one hour of collection as this prevents activation of monocytes. One part of the whole blood is centrifuged at 3,000 x g for 10 min with brake, the thereby generated PPP is taken for further analysis as negative control. The second part is stimulated with 10 µg/mL lipopolysaccharide (LPS) (EMD Millipore Corporation, USA) and agitated for 5 h, 200 rpm at 37 °C. Afterwards the stimulated blood is centrifuged at 3,000 x g for 10 min. The generated plasma is used for further analysis as positive control.

### 3.4.2 Extraction of Extracellular Vesicles

Citrate plasma samples are thawed at RT. Two hundred µL plasma sample is mixed with 1 mL HBSA-buffer and centrifuged at 18,000 x g for 20 min at 4 °C to isolate the EV. Afterwards the supernatant is discarded, leaving approximately 50 µL in the 1.5 mL Eppendorf tube. Next, 1 mL of HBSA buffer is added and after a thorough vortex step for 30 sec, another centrifugation (18,000 x g/20 min/4 °C) is performed to re-pellet the EVs. Next, the supernatant is aspirated and the EVs are resuspended in 200 µL HBSA by vortexing the tubes for at least 30 sec.

### 3.4.3 Measurement of Microparticle Tissue Factor Activity

For each assay 50 µL of EV sample is added in quadruplicates onto a 96-well plate. In total 20 samples, including positive (LPS-stimulated pooled plasma) and negative control (unstimulated pooled plasma) are measured within one run. The sample quadruplicates are divided into two sets of duplicates. One set is treated with 1 µL inhibitory mouse anti-human CD142 TF antibody (clone: HFT-1, BD Biosciences, San Jose, California, USA) per well (stock concentration: 0.5 mg/mL; final concentration: 9.8 µg/mL). The other set receives 1 µL of IgG control (origin: mouse serum; Sigma Aldrich, St. Louis, USA) per well (working stock concentration: 0.5 mg/mL; final concentration: 9.8 µg/mL). Plates are incubated 15 min at RT, meanwhile TF standards are prepared (conc.: 0, 0.215, 0.43, 0.86, 1.72, 3.44, 6.88, 13.75 pg/mL) using re-lipidated recombinant TF (Dade® Innovin®, Siemens, Munich, Germany) and diluting it with HBSA. The TF working concentration is measured by optical density, assuming a concentration of 220 ng TF/mL, the first standard (conc. 13.75 pg/mL) should have OD 1. The eight standards are pipetted in duplicates (50 µL/well) onto the 96-well plate. After incubation, 50 µL of a mixture of HBSA with 10 mM $CaCl_2$ (pH 7.4, final concentration: 8.13 mM), human FVIIa (working concentration: 410 nM, finale concentration: 10 nM, CoaChrom Diagnostica GmbH, Maria Enzersdorf, Austria) and human FX (working concentration: 900 nM, final concentration: 150 nM, CoaChrom Diagnostica GmbH, Maria Enzersdorf, Austria) were added to each well. Then the plate is sealed with adhesive sealing film and incubated for two hours at 37 °C. Afterwards, 25 µL HBSA buffer with 5 mM EDTA is added to each well and incubated for 5 min at RT. In the last step 25 µL Pefachrome (final conc. 4 mM) is added to the reaction. The plate is covered with aluminum foil and again incubated for 15 min at 37 °C.

Finally, the absorbance at 405 nm is measured at the photometer (Multiskan FC, Thermo Fischer Scientific, Vienna, Austria). For calculation of TF-activity, FXa generation is calculated via the standard curve of TF. Following formular to calculate the TF-dependent FXa generation is applied:

$$EV\ TF\text{-}activity\ [pg/mL] = \frac{total\ FXa\ generation\ (control\ IgG\ well)}{TF\ independent\ FXa\ generation\ (HTF - 1\ well)}$$

Mackman and Hisada suggest four classes for EV-TF activity of PPP: (1) zero (0-<0.5 pg/mL), (2) weak (0.5-<1.0 pg/mL), (3) moderate (1-<2.0 pg/ml) and strong (>2.0 pg/mL) (Hisada and Mackman 2019a).

## 3.5 Lectin Binding Assay: AB-Sera Screening with Washed Platelets

### 3.5.1 Washed Platelets

Blood from a healthy donor is collected in a 9 mL ACD-A Vacuette (22.0 g/l Trisodium citrate, 8.0 g/l Citric Acid, 24.5 g/l Dextrose) (Greiner Bio-One International GmbH, Kremsmünster, Austria), the first 3 mL are discarded. Afterwards the Vacuettes are incubated for 20 min at 37 °C, followed by centrifugation at 120 x g for 20 min at 22 °C without brake. Next, 1 mL of platelet rich plasma (PRP) from each tube is transferred into a separate tube and 111 µL/mL warm citrate-dextrose solution (ACD-A) (Merck KGaA, Darmstadt, Germany) as well as 5 µL/mL Apyrase (Merck KGaA, Darmstadt, Germany) are added. The tubes are then centrifuged at 650 x g for 7 min without brake and the supernatant is discarded. After this, the PRP is resuspended in 5 mL wash solution *(see Annex Tables S1-S4 for information regarding constitution and concentration)* and incubated for 15 min at 37 °C. At the end of the incubation another centrifugation at 650 x g for 7 min without brake is done, again the supernatant is discarded. In the final step, the PRP is resuspended in 2 mL of suspension buffer *(see Annex Table S4)*.

Now the platelet count is determined by a routine hematology analyzer (Sysmex-XN350, Sysmex Deutschland GmbH, Nordersted, Germany) and the PRP is adjusted to a cell count of 250,000/µL with suspension buffer. Next, the extracted washed platelets are used for further analysis.

### 3.5.2 Lectin Binding Assay

The ITP patient and AB-control sera (Merck KGaA, Darmstadt, Germany) are heat-inactivated in the water bath for 30 min at 56-57 °C and afterwards centrifuged at 500 x g for 5 min at 19 °C with brake. Then the supernatant is transferred into a separated tube, as it will be used for further analysis. Next, washed platelets at a concentration of 2.5 x $10^5$/µL are split into seven to nine 1.5 mL Eppendorf tubes and incubated 1:1 (v/v) with either 1x phosphate-

buffered saline (PBS) (ThermoFischer Scientific, Vienna Austria), ITP patient serum or AB-control serum for 2 hours at RT in the dark at low rotation. After 1 h 45 min, 1 µL Neuraminidase (Merck KGaA, Darmstadt, Germany) is added to one tube and the incubation is continued. Thereafter, samples are fixed with 4 % paraformaldehyde (final conc. 2 %) and incubated for 20 min at RT in the dark. Afterwards the samples are washed with 1x PBS and centrifuged (650 x g, 7 min, 20 °C), the supernatant is discarded, and the platelets are resuspended in 75 µL of 1x PBS. Next, 40 µL of the cell suspension in the Eppendorf tubes are transferred to FACS tubes according to the pipetting scheme shown in Table 2. The Isotypecontrol (Beckman Coulter GmbH, Krefeld, Germany), platelet marker CD41-APC (Beckman Coulter GmbH, Krefeld, Germany), and Lectin dilutions (Erythrina Cristagalli Lectin (ECL), final conc. 1 µg/mL; Ricinus Communis Agglutinin (RCA), final conc. 0.5 µg/mL; Vector Laboratories, Inc. California, United Stated) were then added according to Table 2 into the respective FACS tube and incubated (30 min/ RT/ dark). Afterwards all samples were washed with 2 mL 1x PBS and centrifuged (650 x g/7 min/ without brake). Again, the supernatant is discarded, and the remaining cell pellet is dissolved in 500 µL 1x PBS. The samples are then analyzed by flow cytometry (Cyto Flex, Beckman Coulter GmbH, Krefeld, Germany) and fold increases regarding Lectin binding between the healthy (blood group AB) and ITP sera are calculated.

**Table 2: Pipetting Scheme for Lectin Staining.**

| Eppi | Constitution | FACS tubes | FACS-List |
|---|---|---|---|
| 1 | 25 µl wPLTs + 25 µl PBS | 1 | Unstained |
| 2 | 25 µl wPLTs + 25 µl PBS | 2 | 1 µl Isotype Control |
| 3 | 25 µl wPLTs + 25 µl PBS | 3 | 1 µl CD41-APC |
| 4 | 25 µl wPLTs + 25 µl PBS | 4 | 1 µl IgG1-APC |
| 5 | 25 µl wPLTs + 25 µl PBS | 5 | 40 µl ECL |
| | | 6 | 40 µl RCA |
| 6 | 25 µl wPLTs + 25 µl PBS + **NEU** 1 µl | 7 | 40 µl ECL |
| | | 8 | 40 µl RCA |
| 7 | 25 µl wPLTs + 25 µl **AB-Serum** | 9 | 40 µl ECL |
| | | 10 | 40 µl RCA |
| 8 | 25 µl wPLTs + 25 µl *Patient 1* | 11 | 40 µl ECL |
| | | 12 | 40 µl RCA |
| 9 | 25 µl wPLTs + 25 µl *Patient 2* | 13 | 40 µl ECL |
| | | 14 | 40 µl RCA |
| 10 | 25µl wPLTs + 25µl *Patient 3* | 15 | 40 µl ECL |
| | | 16 | 40 µl RCA |

*Abbreviations: wPLTs, washed platelets, NEU, neuraminidase; ECL, Erythrina Cristagalli Lectin; RCA, Ricinus Communis Agglutinin; PBS, phosphate-buffered saline; Eppi, Eppendorf tube*

## 3.6 Data Analysis Tools and Statistics

For evaluation of demographic and laboratory patient data the Statistical Package for Social Sciences (SPSS version 27.0, SPSS, Chicago, Illinois, USA) was used. Statistical analysis in the Thrombin generation assay was executed with SPSS, p-values and correlation coefficients were calculated. In the clot formation and lysis assay STATA IC17 (StataCorp LP, College Station, TX, USA) was used to evaluate the individual experimental parameters. Further statistical analysis was performed with SPSS. In the Mackman assay, Microsoft Excel (Version 2016) was used to convert optical densities into absolute values of EV-TF activity. Significance and correlation coefficients were determined with SPSS.

Descriptive statistics were performed to evaluate and describe data. Normally distributed data is depicted as mean ± standard deviation (SD), significant differences between the three cohorts were analyzed using unpaired t-tests. Non-normally distributed data is shown as median, $25^{th}$ and $75^{th}$ quartile to provide a good overview of the data. Differences between the groups of non-normally distributed data were calculated via the Mann-Whitney U test. For

categorical variables (non-numerical) the Pearson's Chi-Square test was executed to show whether the association between two groups arose by chance.

In TGA, CLA and Mackman assay associations between the experimental parameters and test groups were investigated via mixed-effect linear regression models. In this test a 95 % confidence interval was applied, values outside this interval were defined as abnormal. A more complex parameter calculated was the Spearman correlation coefficient. Spearman's rank correlation coefficient is a nonparametric test to evaluate the strength and direction of the monotonic relationship between two ranked variables ('Spearman's Rank-Order Correlation').

The significance level (alpha) applied to all statistical tests was 0.05. P-values below 0.05 indicate a significant difference between the two test cohorts.

# 4 Results

The following chapter shows the experimental results of my Master Thesis.

## 4.1 Patients

Eighty-eight patients with primary ITP, 19 thrombocytopenic controls (TC) and 159 healthy controls (HC) were investigated. Clinical and laboratory characteristics are shown in Table 3. Fifty-seven of the ITP patients (64.8 %), ten out of 19 TC (52.6 %), and 107 of the HC (67.3 %) were women. ITP patients were younger than the group of TC, whereas there was no difference in comparison to the group of HC. The body mass index (BMI) was significantly lower in HC in comparison to ITP patients without any difference between TC and ITP patients.

The bleeding score was equal between ITP patients and TC. Nine (10.9 %) ITP patients and three (16.7 %) TC patients previously suffered from arterial or venous thrombosis.

Platelet and leukocyte counts did not differ between ITP patients and TC. There were significant differences in Hemoglobin and Fibrinogen levels between ITP patients and TC but not in comparison to HC. There was a tendency towards a shorter aPTT and Prothrombin time between ITP patients and HC. In comparison to TC, ITP patients had a similar aPPT and significantly shorter Prothrombin time. The mean platelet volume (MPV) was highest in the ITP patient group and significantly different to the MPV of TC or HC. Thrombopoietin levels, which were not available from HC, were significantly lower in ITP patients than in TC (Table 3).

In total 16 patients (18.6 %) had newly diagnosed ITP, nine patients (10.5 %) had persistent ITP, and 61 (70.9 %) had chronic ITP. The median (IQR) disease duration was 61 (9-130) months. Data on the disease duration is missing from two patients. Nearly half of the recruited patients (47.6 %) were currently under treatment for ITP and 14 (16.5 %) of 85 patients underwent splenectomy (Table 4). Antibody characterization showed that out of 50 patients of whom data was available, 21 (42 %) had antibodies against GP IIb/IIIa, whereas only 6 (12 %) expressed antibodies against GPIb/IX and 23 (46 %) had no antibodies.

**Table 3: Clinical and Laboratory Data of Patients with Primary ITP (n=88) Compared to Thrombocytopenic Controls (TC) (n=19) and Healthy Controls (n=159)**

| | Primary ITP | | TC | | HC | | Primary ITP vs TC | Primary ITP vs HC |
|---|---|---|---|---|---|---|---|---|
| ***Clinical Data*** | | | | | | | | |
| | **n** | **mean (%)** | **n** | **mean (%)** | **n** | **mean (%)** | **p** | **p** |
| Age, years, mean ± SD | 88 | 43.9 ± 17.0 | 19 | 51.7 ± 14.7 | 159 | 44.4 ± 13.8 | 0.042 | 0.497 |
| Female | 88 | 57 (64.8) | 19 | 10 (52.6) | 159 | 107 (67.3) | 0.321 | 0.688 |
| Previous thrombosis | 84 | 9 (10.7) | 18 | 3 (16.7) | 159 | 0 (0) | 0.106 | 0.108 |
| Blood type 0 | 82 | 29 (35.4) | 9 | 1 (11.1) | 159 | 33 (35.1) | 0.142 | 0.971 |
| Smoking | 81 | 20 (24.7) | na | - | 159 | 3 (1.9) | - | 0.066 |
| | **n** | **median (IQR)** | **n** | **median (IQR)** | **n** | **median (IQR)** | **p-value** | **p-value** |
| BMI, kg/m$^2$ | 84 | 25.4 (22.9-29.7) | 18 | 27.3 (22.8-29.9) | 159 | 23.4 (21.1-25.7) | 0.997 | <0.001 |
| Bleeding score | 88 | 1 (0-3) | 18 | 2 (1-4) | 105 | 0 (0-0) | 0.198 | <0.001 |
| Skin bleeding | 86 | 1 (0.2) | 18 | 1.5 (0-2.25) | na | - | 0.864 | - |
| Mucosal bleeding | 86 | 0 (0-1) | 18 | 0 (0-2) | na | - | 0.668 | - |
| Organ bleeding | 86 | 0 (0-0) | 18 | 0 (0-1) | na | - | 0.416 | - |
| ***Laboratory Data*** | | | | | | | | |
| | **n** | **median (IQR)** | **n** | **median (IQR)** | **n** | **median (IQR)** | **p-value** | **p-value** |
| Platelet count, x10$^9$/l | 86 | 58 (30-119) | 19 | 59 (18-71) | 159 | 252 (217-293) | 0.322 | <0.001 |
| Leukocyte count, x10$^9$/l | 86 | 6.8 (5.5-9.3) | 19 | 3 (0.9-17.3) | 122 | 5.7 (5-6.3) | 0.084 | <0.001 |
| Hemoglobin, g/dl | 86 | 13.8 (12.7-14.6) | 18 | 9.2 (8.5-10.8) | 159 | 14.0 (13.1-14.9) | <0.001 | 0.190 |
| Fibrinogen, mg/dl | 85 | 306.0 (272.5-359.0) | 19 | 427 (315-488) | 159 | 297 (258-331) | <0.001 | 0.086 |
| aPTT, s | 74 | 33.2 (30.7-37.7) | 18 | 33.2 (30.4-35.8) | 159 | 34.7 (32.9-36.9) | 0.155 | 0.052 |
| Prothrombin time, % | 69 | 98 (90-105) | 14 | 93.0 (82.5-97.3) | 159 | 100 (93-111) | 0.044 | 0.049 |
| MPV, fl | 58 | 11.5 (10.5-12.7) | 15 | 9.9 (9.7-10.9) | 159 | 10.4 (9.8-11.0) | 0.002 | <0.001 |
| RPF, % | 67 | 12.0 (5.8-17.2) | 14 | 8.6 (4.5-13.5) | na | - | 0.118 | - |
| Thrombopoietin, pg/ml | 74 | 49.0 (49.0-65.5) | 14 | 633.5 (118-1567.5) | na | - | <0.001 | - |

*Abbreviations: IQR, interquartile range [25th-75th percentile]; BMI, Body mass index; aPTT, activated partial thromboplastin time; MPV, mean platelet volume; fl, femtoliter; RPF, reticulated platelet fraction; TC, thrombocytopenic controls; HC, healthy controls*
*Bleeding score was assed according to the ISTH SMOG Index*

***Table 4: ITP Cohort Specific Data (n=88)***

| **Primary ITP** | | |
|---|---|---|
| | **n** | **median (IQR)** |
| Disease duration, months | 86 | 61 (9-130) |
| | **n** | **mean (%)** |
| acute ITP | 86 | 16 (18.6) |
| persistent ITP | 86 | 9 (10.5) |
| chronic ITP | 86 | 61 (70.9) |
| Current ITP treatment | 88 | 39 (44.3) |
| Splenectomy | 85 | 14 (16.5) |

*Note: Acute ITP, 0-3 months; persistent ITP, 3-12 months; chronic ITP, >12 months*

## 4.2 Thrombin Generation Assay (TGA)

In the TGA after adjustment for sex, age, BMI and Fibrinogen levels, Thrombin generation was delayed in ITP patients in comparison to TC, as shown by a prolonged lag time (median (IQR) 11.6 (9.1-14.6) min and 10.1 (8.6-10.6) min, p = 0.015) and TTP (18.6 (15.5-22.1) min and 16.6 (14.6-17.1) min, p = 0.007), whereas there was no difference to HC (Table 5). Thrombin generation was significantly lower in patients with primary ITP than in HC (223.3 (159.7-346.0) nmol/l and 286.9 (179.1-396.5) nmol/l, p = 0.045), without reaching statistical significance in comparison to TC (243.5 (183.4-312.7) nmol/l, p = 0.529). No significant differences in the VI or AUC were detected within the three groups, although levels were lowest in ITP patients.

***Table 5: Thrombin Generation Assay Parameters for Primary ITP Patients (n=88) Compared to Thrombocytopenic (n=19) and Healthy Controls (n=159)***

| | **Primary ITP** | **TC** | **HC** | **Primary ITP vs TC** | **Primary ITP vs HC** |
|---|---|---|---|---|---|
| | **median (IQR)** | **median (IQR)** | **median (IQR)** | **p*** | **p*** |
| Lag time, min | 11.6 (9.1-14.6) | 10.1 (8.6-10.6) | 10.6 (8.6-14.1) | 0.015 | 0.535 |
| VI, nmol/l/min | 32.9 (19.6-58.4) | 37.7 (29.2-63.6) | 41.0 (19.7-75.7) | 0.258 | 0.082 |
| Peak Thrombin, nmol/l | 223.3 (159.7-346.0) | 243.5 (183.4-312.7) | 286.9 (179.1-396.5) | 0.529 | 0.045 |
| TTP, min | 18.6 (15.5-22.1) | 16.6 (14.6-17.1) | 18.1 (14.1-23.6) | 0.007 | 0.860 |
| AUC, nmol/l x min | 3539.81 (2916.9-4595.3) | 4125.3 (3497.8-5007.9) | 3834.3 (3292.4-4239.2) | 0.179 | 0.662 |

*Abbreviations: IQR, interquartile range [25th 75th percentile]; VI, velocity index; TTP, time-to-peak Thrombin generation; AUC, area under the curve; TC, thrombocytopenic controls; HC, healthy controls*
**p-value: adjusted for sex, age, BMI and Fibrinogen levels by multiple linear regression analysis.*

The Spearman's rank correlation coefficient (roh, ρ) was calculated to identify possible associations of clinical and laboratory characteristics with TGA parameters. The correlation tables for each of the three groups are located in the Annex (Tables S5-S8). No strong correlation was found between the clinical and laboratory characteristics with TGA parameters in the ITP patient group. Age weakly correlated with the VI (ρ = 0.262, p = 0.014), peak Thrombin levels (ρ = 0.248, p = 0.007) and AUC (ρ = 0.305, p = 0.004), and the BMI correlated weakly with the lag time (ρ = 0.223, p = 0.044). The bleeding score was found to weakly correlate with TTP (ρ = 0.220, p = 0.041). In specific, mucosal bleeding weakly correlated to VI (ρ = 0.214, p = 0.048) and TTP (ρ = 0.218, p = 0.044) (Table S5). Leucocyte counts showed weak correlation with the lag time (ρ = 0.214, p = 0.048), VI (ρ = 0.217, p = 0.044) and peak Thrombin generation (ρ = 0.232, p = 0.032), and Hemoglobin with TTP (ρ = 0.236, p = 0.030). The aPTT showed weak and moderate correlation with all of the TGA parameters, except for the lag time. Platelet numbers or other laboratory characteristics like TPO levels showed no influence on any of the assessed TGA parameters (Table S6).

Within the TC, no correlation was found between clinical factors and parameters of TGA (Table S7). Moderate correlation between the bleeding score and the lag time (ρ = 0.533, p = 0.017) as well as TTP (ρ = 0.471, p = 0.049) was detected. Skin bleeding showed moderate to strong correlation to VI (ρ = 0.554, p = 0.017), peak Thrombin levels (ρ = 0.646, p = 0.004) and AUC (ρ = 0.547, p = 0.019), whereas no correlation was found for mucosal or organ bleeding. Furthermore, leukocytes and Fibrinogen moderately correlated with the lag time (leukocytes: ρ = 0.503, p = 0.028, Fibrinogen: ρ = 0.537, p = 0.018) and the Prothrombin time had a moderate influence on VI (ρ = 0.700, p = 0.005), peak Thrombin levels (ρ = 0.604, p = 0.002) and AUC (ρ = 0.656, p = 0.011). (Table S7). In HC, female sex weakly correlated with all TGA parameters, while BMI impacted the lag time (ρ = 0.235, p = 0.003) and TTP (ρ = 0.209, p = 0.009) (Table S8). The platelet count and aPTT showed weak correlation with all parameters of Thrombin generation. Leukocytes were associated with peak Thrombin levels (ρ = 0.209, p = 0.021) and AUC (ρ = 0.207, p = 0.022). Hemoglobin had weak correlation with the lag time (ρ = 0.205, p = 0.009), Fibrinogen with TTP (ρ = 0.208, p = 0.009) and the Prothrombin time with AUC (ρ = 0.201, p = 0.011). However, Hemoglobin, Fibrinogen and Prothrombin time exhibited non-significant correlation with other Thrombin generation parameters (Table S8).

## 4.3 Fibrin clot formation and lysis assay (CLA)

Figure 8 depicts the typical clot formation and lysis curve, showing the change in absorbance in correlation to time. For this figure one representative dataset of each cohort was chosen (females in the age range of 23-25 years). The black curve mimics normal clot formation and lysis in a healthy individual (HC), for better understanding some important assay parameters are indicated. The blue dotted curve represents one primary ITP patient, and it is noteworthy that the curve is clearly shifted downwards in comparison to the HC curve. Notably for this thrombocytopenic patient (TC), depicted as dotted orange curve, is the extremely increased time to peak as well as the elevated clot formation. Looking at the graph, it is noticed that delta absorbance is reached in all three cohorts within approximately the first 20 min of reaction time.

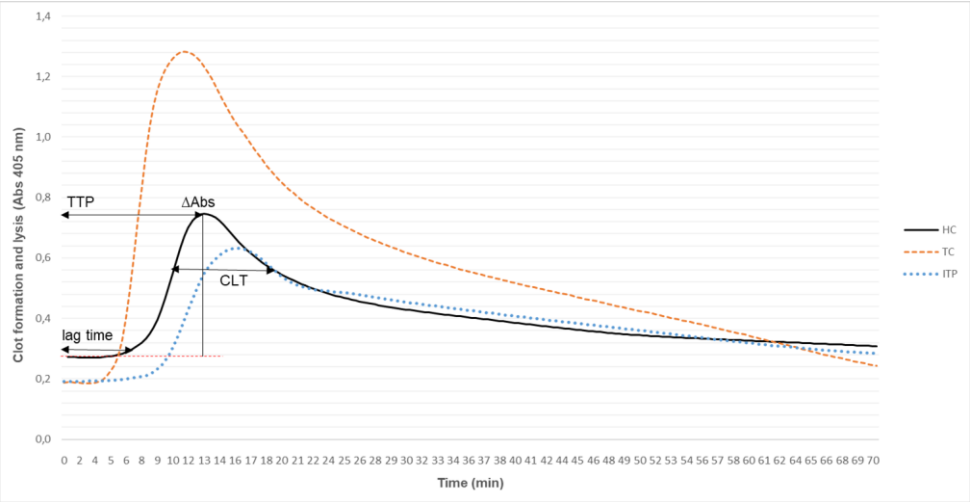



***Figure 8: Representative Fibrin Clot Formation and Lysis Curve*** *of one healthy control (female, 23 years), one primary ITP patient (female, 24 years) and one thrombocytopenic patient (female 25 years). TTP, time-to-peak Thrombin generation; ΔAbs, delta absorbance; Abs, absorbance; CLT, clot lysis time; HC, healthy control; TC, thrombocytopenic control, ITP, primary immune thrombocytopenia.*

Data on clot formation and lysis in ITP patients, TC and HC are shown in Table 6. In comparison to HC, primary ITP patients had a significantly prolonged lag time (median (IQR) 7.0 (5.2-8.7) min and 5.3 (4.7-6.7) min, p = 0.005) and TTP (15.2 (11.3-19.8) min and 11.0 (9.7-14.0) min, p = 0.004) which was equal to TC (lag time: 7.0 (4.7-8.3) min, p = 0.895; TTP: 13.0 (10.0-15.0) min, p = 0.937). The $V_{max}$ was lowest in ITP patients (0.10 (0.07-0.14) OD/min) with a significant difference to both, TC and HC (TC: 0.20 (0.12-0.26) OD/min, p = <0.001; HC: 0.14 (0.10-0.19) OD/min, p = <0.001). Clot

density (ΔAbs) was lower in ITP patients compared to TC (0.42 (0.34-0.54) OD and 0.59 (0.45-0.78) OD, p = 0.026), however no difference to HC was noted.

In clot lysis, clots were lysed slower in ITP patients in comparison to TC and HC with a significantly prolonged clot lysis time (CLT) and time until 50 % of lysis is achieved (t50% lysis), as well as a lower maximum lysis rate (mLR). ITP patients took more than double the time than TC to lyse the formed Fibrin clot (28.0 (17.3-40.3) min and 16.7 (11.0-26.0) min, p = <0.001), and more than three times longer to lyse 50 % of the clot than TC (23.0 (13.3-34.7) min and 7.0 (5.0-16.0) min, p = 0.015).

***Table 6: Clot Formation and Lysis Parameters for Primary ITP Patients (n=88) in Comparison to Thrombocytopenic (n= 19) and Healthy Controls (n= 157)***

| | Primary ITP | TC | HC | Primary ITP vs TC | Primary ITP vs HC |
|---|---|---|---|---|---|
| | median (IQR) | median (IQR) | median (IQR) | p* | p* |
| Baseline OD | 0.23 (0.2-0.3) | 0.19 (0.15-0.28) | 0.2 (0.17-0.26) | 0.301 | 0.452 |
| Lagphase, min | 7.0 (5.2-8.7) | 7.0 (4.7-8.3) | 5.3 (4.7-6.7) | 0.895 | 0.005 |
| $V_{max}$, OD/min | 0.10 (0.07-0.14) | 0.20 (0.12-0.26) | 0.14 (0.10-0.19) | <0.001 | <0.001 |
| ΔAbs, $OD_{405nm}$ | 0.42 (0.34-0.54) | 0.59 (0.45-0.78) | 0.41 (0.34-0.51) | 0.026 | 0.463 |
| TTP, min | 15.2 (11.3-19.8) | 13.0 (10.0-15.0) | 11.0 (9.7-14.0) | 0.937 | 0.004 |
| CLT, min | 28.0 (17.3-40.3) | 11.0 (7.7-19.0) | 16.7 (11.0-26.0) | 0.008 | <0.001 |
| t50% lysis, min | 23.0 (13.3-34.7) | 7.0 (5.0-16.0) | 13 (7.7-21.9) | 0.015 | <0.001 |
| mLR, OD/min | 0.009 (0.011-0.005) | 0.014 (0.033-0.01) | 0.012 (0.018-0.007) | <0.001 | <0.001 |

*Abbreviations: IQR, interquartile range [25th 75th percentile]; OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time to peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; TC, thrombocytopenic control cohort; HC, healthy controls*
**p-value: adjusted for sex, age, BMI and Fibrinogen levels by multiple linear regression analysis.*

Tables S9-S14 (see Annex) show correlations between the CLA parameters and clinical and laboratory data in primary ITP patients, TC and HC. Within the ITP cohort in clot formation, female sex moderately correlated to baseline OD values (ρ = 0.415, p = <0.001) and weakly to the peak absorbance (ρ = 0.259, p = 0.015), whereas smoking behavior was weakly associated with the lag phase (ρ = 0.245, p = 0.038). The platelet count weakly correlated with $V_{max}$ (ρ = 0.212, p = 0.050), Hemoglobin with baseline OD values (ρ = 0.241, p = 0.026). In clot lysis, weak correlation was found between age (ρ = 0.217, p = 0.044) and t50% lysis while BMI correlated to t50% lysis (ρ = 0.263, p-value = 0.018) and CLT (ρ = 0.275, p = 0.013). Fibrinogen correlated weakly with all parameters of clot lysis: CLT (ρ = 0.328, p = 0.002), t50%

lysis (ρ = 0.311, p = 0.004) and mLR (ρ = 0.228, p = 0.037). Furthermore, RPF correlated to TTP (ρ = 0.242, p = 0.048) and mLR (ρ = 0.253, p = 0.039).

In TC, out of the collected clinical data only skin bleeding correlated with TTP (ρ = 0.560, p = 0.016) (Annex Table S11). Fibrinogen levels showed moderate correlation with baseline OD values (ρ = 0.461, p = 0.047) and clot density (ρ = 0.525, p = 0.021) (Annex Table S12). Within the healthy individuals, age weakly correlated with the clot density (ρ = 0.274, p = <0.001) (Annex Table S13). Furthermore, females showed weak correlation with the baseline absorbance (ρ = 0.444, p = <0.001), lag phase (ρ = 0.267, p = <0.001) and peak absorbance (ρ = 0.240, p = 0.002). BMI also showed associations with several plasma clot formation parameters (baseline OD: ρ = 0.320, p = <0.001; $V_{max}$: ρ = 0.242, p = 0.002; peak absorbance: ρ = 0.229, p = 0.004; mLR: ρ = 0.261, p = 0.001). Regarding the laboratory parameters, weak correlation was found between the platelet count and baseline OD (ρ = 0.253, p = 0.001) and lag phase (ρ = 0.221, p = 0.005). Hemoglobin correlated with baseline OD (ρ = 0.303, p = <0.001). Fibrinogen levels showed a moderate correlation to $V_{max}$ (ρ = 0.351, p = <0.001), and a strong correlation to the peak absorbance (ρ = 0.664, p = 0.001) (Annex Table S14). In clot lysis, only BMI and smoking correlated to the mLR (smoking: ρ = 0.401, p = 0.042; BMI: ρ = 0.261, p = 0.001), whereas no other correlation between clinical or laboratory factors and parameters of clot lysis was found (Annex Table S13 and Table S14).

## 4.4 Microparticle Tissue Factor Activity (Mackman Assay)

After adjustment for sex, age, BMI and Fibrinogen, EV associated TF activity did not differ between ITP patients (median (IQR) 0.04 (0.0-0.15) pg/mL) and HC (0.05 (0.0-0.16) pg/mL, p = 0.274) (Table 7). However, significant differences in EV-TF activity were noted in primary ITP compared to TC (0.05 (0.0-16.0) pg/mL, p = 0.003). Still, according to Mackman and Hisada zero activity (0-<0.5 pg/mL) of EV-TF in PPP was detected across all three cohorts except in three ITP patients (Hisada and Mackman 2019a). Furthermore, the Spearman's rank correlation coefficient regarding the influence on EV-TF activity on clinical and laboratory data in primary ITP patient was calculated (Annex Table S15). Only age (ρ = 0.234, p = 0.028) showed weak correlation with EV TF-activity. Within the thrombocytopenic patient cohort several moderate to strong correlations were detected: age: ρ = 0.533, p = 0.019; female sex: ρ = 0.558, p = 0.013; leukocytes: ρ = 0.578, p = 0.010; Prothrombin time: ρ = 0.702,

p = 0.005; thrombopoietin: ρ = 0.536, p = 0.036, whereas no correlation at all appeared in the healthy cohort (Annex Table S15).

***Table 7: Extracellular Vesicle Associated Tissue Factor Activity in Primary ITP Patients (n=88) in Comparison to Thrombocytopenic (n= 19) and Healthy Controls (n= 106)***

| | Primary ITP | TC | HC | Primary ITP vs TC | Primary ITP vs HC |
|---|---|---|---|---|---|
| | median (IQR) | median (IQR) | median (IQR) | p* | p* |
| EV-TF activity, pg/mL | 0.04 (0.0-0.15) | 0.05 (0.0-0.16) | 0.05 (0.0-0.16) | 0.003 | 0.274 |

*Abbreviations: IQR, interquartile range [25th 75th percentile]; EV, extracellular vesicle; TF, Tissue factor; TC, thrombocytopenic control cohort; HC, healthy controls*
**p-value: adjusted for sex, age, BMI and Fibrinogen levels by multiple linear regression analysis.*

## 4.5 ITP Cohort Specific Data

In the cohort of ITP patients, 16 (18.6 %) patients had acute, nine (10.5 %) persistent and 61 (70.9 %) chronic ITP (Table 8). Patients with persistent ITP were on average older (mean: 64 years) than acute ITP (mean: 44.2 years) and significantly older than chronic ITP patients (mean: 39 years). In the distribution of sex between acute, persistent, or chronic ITP no significant difference was detected. A history of thrombosis was recorded in one patient with acute ITP (7.1 %), none with persistent ITP and and three out of 51 patients with chronic ITP (5.9 %). In acute ITP significantly more patients had blood type 0 compared to the chronic form (56.3% vs 28.6%). The bleeding severity was, significantly higher in acute than in persistent ITP, whereas there was no difference to chronic ITP patients. Additionally, the skin bleeding score was notably elevated in acute ITP compared to the two other groups (median, (IQR); acute ITP: 1.5 (1-5.75), persistent ITP: 0 (0-1), chronic ITP: 1 (0-2)). Platelet counts were non-significantly lower in acute ITP patients (median (IQR); 30.5 (8.0-122.5) $x10^9$/L) in comparison to persistent ITP (89 (43-126) $x10^9$/l; p = 0.079) and chronic ITP (61 (32-120) x10^$^9$/l, p = 0.122) (Table 8). In regard to the prevalence of antibodies against GPIIb/IIIa and GPIb/IX no significant differences were found across the three ITP cohorts (Table 8).

Thirty-nine (44.3 %) patients received ITP specific treatment at time of study inclusion and 14 (16.5 %) patients were splenectomized (Table 4). There was no difference in the platelet counts or other laboratory data investigated between patients with or without ITP specific treatment (Annex Table S16) or patients with or without prior splenectomy (Annex Table S17).

Across acute ITP, persistent ITP and chronic ITP no significant differences within Thrombin generation, plasma clot formation and EV associated TF activity were detected (Table 9). Patients with and without treatment show a significant difference in EV-TF activity (median (IQR); treated: 0.06 (0.0-0.26) and untreated: 0.02 (0.0-0.11)), however no differences were found within TGA and CLA parameters (Table 10). Between splenectomized and not splenectomized patients the lag phase in CLA (median (IQR); splenectomized: 8.7 (7.2-11.3) min and not splenectomized: 6.7 (5.0-8.3) min, $p = 0.007$) and also TTP (splenectomized: 19.5 (14.1-24.7) min and not splenectomized: 14.5 (11.3-18.0) min, $p = 0.046$) was significantly prolonged in splenectomized patients (Annex Table S18). Furthermore, no significant difference in patients with antibodies against GPIIb/IIIa in comparison to patients with antibodies against GPIb/IX was detected (Table S19).

***Table 8: Clinical and Laboratory Data Associated with Cohort Specific Parameters of Patients with Primary ITP (n=88)***

| | acute ITP | | persistent ITP | | chronic ITP | | acute vs persistent ITP | acute vs chronic ITP | persistent vs chronic ITP |
|---|---|---|---|---|---|---|---|---|---|
| ***Clinical Data*** | | | | | | | | | |
| | **n** | **mean (%)** | **n** | **mean (%)** | **n** | **mean (%)** | **p** | **p** | **p** |
| Age, years, mean ± SD | 16 | 44.2 ± 18.7 | 9 | 64.0 ± 44-74.5 | 61 | 39.0 ± 28.5-51.5 | 0.061 | 0.656 | 0.008 |
| Female | 16 | 9 (56.3) | 9 | 8 (88.9) | 61 | 39(63.9) | 0.093 | 0.572 | 0.137 |
| Previous thrombosis | 14 | 1 (7.1) | 8 | 0 (0) | 51 | 3 (5.9) | 0.439 | 0.862 | 0.481 |
| Blood type 0 | 16 | 9 (56.3) | 9 | 4 (44.4) | 56 | 16 (28.6) | 0.571 | 0.040 | 0.338 |
| Smoking | 13 | 11 (84.6) | 6 | 5 (83.3) | 47 | 31 (66.0) | 0.943 | 0.194 | 0.391 |
| | **n** | **median (IQR)** | **n** | **median (IQR)** | **n** | **median (IQR)** | **p** | **p** | **p** |
| BMI, $kg/m^2$ | 14 | 26.0 (23-34) | 8 | 29.6 (24.7-34.6) | 58 | 24.8 (22.6-29.3) | 0.706 | 0.451 | 0.099 |
| Bleeding score | 16 | 1.5 (1-8.75) | 9 | 0 (0-1.5) | 60 | 1 (0-3) | 0.042 | 0.168 | 0.189 |
| Skin bleeding | 16 | 1.5 (1-5.75) | 9 | 0 (0-1) | 59 | 1 (0-2) | 0.030 | 0.021 | 0.427 |
| Mucosal bleeding | 16 | 0 (0-3) | 9 | 0 (0-0) | 59 | 0 (0-1) | 0.063 | 0.411 | 0.077 |
| Organ bleeding | 16 | 0 (0-0) | 9 | 0 (0-0) | 59 | 0 (0-0) | 0.690 | 0.718 | 0.497 |
| ***Laboratory Data*** | | | | | | | | | |
| | **n** | **median (IQR)** | **n** | **median (IQR)** | **n** | **median (IQR)** | **p** | **p** | **p** |
| Platelet count, $x10^9/l$ | 16 | 30.5 (8-122.5) | 9 | 89.0 (43-126) | 59 | 61.0 (32-120) | 0.079 | 0.122 | 0.351 |
| | **n** | **mean (%)** | **n** | **mean (%)** | **n** | **mean (%)** | **p** | **p** | **p** |
| Antibodies against GPIIb/IIIa | 9 | 6 (66.7) | 7 | 4 (57.1) | 34 | 11 (32.4) | 0.696 | 0.061 | 0.215 |
| Antibodies against GPIb/IX | 9 | 2 (22.2) | 7 | 0 (0) | 34 | 4 (11.8) | 0.182 | 0.421 | 0.399 |

*Abbreviations: IQR, interquartile range [25th-75th percentile]; BMI, Body mass index; TC, thrombocytopenic controls; HC, healthy controls; Bleeding score was assed according to ISTH SMOG Index*

***Table 9: TGA, CLA and Mackman Assay Results of Primary ITP Cohort (n=88) Specific Parameters***

| | acute ITP | persistent ITP | chronic ITP | Acute ITP vs persistent ITP | Acute ITP vs chronic ITP | Persistent ITP vs chronic ITP |
|---|---|---|---|---|---|---|
| | **median (IQR)** | **median (IQR)** | **median (IQR)** | **p*** | **p*** | **p*** |
| **Thrombin Generation Assay** | | | | | | |
| Lag time, min | 11.2 (9.2-13.1) | 9.1 (7.8-13.6) | 11.6 (9.1-15.6) | 0.670 | 0.249 | 0.568 |
| VI, nmol/l/min | 43.3 (23.9-70.1) | 27.7 (14.4-63.1) | 33 (19.7-54.7) | 0.756 | 0.097 | 0.765 |
| Peak Thrombin, nmol/l | 245 (170.8-457.5) | 201.5 (122.3-293.3) | 234.4 (156-333.2) | 0.595 | 0.093 | 0.935 |
| TTP, min | 17.6 (15-20.7) | 15.1 (12.1-22.1) | 19.6 (16.1-22.8) | 0.947 | 0.156 | 0.280 |
| AUC, nmol/l x min | 3830.8 (2971.9-5449.3) | 3278.4 (1758-4718.3) | 3577.1 (2960.1-4477.8) | 0.298 | 0.286 | 0.495 |
| **Clot Formation and Lysis Assay** | | | | | | |
| Baseline OD | 0.32 (0.21-0.42) | 0.30 (0.20-0.40) | 0.22 (0.18-0.30) | 0.493 | 0.433 | 0.922 |
| Lagphase, min | 7.5 (5.4-9.0) | 6.7 (4.9-8.4) | 7.1 (5.2-8.7) | 0.931 | 0.333 | 0.690 |
| $V_{max}$, OD/min | 0.09 (0.07-0.11) | 0.11 (0.08-0.18) | 0.10 (0.07-0.15) | 0.842 | 0.838 | 0.333 |
| ΔAbs, $OD_{405nm}$ | 0.4 (0.3-0.5) | 0.6 (0.4-0.7) | 0.43 (0.35-0.54) | 0.868 | 0.388 | 0.399 |
| TTP, min | 15.8 (12.4-22.7) | 14.3 (9.8-20.4) | 15.3 (11.3-19.5) | 0.631 | 0.222 | 0.399 |
| t50% lysis, min | 20.8 (12.1-57.1) | 26 (10.2-41) | 23.7 (13.8-34.3) | 0.401 | 0.317 | 0.734 |
| CLT, min | 25.5 (17.1-62.7) | 30.3 (13.7-45.8) | 28.5 (18.3-40.3) | 0.376 | 0.323 | 0.765 |
| mLR | 0.008 (0.011-0.004) | 0.011 (0.018-0.007) | 0.009 (0.112-0.005) | 0.714 | 0.442 | 0.191 |
| **Mackman Assay** | | | | | | |
| EV-TF activity, pg/mL | 0.03 (0-0.14) | 0.02 (0-0.18) | 0.05 (0-0.16) | 0.253 | 0.800 | 0.159 |

*Abbreviations: IQR, interquartile range [25th 75th percentile]; VI, velocity index; TTP, time-to-peak Thrombin generation; AUC, area under the curve; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; CLT, clot lysis time; t50% lysis, time until 50% of lysis is achieved; mLR, maximum of clot lysis rate; EV, extracellular vesicle; TF, Tissue factor*
**p-value: adjusted for sex, age, BMI and Fibrinogen levels by multiple linear regression analysis.*

***Table 10: Results of TGA, CLA and Mackman Assay for Treated (n = 39) and Untreated (n = 49) Primary ITP Patients***

| | Treated primary ITP patients | Untreated primary ITP patients | Treated vs Untreated ITP Patients |
|---|---|---|---|
| | **median (IQR)** | **median (IQR)** | **p*** |
| **Thrombin Generation Assay** | | | |
| Lag time, min | 12.1 (9.1-14.1) | 11.6 (9.1-14.8) | 0.617 |
| VI, nmol/l/min | 36.5 (26.1-69.1) | 26.8 (15.4-53.7) | 0.138 |
| Peak Thrombin, nmol/l | 267.5 (201.5-385.0) | 195.7 (130.2-312.3) | 0.052 |
| TTP, min | 18.6 (15.1-22.1) | 18.6 (15.8-23.6) | 0.384 |
| AUC, nmol/l x min | 4194.2 (3128.9-4716.4) | 3357.1 (2665.1-4396.6) | 0.076 |
| **Clot Formation and Lysis Assay** | | | |
| Baseline OD | 0.27 (0.22-0.39) | 0.2 (0.18-0.3) | 0.176 |
| Lag phase, min | 7.3 (5.7-9.0) | 6.0 (4.9-8.3) | 0.296 |
| $V_{max}$, OD/min | 0.09 (0.06-0.16) | 0.102 (0.075-0.140) | 0.823 |
| ΔAbs, $OD_{405nm}$ | 0.41 (0.35-0.49) | 0.44 (0.33-0.55) | 0.683 |
| TTP, min | 17.0 (11.7-20.7) | 14.7 (11.3-18.4) | 0.651 |
| t50% lysis, min | 22.0 (12.6-34,0) | 23.2 (13.8-38.3) | 0.116 |
| CLT, min | 26.3 (17.0-39.3) | 28.0 (17.6-47.0) | 0.140 |
| mLR, OD/min | 0.009 (0.012-0.004) | 0.009 (0.011-0.005) | 0.333 |
| **Mackman Assay** | | | |
| EV TF-activity, pg/mL | 0.06 (0.0-0.26) | 0.02 (0.0-0.11) | 0.026 |

*Abbreviations: VI, velocity index; OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time-to-peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; EV, extracellular vesicles; TF, Tissue factor*
**p-value: adjusted for sex, age, BMI and Fibrinogen levels by multiple linear regression analysis*

## 4.6 Lectin binding assay (LBA)

Thirty-two primary ITP patient sera (age: mean ± SD, 39.8 ± 16.5 years; female: 22/32, 68.8 %) and 20 healthy control sera (age: 41.6 ± 12.3 years; female: 15/20, 75 %) were incubated with washed platelets from two healthy donors. Out of the 32 ITP patient sera, 18 had antibody characteristics on the platelets and/or in the serum. Three (16.7 %) had antibodies against GPIIb/IIIa + Ib/IX, eight (44.4 %) against GPIIb/IIIa, in 7/18 (38.9 %) no autoantibodies were detected. Within this experiment the cleavage of sialic acid on the washed platelets was investigated via flow cytometry. Therefore, the fold increase (FI) of patient sera in comparison to control sera (AB sera) was calculated. If the resulting FI was bigger than the respective cutoff of ECL or RCA, the patient was identified to have autoantibody-mediated desialylation. ECL and RCA cutoffs were calculated by testing 20 healthy controls, the FI was

determined by comparing each healthy control to AB sera. Afterwards the mean fold increase was calculated, and 2 x SD were added, which resulted in a cutoff value of 1.43 for ECL and 1.10 for RCA.

Out of the 32 investigated ITP sera, two (6.3 %) patients had MFI values above the ECL or RCA cutoff and showed desialylation. Of these two patients, one had no autoantibodies and in the other one the autoantibody status was not known. In the investigated cohort, there was no sign of increased platelet desialylation in patients with those without platelet-specific antibodies. Figure 9 shows a graphical distribution of platelet desialylation ability of autoantibodies from ITP patients, regarding glycoprotein specificity. Patients with unknown autoantibody status are not depicted.

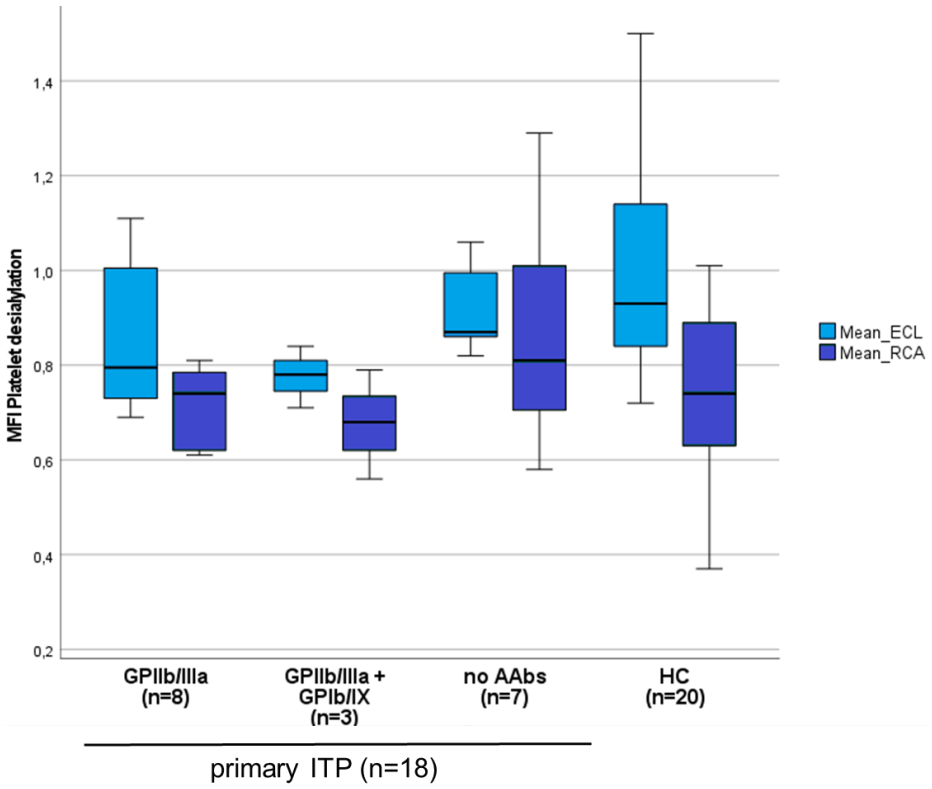



***Figure 9: Platelet Desialylation Ability.*** *Primary ITP sera and healthy control (HC) sera were incubated with washed platelets from two different healthy donors. The fold increase in comparison to AB serum was calculated and the mean value between the two donors was built. The light blue box plots represent the mean fold increase upon ECL binding and the dark blue box plots represent the mean fold increase upon RCA binding. Each box plot marks the median, Q1 and Q2 as well as the whiskers. ECL, Erythrina Cristagalli Lectin; RCA, Ricinus Communis Agglutinin; MFI, mean fold increase*

# 5 Discussion

Primary immune thrombocytopenia is an orphan disease in which autoantibodies lead to the destruction of blood platelets. Typical for patients suffering from this condition are platelet counts <100 x$10^9$/l. Clinically, ITP patients exhibit an increased bleeding tendency, which might vary individually and in part does not correlate with platelet counts. Also, an increased risk for thrombosis has been observed in ITP patients.

In this study, 88 primary ITP patients were investigated regarding their Thrombin generation behavior, Fibrin clot formation and lysis ability as well as microparticle Tissue factor associated activity. Thrombin, Fibrin, and Tissue factor are all important players of the coagulation cascade. Confirming altered behavior in primary ITP patients regarding production, stability or life span and destruction of these three components can give clues on the clinical presentation of ITP and might point out new biomarkers for the risk stratification of ITP patients. To strengthen the findings made in this study, non-immunological thrombocytopenia patients and a large group of age- and sex-matched healthy controls were investigated for the same parameters for comparison.

Here, we observed that Thrombin generation was slightly delayed in ITP patients compared to non-immunological thrombocytopenic patients and lower than in healthy individuals. An impaired Thrombin generation theoretically leads to impaired Fibrin production and subsequently less/less stable cross-linked Fibrin clots. This might indicate a reduced hemostatic potential, which possibly contributes to the elevated bleeding tendency in primary ITP. This is underlined by our results, showing an association of parameters of Thrombin generation with increased bleeding severity, especially in the mucosa, in ITP patients. Our data on delayed Thrombin generation are in line with results from a study by Kjalke et al., who investigated treatment with high-dose FVIIa in a thrombocytopenia-like-cell based model and found an increase of Thrombin generation and promotion of platelet activation after treatment (Kjalke et al. 2001). Contrary to our data, Alvarez-Roman et al. measured Thrombin generation in PPP by calibrated automated thrombogram (CAT) in a study population of 42 patients with chronic ITP and 35 healthy subjects (Alvarez-Roman et al. 2016). They found significantly increased peak Thrombin levels in ITP compared to healthy controls and observed an elevated endogenous Thrombin potential, which would represent the AUC parameter in our TGA. We however did not detect differences in AUC between ITP and healthy controls, or between ITP

and the thrombocytopenic control group. The author suggested that ITP patients have a procoagulant profile, which was not confirmed by our measurements of Thrombin generation or plasma clot formation. In contrast to their findings, we also did not detect abnormal Fibrinogen levels in ITP patients compared to healthy controls. A possible reason why our findings differ to the ones made by the researchers around Alvarez-Roman could be due to the study population size, which was tremendously smaller than ours. Another reason might be due to the fact that two different methods were used. While CAT is based on chromogenic Thrombin substrates which only loosely bind to Thrombin, the TGA kit used by us, monitors Thrombin generation via binding of fluorogenic substrate. Alvarez-Roman and colleagues further tested the impact of platelet poor ITP plasma on the procoagulant capacity of washed platelets from healthy donors and showed a prolongation of the lag time in Thrombin generation in ITP patients compared to control patients. This observation is similar to our data, as it shows delayed Thrombin generation of ITP patients in comparison to non-immunological thrombocytopenic patients and delayed plasma clot formation of ITP patients in comparison to healthy individuals (Alvarez-Roman et al. 2016).

We found that plasma clot formation was delayed in ITP patients in comparison to healthy controls and also impaired and reduced in comparison to thrombocytopenic patients. Larsen and colleagues investigated the effects of recombinant FVIIa and Fibrinogen on clot formation in whole blood measured by rotational thromboelastometry (ROTEM®) in primary ITP patients with low platelet counts (Larsen et al. 2013). Among other things they found that ITP patients had delayed, and impaired clot formation compared to healthy controls prior to Fibrinogen and recombinant FVIIa addition. While only addition of Fibrinogen did not influence clot formation, addition of rFVIIa only and in combination with Fibrinogen improved clotting parameters. Even though the experimental set up was not identical to ours, their findings in whole blood clot formation support our results generated with PPP. Alvarez-Roman et al. used ROTEM® to test the hemostatic potential in chronic ITP patients and found that they exhibited a procoagulant profile in comparison to healthy controls (Alvarez-Roman et al. 2016). This phenomenon was observed due to elevated levels of platelet- and cell-microparticles, enhanced resistance to protein C as well as a firmer clot formation and resistance to fibrinolysis. The researchers correlated this behavior to elevated levels of PAI-1. Furthermore, they suggested that the presence of anti-platelet antibodies in ITP delayed clotting time and lag time in Thrombin generation (Alvarez-Roman et al. 2016). We, however, did not find any differences in ITP patients expressing antibodies against GPIIb/IIIa and GPIb/IX regarding their Thrombin

generation behavior as well as clot formation and lysis ability. Therefore, we cannot confirm this hypothesis. Nevertheless, to the best of our knowledge, clot formation was rarely investigated in ITP patients and thus, more data for comparison are not available, but would be needed to better conclude.

The most significant finding in our CLA measurements was a significantly impaired clot lysis in ITP patients in comparison to both, TC and HC. This on the one hand could counteract the increased bleeding tendency and on the other hand result in a prothrombotic state. Justo Sanz and colleagues investigated the procoagulant effect of TPO-RAs in ITP patients, as patients receiving this therapy have increased risk of thrombosis (Justo Sanz et al. 2018). They analyzed PRP of 40 treated patients with chronic primary ITP, 42 patients with successful TPO-RA therapy and 112 healthy controls. Patients with TPO-RA treatment showed prolonged fibrinolysis in comparison to the two control cohorts, indicating a procoagulant profile. This behavior was linked to elevated PAI-1 activity (Justo Sanz et al. 2018). Our finding of delayed plasma clot lysis in the analyzed ITP cohort are mostly supported by the results of Justo Sanz et al. in TPO-RA treated chronic primary ITP patients and by Alvarez-Roman et al. in chronic ITP patients (Justo Sanz et al. 2018, Alvarez-Roman et al. 2016). As described in Table 9, we did not identify differences in clot lysis between acute, persistent, and chronic ITP patients. Within the scope of this thesis, we also did not look closer into patients who received TPO-RA therapy, but the analysis of treated versus untreated primary ITP patients did not identify significant differences in Fibrin clot lysis. Unfortunately, not much data are available of abnormal plasma clot lysis in primary ITP patients, indicating the need for further research.

To sum up everything that has been stated so far, prolonged clotting time and reduced maximal clot formation rate as well as longer clot lysis time indicated a hypercoagulable profile. Nevertheless, these results were not associated to clinical outcome parameters such as bleeding severity or history of thrombosis.

Contrary to bleeding, elevated thrombosis incidences have been reported in primary ITP. A study by Alvarez-Roman et al. suggested enlarged procoagulant risk due to elevated amounts of platelet-derived MV in chronic ITP patients compared to healthy persons (Alvarez Román et al. 2014). Sewify et al. additionally reported increased amounts of red cell microparticles and platelet microparticles within chronic ITP patients (Sewify et al. 2013). To investigate the procoagulant risk within ITP, we assessed Tissue factor bearing microparticles using a method

established by Hisada and Mackman (Hisada and Mackman 2019a). We did not detect elevated TF-activity in ITP patients and no significant differences to healthy individuals were found. Patients with non-immunological thrombocytopenia after chemotherapy had higher EV-TF activity than ITP patients. These findings go along with the results of a study published by Garabet et al. in 2020. The researchers investigated the procoagulant activity of microvesicles before and after TPO-RA-treatment in eleven ITP patients and 15 healthy controls. They focused on TPO-RA treatment, as it is thought to increase thrombosis risk in ITP patients. The authors found no difference before, but higher MV-associated PS-activity and increased Thrombin generation in a phospholipid dependent manner after treatment with TPO-RA. However, MV-associated TF-activity was equal between ITP patients and controls and did not change before and after TPO-RA-treatment. The authors suggested that TPO-RAs promote phospholipid-dependent MV production, which in turn leads to elevated Thrombin generation and therefore enforces the hypercoagulable state of ITP patients (Garabet et al. 2020). Nevertheless, there was no difference in CLA or Thrombin generation parameters between treated and untreated patients in our study. In line with data of Gabaret et al., patients within our study, with current ITP specific treatment had higher levels of Tissue factor bearing microparticles than untreated patients, as shown in Table 10. Still, the overall activity was below the clinical significant threshold. In total only three out of 88 ITP patients showed EV-TF activity at all. Two out of these three patients received cortison and IVIG treatment, but they were not treated with TPO. The third one did not receive any treatment. Our study and the study of Garabet et al. therefore support each other's findings of not increased TF-EV in ITP patients and our combined studies do not support the hypothesis, that extracellular TF is directly involved in the increased thrombotic risk of ITP patients.

To identify a new biomarker in primary ITP we looked at the desialylation status of washed platelets from healthy donors, which were incubated with ITP patient sera. Previously, Marini et al. confirmed that ITP patients express autoantibodies with the ability to desialylate MKs and platelets, which decreases platelet survival and function. These autoantibodies are believed to especially target GPIIb/IIIa and GPIb/IX present on MK and platelets, resulting in the characteristic low platelet count in ITP. The researchers could confirm that platelet desialylation promotes autoantibody driven elimination of platelets to further promote release of sialidase from platelets, which in turn promotes desialylation. Additionally, they showed that desialylated platelets have reduced functional capacity, which could be relevant for ITP patients with bleeding symptoms. It was observed that especially patients with bleeding

tendency had desialylating autoantibodies (Marini et al. 2019). A novel finding Marini et al. made was, that desialylated MK lose their capacity for adhesion to the bone marrow extracellular matrix, causing reduced cell differentiation and reduced platelet formation. Our research group at the Medical University of Vienna established a cooperation with the research group of Prof. Backoul, Eberhart-Karls University, Tübingen, to share the protocol and investigate the platelet desialylation status in the patients of the Vienna ITP Biobank. The desialylation status of several ITP patients from our biobank, had previously been measured at the Eberhart-Karls University and we tested for reproducibility in our laboratory. We investigated 32 primary ITP patients by flow cytometry. For 17 of them, the autoantibody status was known. Two of the investigated patients had platelet desialylation, both with no autoantibodies or unknown autoantibody status. These two patients were chronic ITP patients, both males and <30 years, and interestingly both had previously suffered from thrombosis and their bleeding scores were zero. One patient, aged 22 years, did not receive treatment, while the older one (29 years) had multirefractory ITP with previous treatments with corticosteroid, IVIG, eltrombopag, romiplostim. We investigated a subpopulation of the samples tested at the Eberhart-Karls University in Tübingen and desialylation seems to be a seldom phenomenon observed in ITP patients. Based on our results it could be linked to thrombosis history and the male sex. The research team in Tübingen however linked platelet desialylation to patients with bleeding symptoms. Unfortunately, no general conclusion on the complete ITP patient population can be drawn from the two males which tested positive in our study and a larger study cohort would be required. Due to our results, we still conclude that platelet desialylation is not a main characteristic of adult patients suffering from primary ITP. The remaining ITP samples of the Vienna ITP biobank wait to be investigated for disease causing or associated vulnerable nodes that we did not yet illuminate, so final conclusions can be drawn.

## 5.1 Conclusion

In conclusion, the global hemostatic potential of primary ITP was characterized through decreased Thrombin generation and delayed and compromised Fibrin clot formation and lysis. Mechanism behind these hypofibrinolytic potential still need to be elucidated. No clear correlation between the investigated hemostatic potential and clinical outcome parameters in respect to bleeding severity was seen.

Even though other studies showed increased extracellular vesicle appearance in ITP, we support the more recent study by Garabet et al. 2020 in which TF-EV activity did not differ in ITP patients compared to healthy controls, indicating that EV-TF activity is not a key driver of thrombosis. Platelet desialylation seems to be rare and not solely dependent on detectable antibodies. Its association with thrombosis or bleeding severity needs to be investigated in a larger cohort.

Evidently the physiological mechanisms behind this orphan disease and its clinical manifestations need further investigations. Especially identifying factors promoting the procoagulant behavior would be of interest for patient risk stratification and the development of individualized treatment strategies. Our results further indicate the high need for individualized therapeutic approaches, taking account of the patients procoagulant profile and other concomitant risk factors. The exact mechanisms behind alterations in the global hemostatic potential of primary ITP patients, especially the reduced plasma clot lysis capability, autoantibody-mediated desialylation and its impact on platelet destruction remain to be investigated.

# 6 Summary

## 6.1 Summary in English

Primary ITP is a rare autoimmune disease, characterized through isolated thrombocytopenia. Its pathological mechanisms are not completely elucidated yet. Hence, diagnosis remains one of exclusion as no reliable, robust, and uniformly used clinical and/or laboratory parameters exist.

Within this study the global hemostatic potential of ITP patients was thoroughly investigated, and it was aimed to link the results to clinical characteristics and especially to the heterogenous bleeding phenotype found in ITP patients. The global hemostatic potential was assed via a Thrombin generation assay, a plasma clot formation and lysis assay as well as the extracellular vesicle (EV)-associated Tissue factor activity. As the low platelet numbers are thought to be caused through autoantibodies targeting desialylated platelets and thereby marking them for destruction, a Lectin binding assay was performed. With this assay the desialylation status of ITP patient platelets was investigated, since previous studies found increased platelet desialylation in primary ITP. In this study three cohorts were investigated, primary ITP patients, patients suffering from non-immunological thrombocytopenia and healthy controls.

In regard to the global hemostatic potential, we found mildly impaired Thrombin generation in ITP patients in comparison to non-immunological thrombocytopenic patients and healthy controls. Furthermore, reduced plasma clot formation was observed in ITP and significantly delayed plasma clot lysis, compared to the two control groups. No significant differences were found in EV-associated Tissue factor activity. In the Lectin binding assay only two out of 32 patients, both with a previous history of thrombosis were identified to show desialylated platelet characteristics.

Summing up our data show mildly impaired Thrombin generation and plasma clot formation and delayed clot lysis in ITP patients compared to non-immunological thrombocytopenia patients and healthy controls. EV-associated TF activity did not differ and no significant association to thrombosis incidence in primary ITP could be drawn. The Lectin binding assay needs to be completed for all available ITP samples in the Vienna ITP biobank to draw final conclusions on the impact of platelet desialylation on clinical manifestations in primary ITP.

## 6.2 Summary in German

Die Immunthrombozytopenie (ITP) ist eine erworbene Autoimmunerkrankung, bei der Autoantikörper gegen Thrombozyten und Megakaryozyten gebildet werden. Der pathophysiologische Mechanismus der primären ITP ist nicht lückenlos bekannt. Die Diagnose der primären ITP gestaltet sich schwierig und erfolgt als Ausschlussdiagnose. Eine Therapie sollte nicht allein von Blutungsneigung und Thrombozytenzahl abhängig gemacht werden, sondern individuell unter Berücksichtigung des Krankheitsstadiums und Krankheitsverlaufes angepasst werden.

Das Ziel dieser Studie war, das hämostatische Potential von ITP Patient*innen, zu analysieren um dessen Einfluss auf die klinische Präsentation der Patient*innen mit individuell unterschiedlicher Blutungsneigung, aber auch Thromboseneigung, zu untersuchen. Um das hämostatische Potential zu erfassen, wurde die Messung der Thrombin-Generierung, der Dynamik der Gerinnselbildung und Auflösung und eine quantitative Messung des zirkulierenden Gewebsthromboplastin durchgeführt. Da die geringe Thrombozytenzahl in primärer ITP teilweise durch Autoantikörper, welche sich an desialylierte Glykoproteinreste an der Thrombozytenoberfläche binden, verursacht wird, wurde der durch Patientenserum verursachte Desialylierungs-Status von Spenderthrombozyten mittels Durchflusszytometrie gemessen. Im Rahmen dieser Studie wurden drei Kohorten untersucht, primäre ITP Patient*innen, Patient*innen mit nicht-immun bedingter Thrombozytopenie und gesunde Kontrollen.

Unsere Daten zeigten, dass ITP PatientInnen eine leicht verzögerte Thrombin-Generierung im Vergleich zu Patient*innen mit nicht-immun bedingter Thrombozytopenie und gesunde Kontrollen hatten. Auch die Gerinnselbildung war schwächer im Vergleich zu den beiden Kohorten. Am deutlichsten zeigte sich allerdings eine verzögerte Auflösung des Plasmagerinnsels (Hypofibrinolyse) bei den ITP Patient*innen im Vergleich zu beiden Kontrollgruppen. Die Menge an zirkulierendem Gewebsthromboplastin hat sich nicht merklich zwischen den Kohorten unterschieden. Im Rahmen der Lektin-Bindungs-Untersuchung konnten zwei Patienten mit verstärkter Thrombozyten-Desialylierung identifiziert werden.

Zusammenfassend zeigen die Daten dieser Studie, dass Thrombin-Generierung und Gerinnselbildung leicht reduziert und die Auflösung des Gerinnsels langsamer in ITP

Patient*innen als in den zwei Kontrollkohorten war. Es wurde keine erhöhte Menge an zirkulierenden Gewebsthromboplastin und auch keine Verbindung dieses Parameters zu klinischer Thrombose-Inzidenz nachgewiesen. Um eine klare Schlussfolgerung zu charakteristischer Thrombozyten-Desialylierung bei Patient*innen mit primärer ITP ziehen zu können, muss die gesamte ITP Kohorte noch weiter untersucht werden.

# 7 Bibliography


Alexaki, V. I., and H. Henneicke. 2021. 'The Role of Glucocorticoids in the Management of COVID-19', *Horm Met Res* 53: 9-15.

Alvarez-Roman, M. T., I. Fernandez-Bello, V. Jimenez-Yuste, M. Martin-Salces, E. G. Arias-Salgado, M. I. Rivas Pollmar, R. Justo Sanz, and N. V. Butta. 2016. 'Procoagulant profile in patients with immune thrombocytopenia', *Br J Haematol*, 175: 925-34.

Alvarez Román, M. T., I. Fernández Bello, E. G. Arias-Salgado, M. I. Rivas Pollmar, V. Jiménez Yuste, M. Martín Salces, and N. V. Butta. 2014. 'Effects of thrombopoietin receptor agonists on procoagulant state in patients with immune thrombocytopenia', *Thromb Haemost*, 112: 65-72.

Audia, S., M. Mahevas, M. Nivet, S. Ouandji, M. Ciudad, and B. Bonnotte. 2021. 'Immune Thrombocytopenia: Recent Advances in Pathogenesis and Treatments', *Hemasphere*, 5: e574.

Audia, S., K. Santegoets, A. G. Laarhoven, G. Vidarsson, O. Facy, P. Ortega-Deballon, M. Samson, N. Janikashvili, P. Saas, B. Bonnotte, and T. R. Radstake. 2017. 'Fcgamma receptor expression on splenic macrophages in adult immune thrombocytopenia', *Clin Exp Immunol*, 188: 275-82.

Blasi, F., J. D. Vassalli, and K. Danø. 1987. 'Urokinase-type plasminogen activator: proenzyme, receptor, and inhibitors', *J Cell Biol*, 104: 801-04.

Bouchard, B. A., M. A. Shatos, and P. B. Tracy. 1997. 'Human brain pericytes differentially regulate expression of procoagulant enzyme complexes comprising the extrinsic pathway of blood coagulation', *Arterioscler Thromb Vasc Biol*, 17: 1-9.

Braester, A. 2003. 'Pseudothrombocytopenia as a pitfall in the treatment of essential thrombocythemia', *Eur J Haematol*, 70: 251-52.

Broos, K., H. B. Feys, S. F. De Meyer, K. Vanhoorelbeke, and H. Deckmyn. 2011. 'Platelets at work in primary hemostasis', *Blood Rev*, 25: 155-67.

Brummel-Ziedins, K. E., and A. S. Wolberg. 2014. 'Global assays of hemostasis', *Curr Opin Hematol*, 21: 395-403.

Butenas, S. 2012. 'Tissue factor structure and function', *Scientifica (Cairo)*, 2012: 964862.

Catani, L., M. E. Fagioli, P. L. Tazzari, F. Ricci, A. Curti, M. Rovito, P. Preda, G. Chirumbolo, M. Amabile, R. M. Lemoli, S. Tura, R. Conte, M. Baccarani, and N. Vianelli. 2006. 'Dendritic cells of immune thrombocytopenic purpura (ITP) show increased capacity to present apoptotic platelets to T lymphocytes', *Exp Hematol*, 34: 879-87.

Cesarman-Maus, G., and K. A. Hajjar. 2005. 'Molecular mechanisms of fibrinolysis', *Br J Haematol*, 129: 307-21.

Chang, M., P. A. Nakagawa, S. A. Williams, M. R. Schwartz, K. L. Imfeld, J. S. Buzby, and D. J. Nugent. 2003. 'Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro', *Blood*, 102: 887-95.

Cines, D. B., J. B. Bussel, H. A. Liebman, and E. T. Luning Prak. 2009. 'The ITP syndrome: pathogenic and clinical diversity', *Blood*, 113: 6511-21.

Cohen, Y. C., B. Djulbegovic, O. Shamai-Lubovitz, and B. Mozes. 2000. 'The bleeding risk and natural history of idiopathic thrombocytopenic purpura in patients with persistent low platelet counts', *Arch Intern Med*, 160: 1630-8.

Colella, M., E. Alves, G. Delmoro, G. Yamaguti-Hayakawa, F. Orsi, E. De Paula, and J. Bizzacchi. 2018. 'A Retrospective Analysis of 122 Immune Thrombocytopenia Patients Treated with Dapsone: Efficacy, Safety and Factors Associated with Treatment Response', *Blood*, 132: 737-37.

De Marco, L., A. Girolami, S. Russell, and Z. M. Ruggeri. 1985. 'Interaction of asialo von Willebrand factor with glycoprotein Ib induces fibrinogen binding to the glycoprotein IIb/IIIa complex and mediates platelet aggregation', *J Clin Invest*, 75: 1198-203.

Del Conde, I., C. N. Shrimpton, P. Thiagarajan, and J. A. Lopez. 2005. 'Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation', *Blood*, 106: 1604-11.

D rmann, D., K. J. Clemetson, and B. E. Kehrel. 2000. 'The GPIb thrombin-binding site is essential for thrombin-induced platelet procoagulant activity', *Blood*, 96: 2469-78.

Engelmann, B., and S. Massberg. 2013. 'Thrombosis as an intravascular effector of innate immunity', *Nat Rev Immunol*, 13: 34-45.

Ferreira, A. A., R. Zulli, S. Soares, Vd Castro, and H. Moraes-Souza. 2011. 'Identification of platelet refractoriness in oncohematologic patients', *Clinics (Sao Paulo)*, 66: 35-40.

Fitzgerald, J., R. McMonnies, A. Sharkey, P. L. Gross, and K. Karkouti. 2020. 'Thrombin generation and bleeding in cardiac surgery: a clinical narrative review', *Can J Anaesth*, 67: 746-53.

Gao, L., M. Decker, H. Chen, and L. Ding. 2021. 'Thrombopoietin from hepatocytes promotes hematopoietic stem cell regeneration after myeloablation', *bioRxiv*: 2021.05.13.443980.

Garabet, L., W. Ghanima, M. Hellum, P. M. Sandset, J. B. Bussel, H. Tran, and C. E. Henriksson. 2020. 'Increased microvesicle-associated thrombin generation in patients with immune thrombocytopenia after initiation of thrombopoietin receptor agonists', *Platelets*, 31: 322-28.

Goette, N. P., A. C. Glembotsky, P. R. Lev, M. Grodzielski, G. Contrufo, M. S. Pierdominici, Y. R. Espasandin, D. Riveros, A. J. Garcia, F. C. Molinas, P. G. Heller, and R. F. Marta. 2016. 'Platelet Apoptosis in Adult Immune Thrombocytopenia: Insights into the Mechanism of Damage Triggered by Auto-Antibodies', *PLoS One*, 11: e0160563.

Gray, L. D., M. A. Hussey, B. M. Larson, K. R. Machlus, R. A. Campbell, G. Koch, M. Ezban, U. Hedner, and A. S. Wolberg. 2011. 'Recombinant factor VIIa analog NN1731 (V158D/E296V/M298Q-FVIIa) enhances fibrin formation, structure and stability in lipidated hemophilic plasma', *Thromb Res*, 128: 570-6.

Gremmel, T., A. L. Frelinger, 3rd, and A. D. Michelson. 2016. 'Platelet Physiology', *Semin Thromb Hemost*, 42: 191-204.

Grover, S. P., and N. Mackman. 2018. 'Tissue Factor: An Essential Mediator of Hemostasis and Trigger of Thrombosis', *Arterioscler Thromb Vasc Biol*, 38: 709-25.

Grozovsky, R., A. J. Begonja, K. Liu, G. Visner, J. H. Hartwig, H. Falet, and K. M. Hoffmeister. 2014. 'The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling', *Nat Med*, 21: 47-54.

Grozovsky, R., S. Giannini, H. Falet, and K. M. Hoffmeister. 2015. 'Regulating billions of blood platelets: glycans and beyond', *Blood*, 126: 1877-84.

Handgretinger, R., and K. Schilbach. 2018. 'The potential role of γδ T cells after allogeneic HCT for leukemia', *Blood*, 131: 1063-72.

Haslacher, H., M. Gerner, P. Hofer, A. Jurkowitsch, J. Hainfellner, R. Kain, O. F. Wagner, and T. Perkmann. 2018. 'Usage Data and Scientific Impact of the Prospectively Established Fluid Bioresources at the Hospital-Based MedUni Wien Biobank', *Biopreserv Biobank*, 16: 477-82.

Heijnen, H. F. G., A. E. Schiel, R. Fijnheer, H. J. Geuze, and J. J. Sixma. 1999. 'Activated Platelets Release Two Types of Membrane Vesicles: Microvesicles by Surface Shedding and Exosomes Derived From Exocytosis of Multivesicular Bodies and ・-Granules', *Blood*, 94: 3791-99.

Hisada, Y., and N. Mackman. 2019a. 'Measurement of tissue factor activity in extracellular vesicles from human plasma samples', *Res Pract Thromb Haemost*, 3: 44-48.
—. 2019b. 'Tissue Factor and Cancer: Regulation, Tumor Growth, and Metastasis', *Semin Thromb Hemost*, 45: 385-95.
Hofer, S., C. Ay, J. Rejto, A. S. Wolberg, H. Haslacher, S. Koder, I. Pabinger, and J. Gebhart. 2019. 'Thrombin-generating potential, plasma clot formation, and clot lysis are impaired in patients with bleeding of unknown cause', *J Thromb Haemost*, 17: 1478-88.
Hoffman, R., E.J. Benz, L.E. Silberstein, H. Heslop, J. Anastasi, and J. Weitz. 2013. 'Hematology: Basic Principles and Practice.' in, *Hematology: Basic Principles and Practice* (Saunders/Elsevier).
Holinstat, M. 2017. 'Normal platelet function', *Cancer Metastasis Rev*, 36: 195-98.
Hou, M., P. O. Andersson, D. Stockelberg, U. H. Mellqvist, B. Ridell, and H. Wadenvik. 1998. 'Plasma thrombopoietin levels in thrombocytopenic states: implication for a regulatory role of bone marrow megakaryocytes', *Br J Haematol*, 101: 420-4.
Huang, J., X. Li, X. Shi, M. Zhu, J. Wang, S. Huang, X. Huang, H. Wang, L. Li, H. Deng, Y. Zhou, J. Mao, Z. Long, Z. Ma, W. Ye, J. Pan, X. Xi, and J. Jin. 2019. 'Platelet integrin alphaIIbbeta3: signal transduction, regulation, and its therapeutic targeting', *J Hematol Oncol*, 12: 26.
Iraqi, M., J. Perdomo, F. Yan, P. Y. Choi, and B. H. Chong. 2015. 'Immune thrombocytopenia: antiplatelet autoantibodies inhibit proplatelet formation by megakaryocytes and impair platelet production in vitro', *Haematologica*, 100: 623-32.
Jackson, S. P. 2011. 'Arterial thrombosis--insidious, unpredictable and deadly', *Nat Med*, 17: 1423-36.
Jansen, A. J., E. C. Josefsson, V. Rumjantseva, Q. P. Liu, H. Falet, W. Bergmeier, S. M. Cifuni, R. Sackstein, U. H. von Andrian, D. D. Wagner, J. H. Hartwig, and K. M. Hoffmeister. 2012. 'Desialylation accelerates platelet clearance after refrigeration and initiates GPIbalpha metalloproteinase-mediated cleavage in mice', *Blood*, 119: 1263-73.
Janus, J., and S. K. Moerschel. 2010. 'Evaluation of anemia in children', *Am Fam Physician*, 81: 1462-71.
Josefsson, E. C., H. H. Gebhard, T. P. Stossel, J. H. Hartwig, and K. M. Hoffmeister. 2005. 'The macrophage alphaMbeta2 integrin alphaM lectin domain mediates the phagocytosis of chilled platelets', *J Biol Chem*, 280: 18025-32.
Justo Sanz, R., E. Monzón Manzano, I. Fernandez-Bello, T. Álvarez-Roman, M. Martín, M. I. Rivas Pollmar, M. A. Canales, V. Jimenez-Yuste, and N. V. Butta. 2018. 'Platelet Apoptosis and PAI-1 Content Are Involved in the Procoagulant Profile of Immune Thrombocytopenia Patients Responders to Agonists of Thrombopoietin Receptor', *Blood*, 132: 3738-38.
Kahn, M. L., M. Nakanishi-Matsui, M. J. Shapiro, H. Ishihara, and S. R. Coughlin. 1999. 'Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin', *J Clin Invest*, 103: 879-87.
Kahn, M. L., Y. W. Zheng, W. Huang, V. Bigornia, D. Zeng, S. Moff, R. V. Farese, Jr., C. Tam, and S. R. Coughlin. 1998. 'A dual thrombin receptor system for platelet activation', *Nature*, 394: 690-4.
Kapur, R., R. Aslam, E. R. Speck, J. M. Rebetz, and J. W. Semple. 2020. 'Thrombopoietin receptor agonist (TPO-RA) treatment raises platelet counts and reduces anti-platelet antibody levels in mice with immune thrombocytopenia (ITP)', *Platelets*, 31: 399-402.
Kile, B. T. 2014. 'The role of apoptosis in megakaryocytes and platelets', *Br J Haematol*, 165: 217-26.
Kistangari, G., and K. R. McCrae. 2013. 'Immune thrombocytopenia', *Hematol Oncol Clin North Am*, 27: 495-520.

Kjalke, M., M. Ezban, D. M. Monroe, M. Hoffman, H. R. Roberts, and U. Hedner. 2001. 'High-dose factor VIIa increases initial thrombin generation and mediates faster platelet activation in thrombocytopenia-like conditions in a cell-based model system', *Br J Haematol*, 114: 114-20.

Kristinsson, S. Y., G. Gridley, R. N. Hoover, D. Check, and O. Landgren. 2014. 'Long-term risks after splenectomy among 8,149 cancer-free American veterans: a cohort study with up to 27 years follow-up', *Haematologica*, 99: 392-8.

Kuwana, M., Y. Okazaki, and Y. Ikeda. 2009. 'Splenic macrophages maintain the anti-platelet autoimmune response via uptake of opsonized platelets in patients with immune thrombocytopenic purpura', *J Thromb Haemost*, 7: 322-9.

Kuwana, M., Y. Okazaki, J. Kaburaki, Y. Kawakami, and Y. Ikeda. 2002. 'Spleen is a primary site for activation of platelet-reactive T and B cells in patients with immune thrombocytopenic purpura', *J Immunol*, 168: 3675-82.

Larsen, O. H., J. Stentoft, D. Radia, J. Ingerslev, and B. Sorensen. 2013. 'Combination of recombinant factor VIIa and fibrinogen corrects clot formation in primary immune thrombocytopenia at very low platelet counts', *Br J Haematol*, 160: 228-36.

Lefrancais, E., G. Ortiz-Munoz, A. Caudrillier, B. Mallavia, F. Liu, D. M. Sayah, E. E. Thornton, M. B. Headley, T. David, S. R. Coughlin, M. F. Krummel, A. D. Leavitt, E. Passegue, and M. R. Looney. 2017. 'The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors', *Nature*, 544: 105-09.

Lete, I., C. Obispo, F. Izaguirre, T. Orte, B. Rivero, M. J. Cornellana, and I. Bermejo. 2008. 'The levonorgestrel intrauterine system (Mirena) for treatment of idiopathic menorrhagia. Assessment of quality of life and satisfaction', *Eur J Contracept Reprod Health Care*, 13: 231-7.

Li, R., K. M. Hoffmeister, and H. Falet. 2016. 'Glycans and the platelet life cycle', *Platelets*, 27: 505-11.

Liebman, Howard A, and Roberto Stasi. 2007. 'Secondary immune thrombocytopenic purpura', *Curr Opin Hematol*, 14: 557-73.

Liu, W., X. Gu, R. Fu, Y. Li, M. Lv, T. Sun, C. Lv, X. Liu, F. Xue, L. Zhang, and R. Yang. 2016. 'The Effect of Danazol in Primary Immune Thrombocytopenia: An Analysis of a Large Cohort From a Single Center in China', *Clin Appl Thromb Hemost*, 22: 727-33.

Loof, T. G., C. Deicke, and E. Medina. 2014. 'The role of coagulation/fibrinolysis during Streptococcus pyogenes infection', *Front Cell Infect Microbiol*, 4: 128.

Mackman, N., R. E. Tilley, and N. S. Key. 2007. 'Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis', *Arterioscler Thromb Vasc Biol*, 27: 1687-93.

Manco-Johnson, M. J, L. Jacobson, D. Thornhill, C. Baird, and B. B. Warren. 2020. 'Application of Thrombomodulin-Thrombin Generation to Diverse Abnormalities of the Protein C System', *Blood*, 136: 31-32.

Marini, I., J. Zlamal, C. Faul, U. Holzer, S. Hammer, L. Pelzl, W. Bethge, K. Althaus, and T. Bakchoul. 2019. 'Autoantibody-mediated desialylation impairs human thrombopoiesis and platelet life span', *Haematologica*, 106 (1): 196-207.

Massey, A. C. 1992. 'Microcytic anemia. Differential diagnosis and management of iron deficiency anemia', *Med Clin North Am*, 76: 549-66.

Matzdorff, A. , S. Holzhauer, T. Kühne, O. Meyer, H. Ostermann, I. Pabinger-Fasching, M.J. Rummel, U. Sachs, and B. Wörmann. 2021. 'Immunthrombozytopenie (ITP)', DGHO, OeGHO, SSMO SSOM SGMO, GPOH, Accessed 30.06.2021. https://www.onkopedia.com/de/onkopedia/guidelines/immunthrombozytopenie-itp/@@guideline/html/index.html.

Matzdorff, A., O. Meyer, H. Ostermann, V. Kiefel, W. Eberl, T. Kuhne, I. Pabinger, and M. Rummel. 2018. 'Immunthrombozytopenie - aktuelle Diagnostik und Therapie: Empfehlungen einer gemeinsamen Arbeitsgruppe der DGHO, OGHO, SGH, GPOH und DGTI', *Oncol Res Treat*, 41 Suppl 2: 5-36.

McMillan, R., L. Wang, A. Tomer, J. Nichol, and J. Pistillo. 2004. 'Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP', *Blood*, 103: 1364-9.

Michel, M. 2013. 'Immune thrombocytopenia nomenclature, consensus reports, and guidelines: what are the consequences for daily practice and clinical research?', *Seminars in hematology*, 50 Suppl 1: S50-4.

Michel, M., O. B. Rauzy, F. R. Thoraval, L. Languille, M. Khellaf, P. Bierling, and B. Godeau. 2011. 'Characteristics and outcome of immune thrombocytopenia in elderly: results from a single center case-controlled study', *Am J Hematol*, 86: 980-4.

Michelson, A. D. 2003. 'How platelets work: platelet function and dysfunction', *J Thromb Thrombolysis*, 16: 7-12.

Miyagi, T., K. Takahashi, K. Hata, K. Shiozaki, and K. Yamaguchi. 2012. 'Sialidase significance for cancer progression', *Glycoconj J*, 29: 567-77.

Miyagi, T., and K. Yamaguchi. 2012. 'Mammalian sialidases: physiological and pathological roles in cellular functions', *Glycobiology*, 22: 880-96.

Mork, M., M. H. Nielsen, R. Baek, M. M. Jorgensen, S. Pedersen, and S. R. Kristensen. 2018. 'Postprandial Increase in Blood Plasma Levels of Tissue Factor-Bearing (and Other) Microvesicles Measured by Flow Cytometry: Fact or Artifact?', *TH Open*, 2: e147-e57.

Moulis, G., A. Palmaro, J. L. Montastruc, B. Godeau, M. Lapeyre-Mestre, and L. Sailler. 2014. 'Epidemiology of incident immune thrombocytopenia: a nationwide population-based study in France', *Blood*, 124: 3308-15.

Nagelkerke, S. Q., and T. W. Kuijpers. 2014. 'Immunomodulation by IVIg and the Role of Fc-Gamma Receptors: Classic Mechanisms of Action after all?', *Front Immunol*, 5: 674.

Negrier, C., M. Shima, and M. Hoffman. 2019. 'The central role of thrombin in bleeding disorders', *Blood Rev*, 38: 100582.

Neunert, C., D. R. Terrell, D. M. Arnold, G. Buchanan, D. B. Cines, N. Cooper, A. Cuker, J. M. Despotovic, J. N. George, R. F. Grace, T. Kuhne, D. J. Kuter, W. Lim, K. R. McCrae, B. Pruitt, H. Shimanek, and S. K. Vesely. 2019. 'American Society of Hematology 2019 guidelines for immune thrombocytopenia', *Blood Adv*, 3: 3829-66.

NIH. 2021. 'Thrombocytopenia', National Institutes of Health, U.S. Department of Health and Human Services, Accessed 01.05.2021. https://www.nhlbi.nih.gov/health-topics/thrombocytopenia.

Niiya, K., E. Hodson, R. Bader, V. Byers-Ward, J. A. Koziol, E. F. Plow, and Z. M. Ruggeri. 1987. 'Increased surface expression of the membrane glycoprotein IIb/IIIa complex induced by platelet activation. Relationship to the binding of fibrinogen and platelet aggregation', *Blood*, 70: 475-83.

Nishioka, T., T. Yamane, T. Takubo, K. Ohta, K. Park, and M. Hino. 2005. 'Detection of various platelet-associated immunoglobulins by flow cytometry in idiopathic thrombocytopenic purpura', *Cytometry B Clin Cytom*, 68: 37-42.

Østerud, B., and E. Bjørklid. 2001. 'The tissue factor pathway in disseminated intravascular coagulation', *Semin Thromb Hemost*, 27: 605-17.

Owens, A. P., 3rd, and N. Mackman. 2011. 'Microparticles in hemostasis and thrombosis', *Circulation research*, 108: 1284-97.

Pabinger, I., G. Gastl, M. Steurer, S. Sormann, M. Fillitz, J. Friedl, D. Geissler, K. Geissler, R. Greil, P. Knobl, S. Kozek-Langenecker, P. Krippl, P. Kyrle, A. Lang, W. Linkesch, H. Ludwig, M. Muller, S. Panzer, E. Pittermann, J. Thaler, and A. Weltermann. 2012.

'[Primary immune thrombocytopenia in adults: diagnostics and treatment consensus statement of the Austrian Society of Hematology and Oncology (OGHO)]', *Wien Klin Wochenschr*, 124: 111-23.

Park, Y. H., H. G. Yi, M. H. Lee, C. S. Kim, and J. H. Lim. 2016. 'Clinical efficacy and tolerability of vincristine in splenectomized patients with refractory or relapsed immune thrombocytopenia: a retrospective single-center study', *Int J Hematol*, 103: 180-8.

Peerschke, E. I., B. Andemariam, W. Yin, and J. B. Bussel. 2010. 'Complement activation on platelets correlates with a decrease in circulating immature platelets in patients with immune thrombocytopenic purpura', *Br J Haematol*, 148: 638-45.

Petty, H. R., and R. R Todd III. 1993. 'Receptor-receptor interactions of complement receptor type 3 in neutrophil membranes', *J Leukoc Biol*, 54: 492-94.

Piel-Julian, M. L., M. Mahevas, J. Germain, L. Languille, T. Comont, M. Lapeyre-Mestre, B. Payrastre, O. Beyne-Rauzy, M. Michel, B. Godeau, D. Adoue, G. Moulis, and Carmen investigators group. 2018. 'Risk factors for bleeding, including platelet count threshold, in newly diagnosed immune thrombocytopenia adults', *J Thromb Haemost*, 16: 1830-42.

Pieters, M., H. Philippou, A. Undas, Z. de Lange, D. C. Rijken, N. J. Mutch, Xiii Subcommittee on Factor, Fibrinogen, and Fibrinolysis the Subcommittee on. 2018. 'An international study on the feasibility of a standardized combined plasma clot turbidity and lysis assay: communication from the SSC of the ISTH', *J Thromb Haemost*, 16: 1007-12.

Portielje, J. E. A., R. G. J. Westendorp, H. C. Kluin-Nelemans, and A. Brand. 2001. 'Morbidity and mortality in adults with idiopathic thrombocytopenic purpura', *Blood*, 97: 2549-54.

Posch, F., S. Hofer, J. Thaler, L. Hell, O. Konigsbrugge, E. Grilz, L. M. Mauracher, J. Gebhart, C. Marosi, B. Jilma, I. Pabinger, and C. Ay. 2020. 'Ex vivo properties of plasma clot formation and lysis in patients with cancer at risk for venous thromboembolism, arterial thrombosis, and death', *Transl Res*, 215: 41-56.

Pravosudov, V. V. 2019. 'Memory, Learning, Hormones and Behavior☆.' in Jae Chun Choe (ed.), *Encyclopedia of Animal Behavior (Second Edition)* (Academic Press: Oxford).

Provan, D., D. M. Arnold, J. B. Bussel, B. H. Chong, N. Cooper, T. Gernsheimer, W. Ghanima, B. Godeau, T. J. Gonzalez-Lopez, J. Grainger, M. Hou, C. Kruse, V. McDonald, M. Michel, A. C. Newland, S. Pavord, F. Rodeghiero, M. Scully, Y. Tomiyama, R. S. Wong, F. Zaja, and D. J. Kuter. 2019. 'Updated international consensus report on the investigation and management of primary immune thrombocytopenia', *Blood Adv*, 3: 3780-817.

Qin, Y., Y. Han, C. L. Xiong, H. G. Li, L. Hu, and L. Zhang. 2015. 'Urokinase-type plasminogen activator: a new target for male contraception?', *Asian J Androl*, 17: 269-73.

Ramamoorthy, S., and J. A. Cidlowski. 2016. 'Corticosteroids: Mechanisms of Action in Health and Disease', *Rheum Dis Clin North Am*, 42: 15-31, vii.

Rao, L. V., H. Kothari, and U. R. Pendurthi. 2012. 'Tissue factor encryption and decryption: facts and controversies', *Thromb Res*, 129 Suppl 2: S13-7.

Rau, J. C., L. M. Beaulieu, J. A. Huntington, and F. C. Church. 2007. 'Serpins in thrombosis, hemostasis and fibrinolysis', *J Thromb Haemost*, 5 Suppl 1: 102-15.

Rauch, U., and Y. Nemerson. 2000. 'Tissue Factor, the Blood, and the Arterial Wall', *Trends Cardiovas Med*, 10: 139-43.

Remiker, A., and C. Neunert. 2020. 'Splenectomy for immune thrombocytopenia: the evolution and preservation of treatment', *Haematologica*, 105: 2507-09.

Rijken, D. C., and H. R. Lijnen. 2009. 'New insights into the molecular mechanisms of the fibrinolytic system', *J Thromb Haemost*, 7: 4-13.

Rodeghiero, F., M. Michel, T. Gernsheimer, M. Ruggeri, V. Blanchette, J. B. Bussel, D. B. Cines, N. Cooper, B. Godeau, A. Greinacher, P. Imbach, M. Khellaf, R. J. Klaassen, T.

Kuhne, H. Liebman, M. G. Mazzucconi, A. Newland, I. Pabinger, A. Tosetto, and R. Stasi. 2013. 'Standardization of bleeding assessment in immune thrombocytopenia: report from the International Working Group', *Blood*, 121: 2596-606.

Rodeghiero, F., R. Stasi, T. Gernsheimer, M. Michel, D. Provan, D. M. Arnold, J. B. Bussel, D. B. Cines, B. H. Chong, N. Cooper, B. Godeau, K. Lechner, M. G. Mazzucconi, R. McMillan, M. A. Sanz, P. Imbach, V. Blanchette, T. Kuhne, M. Ruggeri, and J. N. George. 2009. 'Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group', *Blood*, 113: 2386-93.

Rugeri, L., S. Beguin, C. Hemker, J. C. Bordet, R. Fleury, B. Chatard, C. Negrier, and Y. Dargaud. 2007. 'Thrombin-generating capacity in patients with von Willebrand's disease', *Haematologica*, 92: 1639-46.

Saes, J. L., S. E. M. Schols, W. L. van Heerde, and M. R. Nijziel. 2018. 'Hemorrhagic disorders of fibrinolysis: a clinical review', *J Thromb Haemost*, 16: 1498– 509.

Sajjan, M., G. B. Morgan, J. C. Dominic, N. B. Robert, and A. V. Mark. 2008. 'A high rate of CLL phenotype lymphocytes in autoimmune hemolytic anemia and immune thrombocytopenic purpura', *Haematologica*, 93: 151-52.

Schecter, A. D., B. Spirn, M. Rossikhina, P. L. Giesen, V. Bogdanov, J. T. Fallon, E. A. Fisher, L. M. Schnapp, Y. Nemerson, and M. B. Taubman. 2000. 'Release of active tissue factor by human arterial smooth muscle cells', *Circulation research*, 87: 126-32.

Sewify, E. M., D. Sayed, R. F. Abdel Aal, H. M. Ahmad, and M. A. Abdou. 2013. 'Increased circulating red cell microparticles (RMP) and platelet microparticles (PMP) in immune thrombocytopenic purpura', *Thromb Res*, 131: e59-63.

Smith, S. A., R. J. Travers, and J. H. Morrissey. 2015. 'How it all starts: Initiation of the clotting cascade', *Crit Rev Biochem Mol Biol*, 50: 326-36.

'Spearman's Rank-Order Correlation'. 2018 Lund Research Ltd. https://statistics.laerd.com/statistical-guides/spearmans-rank-order-correlation-statistical-guide

Springer, T. A. 1994. 'Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm', *Cell*, 76: 301-14.

Stasi, R. 2012. 'Immune thrombocytopenia: pathophysiologic and clinical update', *Semin Thromb Hemost*, 38: 454-62.

Stasi, R., and A. C. Newland. 2011. 'ITP: a historical perspective', *Br J Haematol,* 153: 437-50.

Stasi, R., A. Sarpatwari, J. B. Segal, J. Osborn, M. L. Evangelista, N. Cooper, D. Provan, A. Newland, S. Amadori, and J. B. Bussel. 2009. 'Effects of eradication of Helicobacter pylori infection in patients with immune thrombocytopenic purpura: a systematic review', *Blood*, 113: 1231-40.

Swinkels, M., M. Rijkers, J. Voorberg, G. Vidarsson, F. W. G. Leebeek, and A. J. G. Jansen. 2018. 'Emerging Concepts in Immune Thrombocytopenia', *Front Immunol*, 9: 880.

Taylor, A., L. Neave, S. Solanki, J. P. Westwood, I. Terrinonive, S. McGuckin, J. Kothari, N. Cooper, R. Stasi, and M. Scully. 2015. 'Mycophenolate mofetil therapy for severe immune thrombocytopenia', *Br J Haematol*, 171: 625-30.

Terrell, D. R., L. A. Beebe, S. K. Vesely, B. R. Neas, J. B. Segal, and J. N. George. 2010. 'The incidence of immune thrombocytopenic purpura in children and adults: A critical review of published reports', *Am J Hematol*, 85: 174-80.

Thai, L. H., M. Mahevas, F. Roudot-Thoraval, N. Limal, L. Languille, G. Dumas, M. Khellaf, P. Bierling, M. Michel, and B. Godeau. 2016. 'Long-term complications of splenectomy in adult immune thrombocytopenia', *Medicine (Baltimore)*, 95: e5098.

Thiagarajan, P., and J. F. Tait. 1991. 'Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles', *J Biol Chem*, 266: 24302-07.
Tringali, C., B. Lupo, F. Cirillo, N. Papini, L. Anastasia, G. Lamorte, P. Colombi, R. Bresciani, E. Monti, G. Tettamanti, and B. Venerando. 2009. 'Silencing of membrane-associated sialidase Neu3 diminishes apoptosis resistance and triggers megakaryocytic differentiation of chronic myeloid leukemic cells K562 through the increase of ganglioside GM3', *Cell Death Differ*, 16: 164-74.
Undas, A. 2014. 'Fibrin clot properties and their modulation in thrombotic disorders', *Thromb Haemost*, 112: 32-42.
Undas, A., K. Zawilska, M. Ciesla-Dul, A. Lehmann-Kopydlowska, A. Skubiszak, K. Ciepluch, and W. Tracz. 2009. 'Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives', *Blood*, 114: 4272-8.
Uzan, G., M. Prenant, M. H. Prandini, F. Martin, and G. Marguerie. 1991. 'Tissue-specific expression of the platelet GPIIb gene', *J Biol Chem.*, 266: 8932-39.
Varga-Szabo, D., I. Pleines, and B. Nieswandt. 2008. 'Cell adhesion mechanisms in platelets', *Arterioscler Thromb Vasc Biol*, 28: 403-12.
Vayne, C., E. A. Guery, J. Rollin, T. Baglo, R. Petermann, and Y. Gruel. 2020. 'Pathophysiology and Diagnosis of Drug-Induced Immune Thrombocytopenia', *J Clin Med*, 9: n. pa.
Winter, O., K. Moser, E. Mohr, D. Zotos, H. Kaminski, M. Szyska, K. Roth, D. M. Wong, C. Dame, D. M. Tarlinton, H. Schulze, I. C. MacLennan, and R. A. Manz. 2010. 'Megakaryocytes constitute a functional component of a plasma cell niche in the bone marrow', *Blood*, 116: 1867-75.
Wolberg, A. S. 2007. 'Thrombin generation and fibrin clot structure', *Blood Rev*, 21: 131-42.
Wolberg, A. S., G. A. Allen, D. M. Monroe, U. Hedner, H. R. Roberts, and M. Hoffman. 2005. 'High dose factor VIIa improves clot structure and stability in a model of haemophilia B', *Br J Haematol*, 131: 645-55.
Xia, Y., G. Borland, J. Huang, I. F. Mizukami, H. R. Petty, R. F. Todd, 3rd, and G. D. Ross. 2002. 'Function of the lectin domain of Mac-1/complement receptor type 3 (CD11b/CD18) in regulating neutrophil adhesion', *J Immunol*, 169: 6417-26.
Xu, J., L. Zhao, Y. Zhang, Q. Guo, and H. Chen. 2016. "CD16 and CD32 Gene Polymorphisms May Contribute to Risk of Idiopathic Thrombocytopenic Purpura." In *Med Sci Monit.*, 2086-96.
Zufferey, A., R. Kapur, and J. W. Semple. 2017. 'Pathogenesis and Therapeutic Mechanisms in Immune Thrombocytopenia (ITP)', *J Clin Med*, 6: n. pa.

# 8 List of Tables

# 9 List of Figures

# 10 List of Abbreviations

| | |
|---|---|
| ACD-A | Citrate-dextrose solution |
| ACT | Activated clotting time |
| ADP | Adenosine diphosphate |
| AMR | Ashwell-Morell Receptor |
| aPTT | Activated partial thromboplastin time |
| AUC | Area under the curve |
| BMI | Body mass index |
| BSA | Bovine serum albumin |
| BUC | Bleeding of unknown cause |
| CAT | Calibrated automated thrombogram |
| CI | Confidence interval |
| CLA | Clot formation and lysis assay |
| CLT | Clot lysis time |
| cMpl | Thrombopoietin receptor |
| DANA | 2,3-dehydro-2-deoxy- N-acetylneuraminic acid |
| DC | Dendritic cell |
| DIC | Disseminated intravascular coagulation |
| ECL | Erythrina Cristagalli Lectin |
| EMA | European Medicines Agency |
| EV | Extracellular vesicle |
| F | Factor |
| Fc-gamma-R | Fc-gamma-receptor |
| FDA | US Food and Drug Administration |
| FDP | Fibrin degradation products |
| FI | Fibrinogen |
| FII | Pro-thrombin |
| FIIa | Thrombin |
| FIII | Tissue factor, Thromboplastin, CD142 |
| FIV | Calcium |
| FIX | Antihemophilic Globulin B/Christmas Factor |
| FV | Proaccelerin |
| FVII | Proconvertin |

| | |
|---|---|
| FVIII | Antihemophilic Globulin A |
| FX | Stuart-Prower-Factor |
| FXI | Serine proteinase factor |
| FXII | Hageman-Factor |
| FXIII | Fibrin stabilizing factor |
| GlcNAc | N-Acetylglucosamine |
| GP | Glycoprotein |
| GPIb-IX-V | Glycoprotein receptor for von Willebrand factor |
| GPIIb/IIIa | Integrin alpha IIb/beta 3 |
| GPVI | Glycoprotein receptor for collagen |
| HBS | HEPES buffer saline |
| HBSA | HEPES buffer saline with bovine serum albumin |
| HBV | Hepatitis B virus |
| HC | Healthy control |
| HCV | Hepatitis C virus |
| HHV3 | Human alphaherpesvirus 3 |
| HIV | Human immunodeficiency virus |
| HK | High molecular weight kininogen |
| Ig | Immunoglobulin |
| IQR | Interquartile range |
| ISTH | International Society on Thrombosis and Haemostasis |
| ITP | Immune thrombocytopenia |
| ITP-BAT | ITP-specific Bleeding Assessment Tool |
| IVIg | Intravenous immunoglobulin G |
| IWG | International Working Group |
| LBA | Lectin binding assay |
| LPS | Lipopolysaccharide |
| MK | Megakaryocytes |
| mLR | Maximum of clot lysis rate |
| MPV | Mean platelet volume |
| MV | Microvesicles |
| NEU | Neuraminidase |
| NIH | National Heart, Lung and Blood Institute |

| | |
|---|---|
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| OD | Optical density |
| OR | Odds ratio |
| PAI-1 | Plasminogen activator inhibitor |
| PAR | Protease-activated surface receptor |
| PBS | Phosphate-buffered saline |
| PC | Plasma cell |
| PLG | Plasminogen |
| PPP | Platelet poor plasma |
| PRP | Platelet rich plasma |
| PS | Phosphatidylserine |
| PT | Prothrombin time |
| RCA | Ricinus Communis Agglutinin |
| RFU | Relative fluorescence units |
| RPF | Reticulated platelet fraction |
| RT | Room temperature |
| SD | Standard deviation |
| SSC | Scientific and Standardization Committee |
| SYK | Spleen tyrosine kinase |
| t50% lysis | Time until 50% of lysis is achieved |
| TAFI | Thrombin-activatable fibrinolysis inhibitor |
| TC | Thrombocytopenic controls |
| TF | Tissue factor |
| TFPI | Tissue factor pathway inhibitor |
| TGA | Thrombin generation assay |
| TGF-beta 1 | Tumor growth factor beta-1 |
| Th cell | T helper cell |
| tPA | Tissue-type plasminogen activator |
| TPO | Thrombopoietin |
| TPO-RA | Thrombopoietin receptor agonist |
| TP alpha receptor | Thromboxane proteinoid alpha |
| Treg | Regulatory T cell |
| TTP | Time-to-Peak absorbance |

| | |
|---|---|
| TXA2 | Thromboxane A2 |
| uPA | Urokinase-type plasminogen activator |
| Vmax | Maximum clot formation rate |
| VWD | von Willebrand disease |
| VWF | von Willebrand Factor |
| α2-AP | alpha2-antiplasmin |
| ΔAbs | Peak absorbance |

# 11 Annex

## 11.1 Buffer LBA

The four tables depicted below show the buffer preparation for the washed platelet protocol in the Lectin binding assay.

**Table S1: Preparation Bicarbonate Buffer**

| Bicarbonate Buffer | Amount | Final Conc. |
|---|---|---|
| NaCl | 8.0 g | 144.3 mM |
| KCl | 0.2 g | 2.7 mM |
| $NaHCO_3$ | 1.0 g | 11.9 mM |
| $NaH_2PO_4$ | 0.05 g | 0.42 mM |
| Distilled $H_2O$ | fill up to 50 mL | |

**Table S2: Preparation Pre-Wash-Solution**

| Pre-Wash-Solution | Amount | Final Conc. |
|---|---|---|
| 20 % BSA | 1.8 mL | 0,36 % |
| 10 % Glucose Solution | 1 mL | 0,1 % |
| Bicarbonate Buffer | 5 mL | |
| Ampuwa | fill up to 100 mL | |

**Table S3: Preparation Wash-Solution**

| Wash Solution, pH 6.2-6.5 | Amount | Final Conc. |
|---|---|---|
| Fresh Pre-Wash-Solution | 20 mL | |
| Hirduin (1000 U/mL) | 20 µL | 1 U/mL |
| Apyrase (1000 U/mL) | 50 µL | 2.5 U/mL |

**Table S4: Preparation Suspension Solution**

| Suspension Solution, pH 7.2-7.4 | Amount | Final Conc. |
|---|---|---|
| Fresh Pre-Wash-Solution | 50 mL | |
| $CaCl_2$ | 1000 µL | 0.0022 M |
| $MgCl_2$ | 500 µL | 0.002 M |

## 11.2 Thrombin Generation Assay

**Table S5: Correlation of TGA Parameters with Clinical Data of ITP Patients**

| | Lag time, min | | VI, nmol/l/min | | Peak Thrombin, nmol/l | | TTP, min | | AUC, nmol/l x min | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** |
| ***Clinical Data*** | | | | | | | | | | |
| Age, years | 0.007 | 0.946 | 0.262* | 0.014 | 0.248** | 0.007 | 0.029 | 0.786 | 0.305** | 0.004 |
| Female | 0.062 | 0.567 | 0.069 | 0.521 | 0.053 | 0.621 | 0.050 | 0.643 | 0.044 | 0.684 |
| Previous thrombosis | 0.056 | 0.635 | 0.131 | 0.264 | 0.129 | 0.274 | 0.045 | 0.705 | 0.129 | 0.274 |
| Blood type 0 | 0.101 | 0.365 | 0.091 | 0.419 | 0.100 | 0.370 | 0.084 | 0.455 | 0.151 | 0.175 |
| Smoking | 0.071 | 0.568 | 0.013 | 0.919 | 0.011 | 0.930 | 0.083 | 0.506 | 0.005 | 0.968 |
| BMI, kg/m$^2$ | 0.223* | 0.044 | 0.106 | 0.345 | 0.101 | 0.365 | 0.183 | 0.100 | 0.120 | 0.281 |
| Bleeding score | 0.161 | 0.137 | 0.147 | 0.176 | 0.15 | 0.165 | 0.220* | 0.041 | 0.119 | 0.271 |
| Skin bleeding | 0.083 | 0.449 | 0.032 | 0.769 | 0.021 | 0.851 | 0.137 | 0.208 | 0.005 | 0.962 |
| Mucosal bleeding | 0.206 | 0.057 | 0.214* | 0.048 | 0.201 | 0.063 | 0.218* | 0.044 | 0.144 | 0.185 |
| Organ bleeding | 0.159 | 0.143 | 0.165 | 0.128 | 0.163 | 0.133 | 0.204 | 0.060 | 0.207 | 0.056 |

*Abbreviations: VI, velocity index; TTP, time-to-peak Thrombin levels; AUC, area under the curve; BMI, body mass index; ρ = Spearman's rank correlation coefficient*
*Bleeding score was assed according to the ISTH SMOG Index.*
*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

**Table S6: Correlation of TGA Parameters with Laboratory data of ITP Patients**

| | Lag time, min | | VI, nmol/l/min | | Peak Thrombin, nmol/l | | TTP, min | | AUC, nmol/l x min | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value |
| ***Laboratory Data*** | | | | | | | | | | |
| Platelet count, $x10^9$/l | 0.007 | 0.948 | 0.065 | 0.555 | 0.093 | 0.394 | 0.052 | 0.634 | 0.037 | 0.735 |
| Leukocyte count, $x10^9$/l | 0.214* | 0.048 | 0.217* | 0.044 | 0.232* | 0.032 | 0.169 | 0.120 | 0.182 | 0.094 |
| Hemoglobin, g/dl | 0.192 | 0.079 | 0.096 | 0.381 | 0.120 | 0.274 | 0.236* | 0.030 | 0.200 | 0.067 |
| Fibrinogen, mg/dl | 0.045 | 0.682 | 0.109 | 0.320 | 0.088 | 0.423 | 0.054 | 0.620 | 0.162 | 0.140 |
| aPTT, s | 0.171 | 0.146 | 0.411** | <0.001 | 0.408** | <0.001 | 0.259* | 0.026 | 0.251* | 0.031 |
| Prothrombin time, % | 0.139 | 0.256 | 0.145 | 0.235 | 0.165 | 0.176 | 0.103 | 0.401 | 0.036 | 0.770 |
| MPV, fl | 0.211 | 0.113 | 0.095 | 0.480 | 0.067 | 0.620 | 0.112 | 0.402 | 0.025 | 0.852 |
| RPF, % | 0.176 | 0.156 | 0.169 | 0.170 | 0.194 | 0.116 | 0.168 | 0.173 | 0.071 | 0.567 |
| Thrombopoietin, pg/ml | 0.022 | 0.852 | 0.108 | 0.361 | 0.102 | 0.386 | 0.080 | 0.496 | 0.078 | 0.510 |

*Abbreviations: VI, velocity index; TTP, time-to-peak Thrombin levels; AUC, area under the curve; aPPT, activated partial thromboplastin time; MPV, mean platelet volume; RPF, reticulated platelet fraction; ρ = Spearman's rank correlation coefficient*
*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

**Table S7: Correlation of TGA Parameters with Clinical and Laboratory Data of Thrombocytopenic Patients**

| | Lag time, min | | VI, nmol/l/min | | Peak Thrombin, nmol/l | | TTP, min | | AUC, nmol/l x min | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value |
| ***Clinical Data*** | | | | | | | | | | |
| Age, years | 0.032 | 0.896 | 0.139 | 0.571 | 0.223 | 0.358 | 0.050 | 0.840 | 0.250 | 0.303 |
| Female | 0.010 | 0.969 | 0.096 | 0.695 | 0.038 | 0.876 | 0.165 | 0.501 | 0.135 | 0.582 |
| Previous thrombosis | 0.043 | 0.864 | 0.014 | 0.955 | 0.043 | 0.865 | 0.043 | 0.864 | 0.244 | 0.329 |
| Blood type 0 | 0.275 | 0.474 | 0.137 | 0.725 | 0.000 | 1.000 | 0.069 | 0.860 | 0.137 | 0.725 |
| Smoking | - | - | - | - | - | - | - | - | - | - |
| BMI, kg/m$^2$ | 0.054 | 0.832 | 0.170 | 0.499 | 0.209 | 0.404 | 0.019 | 0.941 | 0.267 | 0.284 |
| Bleeding score | 0.533* | 0.017 | 0.298 | 0.229 | 0.314 | 0.204 | 0.471* | 0.049 | 0.175 | 0.487 |
| Skin bleeding | 0.419 | 0.083 | 0.554* | 0.017 | 0.646** | 0.004 | 0.385 | 0.115 | 0.547* | 0.019 |
| Mucosal bleeding | 0.245 | 0.326 | 0.126 | 0.619 | 0.160 | 0.526 | 0.139 | 0.584 | 0.257 | 0.303 |
| Organ bleeding | 0.339 | 0.169 | 0.020 | 0.938 | 0.099 | 0.695 | 0.325 | 0.188 | 0.126 | 0.620 |
| ***Laboratory Data*** | | | | | | | | | | |
| Platelet count, x10$^9$/l | 0.265 | 0.273 | 0.241 | 0.320 | 0.326 | 0.174 | 0.112 | 0.649 | 0.392 | 0.097 |
| Leukocyte count, x10$^9$/l | 0.503* | 0.028 | 0.304 | 0.207 | 0.304 | 0.207 | 0.438 | 0.061 | 0.307 | 0.201 |
| Hemoglobin, g/dl | 0.128 | 0.612 | 0.303 | 0.222 | 0.236 | 0.347 | 0.022 | 0.930 | 0.249 | 0.319 |
| Fibrinogen, mg/dl | 0.537* | 0.018 | 0.157 | 0.521 | 0.162 | 0.507 | 0.431 | 0.065 | 0.088 | 0.721 |
| aPTT, s | 0.154 | 0.542 | 0.006 | 0.981 | 0.022 | 0.932 | 0.072 | 0.776 | 0.158 | 0.531 |
| Prothrombin time, % | 0.290 | 0.314 | 0.700** | 0.005 | 0.604* | 0.002 | 0.527 | 0.053 | 0.656* | 0.011 |
| MPV, fl | 0.299 | 0.279 | 0.201 | 0.474 | 0.159 | 0.571 | 0.126 | 0.655 | 0.201 | 0.474 |
| RPF, % | 0.391 | 0.108 | 0.069 | 0.785 | 0.015 | 0.951 | 0.290 | 0.244 | 0.108 | 0.669 |
| Thrombopoietin, pg/ml | 0.507 | 0.064 | 0.292 | 0.311 | 0.182 | 0.533 | 0.487 | 0.078 | 0.095 | 0.748 |

*Abbreviations: VI, velocity index; TTP, time-to-peak Thrombin levels; AUC, area under the curve; BMI, body mass index; aPPT, activated partial thromboplastin time; MPV, mean platelet volume; RPF, reticulated platelet fraction; ρ = Spearman's rank correlation coefficient; Bleeding score was assed according to the ISTH SMOG Index.*
*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

**Table S8: Correlation of TGA Parameters with Clinical and Laboratory Data of Healthy Controls**

| | Lag time, min | | VI, nmol/l/min | | Peak Thrombin, nmol/l | | TTP, min | | AUC, nmol/l x min | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value |
| ***Clinical Data*** | | | | | | | | | | |
| Age, years | 0.155 | 0.052 | 0.092 | 0.249 | 0.084 | 0.293 | 0.140 | 0.079 | 0.004 | 0.957 |
| Female | 0.225** | 0.004 | 0.179* | 0.024 | 0.200* | 0.012 | 0.204** | 0.010 | 0.205** | 0.010 |
| Previous thrombosis | - | - | - | - | - | - | - | - | - | - |
| Blood type 0 | 0.075 | 0.475 | 0.167 | 0.107 | 0.111 | 0.286 | 0.144 | 0.165 | 0.024 | 0.817 |
| Smoking | 0.048 | 0.815 | 0.024 | 0.907 | 0.088 | 0.668 | 0.048 | 0.815 | 0.056 | 0.785 |
| BMI, kg/m$^2$ | 0.235** | 0.003 | 0.116 | 0.148 | 0.075 | 0.353 | 0.209** | 0.009 | 0.046 | 0.569 |
| Bleeding score | 0.058 | 0.558 | 0.036 | 0.717 | 0.048 | 0.628 | 0.046 | 0.644 | 0.042 | 0.669 |
| Skin bleeding | - | - | - | - | - | - | - | - | - | - |
| Mucosal bleeding | - | - | - | - | - | - | - | - | - | - |
| Organ bleeding | - | - | - | - | - | - | - | - | - | - |
| ***Laboratory Data*** | | | | | | | | | | |
| Platelet count, x10$^9$/l | 0.225** | 0.004 | 0.210** | 0.008 | 0.214** | 0.007 | 0.213** | 0.007 | 0.127 | 0.112 |
| Leukocyte count, x10$^9$/l | 0.126 | 0.167 | 0.163 | 0.072 | 0.209* | 0.021 | 0.122 | 0.179 | 0.207* | 0.022 |
| Hemoglobin, g/dl | 0.205** | 0.009 | 0.140 | 0.079 | 0.171* | 0.031 | 0.175* | 0.028 | 0.152 | 0.056 |
| Fibrinogen, mg/dl | 0.195* | 0.014 | 0.138 | 0.083 | 0.085 | 0.285 | 0.208** | 0.009 | 0.060 | 0.455 |
| aPTT, s | 0.211** | 0.007 | 0.252** | 0.001 | 0.274** | <0.001 | 0.213** | 0.007 | 0.310** | <0.001 |
| Prothrombin time, % | 0.180* | 0.023 | 0.145 | 0.069 | 0.178* | 0.025 | 0.148 | 0.062 | 0.201* | 0.011 |
| MPV, fl | 0.016 | 0.837 | 0.010 | 0.897 | 0.019 | 0.816 | 0.014 | 0.862 | 0.064 | 0.420 |
| RPF, % | - | - | - | - | - | - | - | - | - | - |
| Thrombopoietin, pg/ml | - | - | - | - | - | - | - | - | - | - |

*Abbreviations: VI, velocity index; TTP, time-to-peak Thrombin levels; AUC, area under the curve; BMI, body mass index; aPPT, activated partial thromboplastin time; MPV, mean platelet volume; RPF, reticulated platelet fraction; ρ = Spearman's rank correlation coefficient; Bleeding score was assed according to the ISTH SMOG Index.*
*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

## 11.3 Clot Formation and Lysis Assay

**Table S9: Correlation of CLA Parameters with Clinical Data of ITP Patients**

| | Baseline OD | | Lag phase, min | | $V_{max}$, OD/min | | $\Delta$Abs, $OD_{405nm}$ | | TTP, min | | CLT, min | | t50% lysis, min | | mLR, OD/min | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value |
| ***Clinical Data*** | | | | | | | | | | | | | | | | |
| Age, years | 0.206 | 0.055 | 0.066 | 0.539 | 0.086 | 0.425 | 0.149 | 0.165 | 0.130 | 0.227 | 0.204 | 0.059 | 0.217* | 0.044 | 0.069 | 0.528 |
| Female | 0.415** | <0.001 | 0.137 | 0.204 | 0.027 | 0.805 | 0.259* | 0.015 | 0.083 | 0.442 | 0.174 | 0.107 | 0.204 | 0.059 | 0.157 | 0.147 |
| Previous thrombosis | 0.080 | 0.499 | 0.046 | 0.696 | 0.090 | 0.448 | 0.017 | 0.887 | 0.062 | 0.602 | 0.006 | 0.962 | 0.015 | 0.896 | 0.108 | 0.361 |
| Blood type 0 | 0.092 | 0.410 | 0.059 | 0.597 | 0.030 | 0.788 | 0.058 | 0.607 | 0.041 | 0.715 | 0.009 | 0.934 | 0.0 | 1.0 | 0.009 | 0.934 |
| Smoking | 0.162 | 0.191 | 0.245* | 0.038 | 0.017 | 0.892 | 0.116 | 0.348 | 0.145 | 0.241 | 0.098 | 0.431 | 0.082 | 0.510 | 0.003 | 0.984 |
| BMI, $kg/m^2$ | 0.067 | 0.552 | 0.031 | 0.418 | 0.144 | 0.198 | 0.019 | 0.866 | 0.209 | 0.059 | 0.275* | 0.013 | 0.263* | 0.018 | 0.044 | 0.699 |
| Bleeding score | 0.094 | 0.388 | 0.093 | 0.392 | 0.041 | 0.707 | 0.027 | 0.805 | 0.006 | 0.953 | 0.171 | 0.115 | 0.156 | 0.153 | 0.114 | 0.297 |
| Skin bleeding | 0.151 | 0.165 | 0.063 | 0.566 | 0.109 | 0.316 | 0.050 | 0.646 | 0 | 0.998 | 0.187 | 0.087 | 0.170 | 0.121 | 0.207 | 0.057 |
| Mucosal bleeding | 0.023 | 0.832 | 0.184 | 0.091 | 0.097 | 0.373 | 0.088 | 0.422 | 0.115 | 0.291 | 0.012 | 0.915 | 0.003 | 0.978 | 0.018 | 0.870 |
| Organ bleeding | 0.022 | 0.841 | 0.086 | 0.430 | 0.016 | 0.886 | 0.105 | 0.335 | 0.066 | 0.547 | 0.039 | 0.722 | 0.022 | 0.845 | 0.047 | 0.672 |

*Abbreviations: OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time-to-peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; BMI, body mass index; ρ = Spearman's rank correlation coefficient; Bleeding score was assed according to the ISTH SMOG Index.*
*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

**Table S10: Correlation of CLA Parameters with Laboratory Data of ITP Patients**

| | Baseline OD | | Lag phase, min | | $V_{max}$, OD/min | | ΔAbs, $OD_{405nm}$ | | TTP, min | | CLT, min | | t50% lysis, min | | mLR, OD/min | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value |
| ***Laboratory Data*** | | | | | | | | | | | | | | | | |
| Platelet count, $x10^9$/l | 0.070 | 0.520 | 0.086 | 0.429 | 0.212* | 0.050 | 0.010 | 0.926 | 0.197 | 0.070 | 0.152 | 0.163 | 0.128 | 0.241 | 0.191 | 0.078 |
| Leukocyte count, $x10^9$/l | 0.139 | 0.201 | 0.128 | 0.239 | 0.086 | 0.431 | 0.040 | 0.716 | 0.045 | 0.684 | 0.163 | 0.135 | 0.156 | 0.150 | 0.070 | 0.519 |
| Hemoglobin, g/dl | 0.241* | 0.026 | 0.113 | 0.305 | 0.038 | 0.731 | 0.166 | 0.129 | 0.014 | 0.897 | 0.179 | 0.102 | 0.190 | 0.081 | 0.092 | 0.401 |
| Fibrinogen, mg/dl | 0.143 | 0.191 | 0.004 | 0.970 | 0.184 | 0.092 | 0.060 | 0.586 | 0.030 | 0.784 | 0.328** | 0.002 | 0.311** | 0.004 | 0.228* | 0.037 |
| aPTT, s | 0.112 | 0.342 | 0.116 | 0.327 | 0.027 | 0.822 | 0.068 | 0.562 | 0.171 | 0.145 | 0.085 | 0.471 | 0.062 | 0.600 | 0.186 | 0.114 |
| Prothrombin time, % | 0.068 | 0.581 | 0.171 | 0.161 | 0.208 | 0.087 | 0.065 | 0.593 | 0.138 | 0.257 | 0.009 | 0.940 | 0.026 | 0.832 | 0.105 | 0.391 |
| MPV, fl | 0.146 | 0.275 | 0.11 | 0.411 | 0.124 | 0.353 | 0.013 | 0.921 | 0.216 | 0.130 | 0.153 | 0.252 | 0.121 | 0.367 | 0.187 | 0.159 |
| RPF, % | 0.021 | 0.865 | 0.097 | 0.436 | 0.104 | 0.401 | 0.023 | 0.853 | 0.242* | 0.048 | 0.207 | 0.093 | 0.148 | 0.233 | 0.253* | 0.039 |
| TPO, pg/ml | 0.063 | 0.596 | 0.023 | 0.843 | 0.128 | 0.277 | 0.092 | 0.435 | 0.025 | 0.829 | 0.089 | 0.452 | 0.095 | 0.432 | 0.091 | 0.441 |

*Abbreviations: OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time-to-peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; aPPT, activated partial thromboplastin time; MPV, mean platelet volume; RPF, reticulated platelet fraction; TPO, Thrombopoietin; ρ = Spearman's rank correlation coefficient*

*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

**Table S11: Correlation of CLA Parameters with Clinical Data of Thrombocytopenic Patients**

| | Baseline OD | | Lag phase, min | | $V_{max}$, OD/min | | ΔAbs, $OD_{405nm}$ | | TTP, min | | CLT, min | | t50% lysis, min | | mLR, OD/min | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value |
| ***Clinical Data*** | | | | | | | | | | | | | | | | |
| Age, years | 0.267 | 0.269 | 0.131 | 0.594 | 0.204 | 0.402 | 0.265 | 0.274 | 0.011 | 0.964 | 0.072 | 0.769 | 0.002 | 0.994 | 0.235 | 0.333 |
| Female | 0.250 | 0.302 | 0.116 | 0.636 | 0.231 | 0.341 | 0.019 | 0.937 | 0.203 | 0.405 | 0.087 | 0.724 | 0.048 | 0.854 | 0.154 | 0.529 |
| Previous thrombosis | 0.043 | 0.865 | 0.043 | 0.864 | 0.273 | 0.273 | 0.187 | 0.458 | 0.072 | 0.776 | 0.302 | 0.223 | 0.302 | 0.223 | 0.014 | 0.955 |
| Blood type 0 | 0.274 | 0.476 | 0.276 | 0.472 | 0.274 | 0.476 | 0.274 | 0.476 | 0.207 | 0.593 | 0.274 | 0.476 | 0.138 | 0.724 | 0.274 | 0.476 |
| Smoking | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| BMI, $kg/m^2$ | 0.079 | 0.754 | 0.409 | 0.092 | 0.176 | 0.484 | 0.075 | 0.766 | 0.356 | 0.147 | 0.013 | 0.958 | 0.029 | 0.909 | 0.042 | 0.868 |
| Bleeding score | 0.031 | 0.904 | 0.282 | 0.257 | 0.285 | 0.252 | 0.110 | 0.665 | 0.446 | 0.063 | 0.126 | 0.619 | 0.080 | 0.752 | 0.062 | 0.806 |
| Skin bleeding | 0.056 | 0.825 | 0.360 | 0.142 | 0.396 | 0.104 | 0.119 | 0.639 | 0.560* | 0.016 | 0.269 | 0.281 | 0.198 | 0.431 | 0.432 | 0.074 |
| Mucosal bleeding | 0.072 | 0.776 | 0.187 | 0.457 | 0.240 | 0.337 | 0.026 | 0.918 | 0.164 | 0.514 | 0.200 | 0.426 | 0.213 | 0.397 | 0.238 | 0.342 |
| Organ bleeding | 0.018 | 0.942 | 0.125 | 0.621 | 0.124 | 0.623 | 0.400 | 0.100 | 0.016 | 0.951 | 0.121 | 0.632 | 0.143 | 0.570 | 0.196 | 0.435 |

*Abbreviations: OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time-to-peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; BMI, body mass index; ρ = Spearman's rank correlation coefficient; Bleeding score was assed according to the ISTH SMOG Index.*
*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

**Table S12: Correlation of CLA Parameters with Laboratory Data of Thrombocytopenic Patients**

| | Baseline OD | | Lag phase, min | | $V_{max}$, OD/min | | ΔAbs, $OD_{405nm}$ | | TTP, min | | CLT, min | | t50% lysis, min | | mLR, OD/min | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value |
| ***Laboratory Data*** | | | | | | | | | | | | | | | | |
| Platelet count, $x10^9$/l | 0.158 | 0.518 | 0.109 | 0.658 | 0.305 | 0.205 | 0.009 | 0.972 | 0.197 | 0.420 | 0.220 | 0.366 | 0.201 | 0.409 | 0.054 | 0.825 |
| Leukocyte count, $x10^9$/l | 0.425 | 0.070 | 0.073 | 0.766 | 0.014 | 0.955 | 0.205 | 0.399 | 0.051 | 0.836 | 0.159 | 0.516 | 0.204 | 0.403 | 0.102 | 0.679 |
| Hemoglobin, g/dl | 0.228 | 0.362 | 0.417 | 0.085 | 0.088 | 0.729 | 0.013 | 0.958 | 0.304 | 0.221 | 0.025 | 0.920 | 0.053 | 0.834 | 0.103 | 0.683 |
| Fibrinogen, mg/dl | 0.461* | 0.047 | 0.193 | 0.430 | 0.241 | 0.320 | 0.525* | 0.021 | 0.339 | 0.156 | 0.133 | 0.588 | 0.143 | 0.559 | 0.021 | 0.932 |
| aPTT, s | 0.075 | 0.766 | 0.433 | 0.073 | 0.030 | 0.906 | 0.04 | 0.874 | 0.434 | 0.072 | 0.172 | 0.494 | 0.201 | 0.425 | 0.019 | 0.942 |
| Prothrombin time, % | 0.176 | 0.547 | 0.383 | 0.176 | 0.115 | 0.697 | 0.095 | 0.747 | 0.214 | 0.463 | 0.333 | 0.245 | 0.397 | 0.160 | 0.267 | 0.357 |
| MPV, fl | 0.389 | 0.152 | 0.164 | 0.560 | 0.025 | 0.929 | 0.150 | 0.593 | 0.039 | 0.891 | 0.154 | 0.583 | 0.152 | 0.588 | 0.004 | 0.990 |
| RPF, % | 0.273 | 0.272 | 0.196 | 0.435 | 0.067 | 0.791 | 0.420 | 0.083 | 0.345 | 0.161 | 0.051 | 0.842 | 0.031 | 0.903 | 0.216 | 0.390 |
| TPO pg/ml | 0.244 | 0.401 | 0.064 | 0.827 | 0.257 | 0.375 | 0.336 | 0.240 | 0.192 | 0.510 | 0.183 | 0.532 | 0.204 | 0.483 | 0.090 | 0.759 |

*Abbreviations: OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time-to-peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; aPPT, activated partial thromboplastin time; MPV, mean platelet volume; RPF, reticulated platelet fraction; TPO, Thrombopoietin; ρ = Spearman's rank correlation coefficient*

*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

**Table S13: Correlation of CLA Parameters with Clinical Data of Healthy Controls**

| | Baseline OD | | Lag phase, min | | $V_{max}$, OD/min | | ΔAbs, $OD_{405nm}$ | | TTP, min | | CLT, min | | t50% lysis, min | | mLR, OD/min | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** |
| ***Clinical Data*** | | | | | | | | | | | | | | | | |
| Age, years | 0.109 | 0.175 | 0.004 | 0.964 | 0.128 | 0.110 | 0.274** | <0.001 | 0.034 | 0.669 | 0.159* | 0.047 | 0.159* | 0.047 | 0.041 | 0.606 |
| Female | 0.444** | <0.001 | 0.267** | <0.001 | 0.110 | 0.171 | 0.240** | 0.002 | 0.013 | 0.874 | 0.107 | 0.183 | 0.099 | 0.218 | 0.068 | 0.400 |
| Previous thrombosis | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Blood type 0 | 0.033 | 0.753 | 0.019 | 0.853 | 0.041 | 0.697 | 0.053 | 0.612 | 0.088 | 0.399 | 0.012 | 0.912 | 0.027 | 0.795 | 0.197 | 0.057 |
| Smoking | 0.072 | 0.726 | 0.225 | 0.268 | 0.136 | 0.506 | 0.040 | 0.846 | 0.161 | 0.431 | 0.072 | 0.726 | 0.096 | 0.640 | 0.401* | 0.042 |
| BMI, $kg/m^2$ | 0.320** | <0.001 | 0.084 | 0.297 | 0.242** | 0.002 | 0.229** | 0.004 | 0.010 | 0.901 | 0.073 | 0.366 | 0.069 | 0.397 | 0.261** | 0.001 |
| Bleeding score | 0.050 | 0.610 | 0.011 | 0.910 | 0.118 | 0.230 | 0.063 | 0.521 | 0.096 | 0.332 | 0.098 | 0.319 | 0.088 | 0.372 | 0.107 | 0.276 |
| Skin bleeding | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Mucosal bleeding | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Organ bleeding | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

*Abbreviations: OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time-to-peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; BMI, body mass index; ρ = Spearman's rank correlation coefficient; Bleeding score was assed according to the ISTH SMOG Index.*

*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

**Table S14: Correlation of CLA Parameters with Laboratory Data of Healthy Controls**

| | Baseline OD | | Lag phase, min | | $V_{max}$, OD/min | | ΔAbs, $OD_{405nm}$ | | TTP, min | | CLT, min | | t50% lysis, min | | mLR, OD/min | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value | ρ | p-value |
| ***Laboratory Data*** | | | | | | | | | | | | | | | | |
| Platelet count, $x10^9$/l | 0.253** | 0.001 | 0.221** | 0.005 | 0.075 | 0.348 | 0.099 | 0.218 | 0.032 | 0.694 | 0.081 | 0.313 | 0.079 | 0.327 | 0.105 | 0.191 |
| Leukocyte count, $x10^9$/l | 0.145 | 0.114 | 0.004 | 0.967 | 0.128 | 0.162 | 0.171 | 0.061 | 0.001 | 0.987 | 0.056 | 0.543 | 0.061 | 0.509 | 0.130 | 0.157 |
| Hemoglobin, g/dl | 0.303** | <0.001 | 0.197* | 0.013 | 0.047 | 0.557 | 0.171* | 0.032 | 0.010 | 0.896 | 0.029 | 0.722 | 0.032 | 0.691 | 0.000 | 0.999 |
| Fibrinogen, mg/dl | 0.029 | 0.717 | 0.082 | 0.309 | 0.351** | <0.001 | 0.664** | <0.001 | 0.083 | 0.301 | 0.078 | 0.333 | 0.066 | 0.414 | 0.190* | 0.017 |
| aPTT, s | 0.055 | 0.494 | 0.014 | 0.861 | 0.120 | 0.136 | 0.075 | 0.351 | 0.002 | 0.976 | 0.082 | 0.310 | 0.077 | 0.338 | 0.094 | 0.239 |
| Prothrombin time, % | 0.088 | 0.272 | 0.002 | 0.975 | 0.097 | 0.225 | 0.181* | 0.024 | 0.038 | 0.634 | 0.079 | 0.327 | 0.094 | 0.244 | 0.107 | 0.182 |
| MPV, fl | 0.014 | 0.862 | 0.131 | 0.102 | 0.077 | 0.336 | 0.086 | 0.283 | 0.042 | 0.600 | 0.008 | 0.916 | 0.017 | 0.837 | 0.059 | 0.459 |
| RPF, % | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| TPO, pg/ml | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

*Abbreviations: OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time-to-peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; aPPT, activated partial thromboplastin time; MPV, mean platelet volume; RPF, reticulated platelet fraction; TPO, Thrombopoietin; ρ = Spearman's rank correlation coefficient*

*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

## 11.4 Mackman Assay

**Table S15: Correlation of EV TF-activity with Clinical and Laboratory Parameters of Primary ITP, Thrombocytopenic Patients (TC) and Healthy Controls (HC)**

| | primary ITP | | TC | | HC | |
|---|---|---|---|---|---|---|
| | **ρ** | **p-value** | **ρ** | **p-value** | **ρ** | **p-value** |
| ***Clinical Data*** | | | | | | |
| Age, years | 0.234* | 0.028 | 0.533* | 0.019 | 0.037 | 0.709 |
| Female | 0.032 | 0.766 | 0.558* | 0.013 | 0.030 | 0.763 |
| Previous thrombosis | 0.774 | 0.186 | 0.349 | 0.156 | - | - |
| Blood type 0 | 0.166 | 0.135 | 0.418 | 0.263 | 0.005 | 0.968 |
| Smoking | 0.132 | 0.288 | - | - | - | - |
| BMI, kg/m$^2$ | 0.017 | 0.877 | 0.387 | 0.112 | 0.107 | 0.277 |
| Bleeding score | 0.031 | 0.155 | 0.376 | 0.124 | 0.197 | 0.086 |
| Skin bleeding | 0.028 | 0.799 | 0.062 | 0.808 | - | - |
| Mucosal bleeding | 0.046 | 0.674 | 0.439 | 0.068 | - | - |
| Organ bleeding | 0.148 | 0.173 | 0.355 | 0.149 | - | - |
| ***Laboratory Data*** | | | | | | |
| Platelet count, x10$^9$/l | 0.104 | 0.34 | 0.046 | 0.852 | 0.111 | 0.259 |
| Leukocyte count, x10$^9$/l | 0.163 | 0.134 | 0.578** | 0.010 | 0.030 | 0.799 |
| Hemoglobin, g/dl | 0.078 | 0.477 | 0.014 | 0.956 | 0.053 | 0.592 |
| Fibrinogen, mg/dl | 0.067 | 0.543 | 0.152 | 0.535 | 0.054 | 0.585 |
| aPTT, s | 0.111 | 0.347 | 0.016 | 0.95 | 0.058 | 0.557 |
| Prothrombin time, % | 0.071 | 0.560 | 0.702** | 0.005 | 0.020 | 0.840 |
| MPV, fl | 0.015 | 0.908 | 0.197 | 0.481 | 0.091 | 0.353 |
| RPF, % | 0.104 | 0.403 | 0.459 | 0.056 | - | - |
| Thrombopoietin, pg/ml | 0.118 | 0.315 | 0.536* | 0.036 | - | - |

*Abbreviations: BMI, body mass index; aPPT, activated partial thromboplastin time; MPV, mean platelet volume; RPF, reticulated platelet fraction; ρ = Spearman's rank correlation coefficient; TC, thrombocytopenic controls; HC, healthy controls; Bleeding score was assed according to the ISTH SMOG Index.*
*Correlation conditions: weak (**ρ** = 0.2-0.4), moderate (**ρ** = 0.4-0.6), strong (**ρ** = 0.6-0.8), and very strong (**ρ** = 0.8-1.0) correlation; * 0.05 significance level; ** 0.01 significance level*

## 11.5 ITP Cohort Specific Data

**Table S16: Clinical and Laboratory Data of Treated (n = 39) and Untreated (n = 49) Patients with Primary ITP**

| | Treated primary ITP patients | | Untreated primary ITP patients | | Treated vs Untreated ITP Patients |
|---|---|---|---|---|---|
| ***Clinical Data*** | | | | | |
| | **n** | **mean (%)** | **n** | **mean (%)** | **p** |
| Age, years, mean ± SD | 39 | 41.44 ± 19.3 | 49 | 45.9 ± 14.9 | 0.066 |
| Female | 39 | 21 (53.8) | 49 | 36 (78.3) | 0.056 |
| Previous thrombosis | 31 | 2 (6.5) | 43 | 2 (4.7) | 0.735 |
| Blood type 0 | 37 | 16 (43.2) | 45 | 13 (28.9) | 0.176 |
| Smoking | 27 | 10 (37.0) | 40 | 10 (25.0) | 0.291 |
| | **n** | **median (IQR)** | **n** | **median (IQR)** | **p** |
| BMI, $kg/m^2$ | 37 | 25.4 (22.3-29.8) | 45 | 25.4 (23.1-29.6) | 0.641 |
| Bleeding score | 39 | 1 (0-7) | 48 | 1 (0-2) | 0.240 |
| Skin bleeding | 38 | 1 (0-3) | 48 | 1 (0-1) | 0.122 |
| Mucosal bleeding | 38 | 0 (0-2) | 48 | 0 (0-1) | 0.214 |
| Organ bleeding | 38 | 0 (0-0) | 48 | 0 (0-0) | 0.910 |
| ***Laboratory Data*** | | | | | |
| | **n** | **median (IQR)** | **n** | **median (IQR)** | **p** |
| Platelet count, $x10^9/l$ | 38 | 48.0 (17.0-117.8) | 48 | 66.5 (37.3-124.0) | 0.186 |

*Abbreviations: SD, standard deviation; IQR, interquartile range [25th-75th percentile]; BMI, body mass index; Bleeding score was assed according to the ISTH SMOG Index*

**Table S17: Clinical and Laboratory Data of Primary ITP Patients with Splenectomy (n = 14) and without Splenectomy (n = 74)**

| | Primary ITP patients with splenectomy | | Primary ITP patients without splenectomy | | Splenectomy vs No Splenectomy |
|---|---|---|---|---|---|
| ***Clinical Data*** | | | | | |
| | **n** | **mean (%)** | **n** | **mean (%)** | **p** |
| Age, years, mean ± SD | 14 | 43.9 ± 16.5 | 74 | 40.5 ± 17.2 | 0.954 |
| Female | 14 | 10 (71.4) | 74 | 47 (63.5) | 0.570 |
| Previous thrombosis | 13 | 1 (7.7) | 61 | 3 (4.9) | 0.688 |
| Blood type 0 | 13 | 6 (46.2) | 69 | 23 (33.3) | 0.375 |
| Smoking | 11 | 9 (81.8) | 56 | 11 (19.6) | <0.001 |
| | **n** | **median (IQR)** | **n** | **median (IQR)** | **p** |
| BMI, $kg/m^2$ | 14 | 24.5 (21.2-29.5) | 68 | 25.7 (23.0-29.9) | 0.416 |
| Bleeding score | 14 | 1,5 (1-7,3) | 73 | 1 (0-3) | 0.147 |
| Skin bleeding | 14 | 1 (1-4.3) | 72 | 1 (0-2) | 0.033 |
| Mucosal bleeding | 14 | 0 (0-0-3.5) | 72 | 0 (0-1) | 0.512 |
| Organ bleeding | 14 | 0 (0-2) | 72 | 0 (0-0) | 0.075 |
| ***Laboratory Data*** | | | | | |
| | **n** | **median (IQR)** | **n** | **median (IQR)** | **p** |
| Platelet count, $x10^9/l$ | 14 | 39.5 (7.5-81.8) | 72 | 64.5 (32.0-124.0) | 0.131 |

*Abbreviations: SD, standard deviation; IQR, interquartile range [25th-75th percentile]; BMI, body mass index; Bleeding score was assed according to the ISTH SMOG Index*

**Table S18: Results of TGA, CLA and Mackman Assay for Primary ITP Patients with (n = 14) and without Splenectomy (n = 74)**

| | Primary ITP patients with splenectomy | Primary ITP patients without splenectomy | Splenectomy vs No Splenectomy |
|---|---|---|---|
| | **median (IQR)** | **median (IQR)** | **p*** |
| **Thrombin Generation Assay** | | | |
| Lag time, min | 12.6 (10.2-14.1) | 11.3 (0.1-15.1) | 0.784 |
| VI, nmol/l/min | 34.0 (21.9-36.3) | 32.6 (19.0-69.1) | 0.096 |
| Peak Thrombin, nmol/l | 224.9 (169.3-275.1) | 223.3 (151.9-366.3) | 0.197 |
| TTP, min | 19.8 (17.8-21.7) | 18.6 (14.6-22.2) | 0.357 |
| AUC, nmol/l x min | 3752.8 (3052.1-4577.1) | 3539.8 (2912.1-4625.5) | 0.786 |
| **Clot Formation and Lysis Assay** | | | |
| Baseline OD | 0.3 (0.2-0.4) | 0.23 (0.18-0.31) | 0.344 |
| Lag phase, min | 8.7 (7.2-11.3) | 6.7 (5.0-8.3) | 0.007 |
| $V_{max}$, OD/min | 0.09 (0.07-0.14) | 0.1 (0.07-0.14) | 0.705 |
| ΔAbs, $OD_{405nm}$ | 0.45 (0.4-0.6) | 0.42 (0.33-0.52) | 0.113 |
| TTP, min | 19.5 (14.1-24.7) | 14.5 (11.3-18.0) | 0.046 |
| t50% lysis, min | 25.7 (13.2-34.1) | 23.0 (13.0-35.5) | 0.596 |
| CLT, min | 32.8 (18.2-39.3) | 27.0 (17.3-43.3) | 0.645 |
| mLR, OD/min | 0.009 (0.009-0.004) | 0.009 (0.012-0.005) | 0.817 |
| **Mackman Assay** | | | |
| EV TF-activity, pg/mL | 0.06 (0.0-0.23) | 0.04 (0.0-0.13) | 0.645 |

*Abbreviations: VI, velocity index; OD, optical density; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; TTP, time to peak absorbance; CLT, clot lysis time; t50% lysis, time until 50 % of lysis is achieved; mLR, maximum of clot lysis rate; EV, extracellular vesicles; TF, Tissue factor*
**p-value: adjusted for sex, age, BMI and Fibrinogen levels by multiple linear regression analysis*

**Table S19: Results of TGA, CLA and Mackman Assay in Regard Antibody Expression within the Primary ITP Cohort (n = 88)**

| ITP specific data | Antibodies against GPIIb/IIIa | Antibodies against GPIb/IX | AB against GPIIb/IIIa vs AB against GPIb/IX |
|---|---|---|---|
| | **median (IQR)** | **median (IQR)** | **p*** |
| **Thrombin Generation Assay** | | | |
| Lag time, min | 13.1 (9.1-14.3) | 13.6 (11.3-16.0) | 0.330 |
| VI, nmol/l/min | 32.2 (19.8-51.8) | 37.8 (30.9-76.2) | 0.529 |
| Peak Thrombin, nmol/l | 207.2 (151.1-273.5) | 252.2 (197.0-425.1) | 0.595 |
| TTP, min | 19.6 (15.6-21.8) | 20.6 (17.1-22.8) | 0.651 |
| AUC, nmol/l x min | 3130.6 (2814.1-4553.3) | 3775.6 (2925.7-4504.6) | 0.643 |
| **Clot Formation and Lysis Assay** | | | |
| Baseline OD | 0.27 (0.21-0.41) | 0.38 (0.25-0.43) | 0.912 |
| Lag phase, min | 7.0 (5.4-9.4) | 10.0 (7.1-11.3) | 0.104 |
| $V_{max}$, OD/min | 0.11 (0.07-0.13) | 0.08 (0.06-0.13) | 0.731 |
| ΔAbs, OD405nm | 0.44 (0.38-0.55) | 0.43 (0.37-0.55) | 0.655 |
| TTP, min | 15.3 (12.0-19.0) | 17.7 (14.4-21.0) | 0.783 |
| t50% lysis, min | 23.0 (14.2-36.7) | 15.8 (10.3-31.7) | 0.256 |
| CLT, min | 27.0 (18.8-42.2) | 21.5 (14.8-37.8) | 0.231 |
| mLR, OD/min | 0.009 (0.011-0.005) | 0.009 (0.012-0.006) | 0.233 |
| **Mackman Assay** | | | |
| EV-TF activity, pg/mL | 0.05 (0.0-0.19) | 0.07 (0.03-0.09) | 0.162 |

*Abbreviations: IQR, interquartile range [25th 75th percentile]; VI, velocity index; TTP, time-to-peak Thrombin generation; AUC, area under the curve; $V_{max}$, maximal clot formation rate; ΔAbs, peak absorbance; CLT, clot lysis time; t50% lysis, time until 50% of lysis is achieved; mLR, maximum of clot lysis rate; EV, extracellular vesicle; TF, Tissue factor; AB, antibodies*

**p-value: adjusted for sex, age, BMI and Fibrinogen levels by multiple linear regression analysis*