Universiteit
Leiden
The Netherlands

The diagnostic value of plasma thrombopoietin levels and platelet autoantibodies

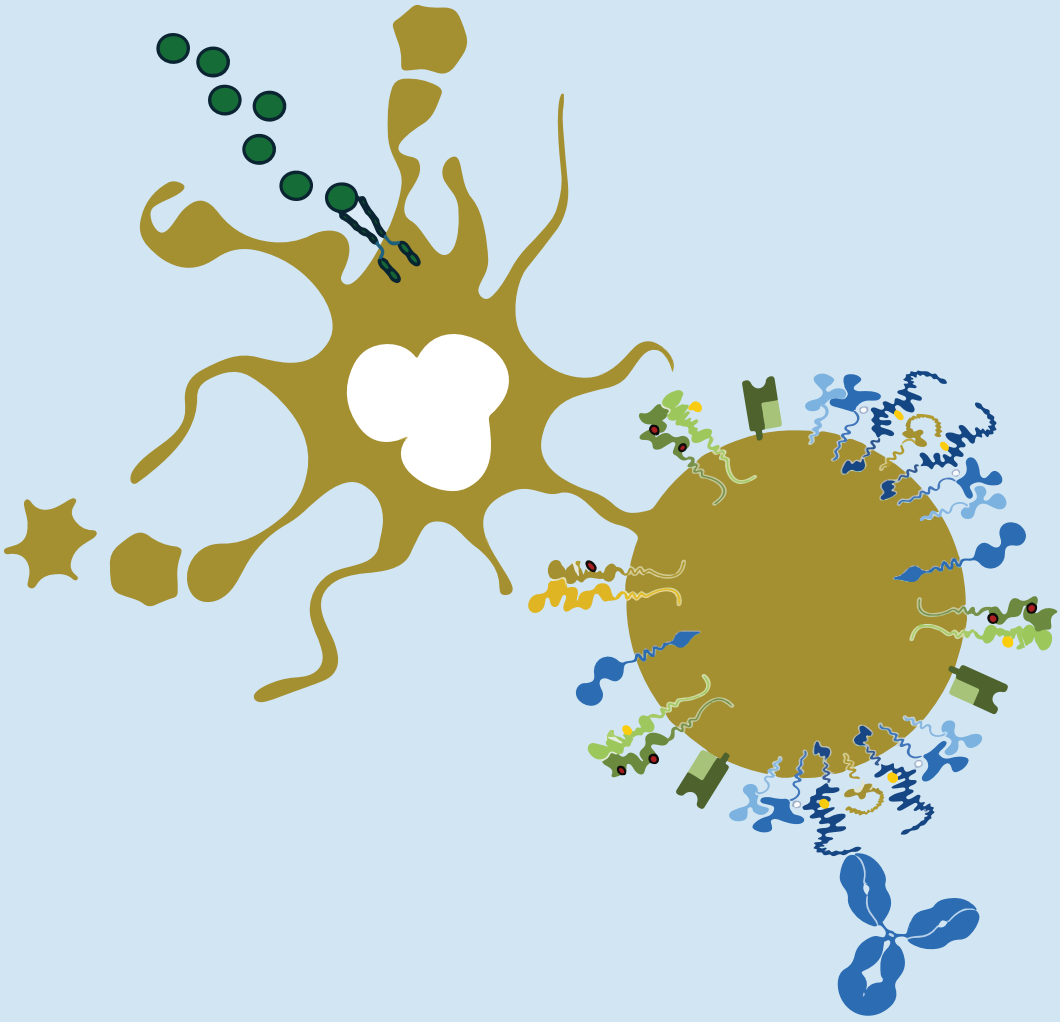
Porcelijn, L.

Citation
Porcelijn, L. (2024, December 17). *The diagnostic value of plasma thrombopoietin levels and platelet autoantibodies*. Retrieved from https://hdl.handle.net/1887/4172615

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/4172615

Note: To cite this publication please use the final published version (if applicable).

THE DIAGNOSTIC VALUE OF PLASMA THROMBOPOIETIN LEVELS AND PLATELET AUTOANTIBODIES



LEENDERT PORCELIJN

THE DIAGNOSTIC VALUE OF PLASMA THROMBOPOIETIN LEVELS AND PLATELET AUTOANTIBODIES

LEENDERT PORCELIJN

The research presented in this thesis was performed at Sanquin Diagnostic Services and Sanquin Research, Amsterdam, The Netherlands.

All rights reserverd. No part of this publication may be produced or transmitted in any form or by any means, electronically or mechanically, including photocopy, recording, or any other storage or retrieval system, without permission from the author.

ISBN 978-94-6506-585-4

© Copyright 2024 Leendert Porcelijn, Hillegom, The Netherlands
Illustrations: Joost M. Bakker, SCICOMVISUALS, www.scicomvisuals.com
Printing: Ridderprint | www.ridderprint.nl
Design by: Cheryl Porcelijn | www.cprecision.nl

THE DIAGNOSTIC VALUE OF PLASMA THROMBOPOIETIN LEVELS AND PLATELET AUTOANTIBODIES

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op dinsdag 17 december 2024
klokke 16.00 uur

door

Leendert Porcelijn

geboren op 19 september 1959
te Hillegom

PROMOTORES EN PROMOTIECOMMISSIE

Promotoren
Prof. dr. J.J. Zwaginga
Prof. dr. M. de Haas

Co-promotor

Dr. R. Kapur	Sanquin

Beoordelingscommissie

Prof. dr. J.G. van der Bom	
Prof. dr. J.W. Semple	Lund University, Lund, Sweden
Prof. dr. L.A. Trouw	
Prof. dr. J.H. Veelken	
dr. V. Novotny	Sanquin

TABLE OF CONTENTS

CHAPTER 1 **7**
Aims and outlines of this thesis and general introduction

CHAPTER 2 **29**
The diagnostic value of thrombopoietin level measurements in thrombocytopenia

CHAPTER 3 **45**
Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia

CHAPTER 4 **55**
Plasma thrombopoietin levels as additional tool in clinical management of thrombocytopenic neonates

CHAPTER 5 **75**
Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art

CHAPTER 6 **89**
Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients

CHAPTER 7 **101**
Anti-glycoprotein Ibα autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients

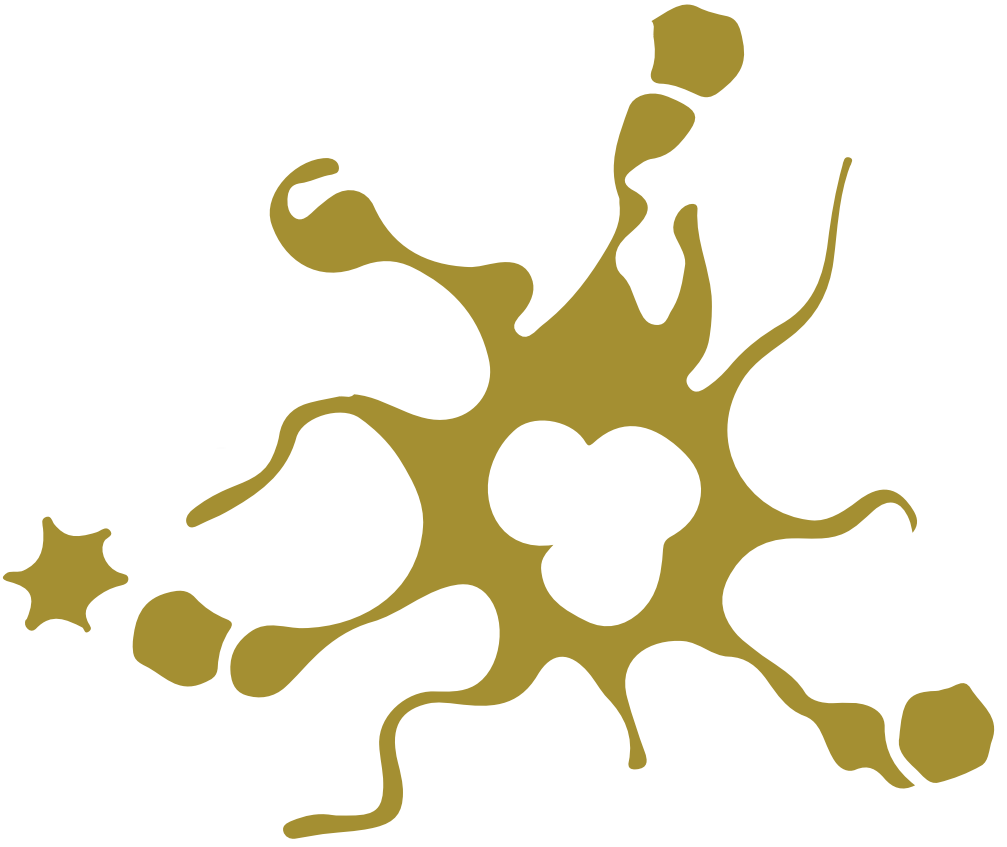
CHAPTER 8 **109**
Evolution and utility of antiplatelet autoantibody testing in patients with immune thrombocytopenia

CHAPTER 9 **143**
Antibodies against platelet glycoproteins in clinically-suspected VITT patients

CHAPTER 10 **167**
General discussion

CHAPTER 11 **191**
Nederlandse samenvatting voor leken

APPENDIX **205**
List of publications
Curriculum Vitae
Dankwoord



01

CHAPTER 1

Aims and outlines of this thesis and general introduction

Aims and outlines of the thesis

The overarching theme of our work described in this thesis is development of diagnostic laboratory tests and testing algorithms to enable accurate diagnosis of antibody-mediated platelet destruction. This will be focused on adults suspected of the autoimmune bleeding disorder immune thrombocytopenia (ITP), and also on neonates suffering from thrombocytopenia. First, the hematopoietic growth hormone thrombopoietin and the diagnostic value of plasma thrombopoietin (TPO) levels for both adults and neonates with thrombocytopenia are described, after which we discuss various aspects of platelet-specific autoantibodies and the introduction in the Netherlands of a reliable platelet autoantibody detection method.

In 1997, we were one of the first who developed a sensitive ELISA to quantify thrombopoietin levels.[1] In that study, several cohorts of thrombocytopenic patients, were evaluated to judge the value of plasma TPO level measurements as part of the diagnostic work-up of thrombocytopenia. We hypothesized that thrombocytopenia caused by antibody-mediated clearance and destruction would not lead to increased plasma TPO levels, in contrast to thrombocytopenia resulting from reduced platelet production. This hypothesis was indeed already confirmed in 1998 for ITP patients *(Chapter 2)* and in 2002 *(Chapter 3)* for patients suffering from fetal/neonatal alloimmune thrombocytopenia (FNAIT). Subsequently, we studied plasma TPO levels in neonates with low platelet counts to see if this could be beneficial for early diagnosis of the underlying cause of their thrombocytopenia *(Chapter 4)*.

To improve platelet autoantibody detection for diagnosing ITP and to gain more insight into the glycoprotein specificity of the antibodies, we specifically optimized the so called monoclonal antibody immobilization of platelet antigens assay (MAIPA) for this purpose *(Chapter 5 and 8)*; an assay that not only detects antibodies binding to platelet glycoproteins but also to which GP. We then used the optimized MAIPA for investigating the clinical relevance for 1) detection of platelet-autoantibodies and the response to the anti-CD20 agent rituximab in ITP patients (*Chapter 6) and 2)* the relationship between the glycoprotein specificity of the platelet-autoantibodies and the TPO levels. The latter to see if GPIb-specific platelet-autoantibodies would increase platelet destruction via the Ashwell-Morell receptor resulting in increased TPO production *(Chapter 7)*. We also used the optimized MAIPA to assess the presence of platelet-autoantibodies and the glycoprotein specificity of these antibodies in a cohort of patients with suspected vaccine-induced thrombotic thrombocytopenia (VITT) *(Chapter 9)*.

General introduction

Platelets are present in relatively high numbers in blood (150-400 x 109/L) and have a main function of regulating hemostasis to prevent bleeding.[2] If there is a shortage of blood platelets (thrombocytopenia) a bleeding tendency can occur. The first signs of bleeding may be ecchymoses, smaller or larger bleeding spots in the skin, called petechiae, purpura and hematoma, or the more alarming bleeding in the soft tissues in the mouth, nose or gut. Thrombocytopenia-related bleeding can also be life-threatening, especially if intracranial bleeding occurs.[2–4] Thrombocytopenia can be caused by a failure to produce sufficient platelets by congenital disorders that can coincide with also dysfunctional platelets or by acquired bone marrow diseases or therapies. Furthermore, thrombocytopenia can be caused by pooling e.g. in an enlarged spleen or liver or by the abundant use of platelets in bleeding, dilution or by their destruction and/ or clearance in disorders such as diffuse intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP) or haemolytic uraemic syndrome (HUS) belonging to the thrombotic microangiopathies (TMA). Finally, thrombocytopenia can be caused by the maternal immune system attacking paternal antigens on the platelets of the foetus or the patient's own immune system attacking its own platelets and their parental cells the megakaryocytes.[3,4] For long the latter has been called "idiopathic thrombocytopenic purpura (ITP)", because the diagnosis of this bleeding disorder could only be made after excluding other causes of thrombocytopenia, but currently ITP is defined as "immune thrombocytopenia".[5]

To start the correct treatment of thrombocytopenia in general, it is evidently important to differentiate between these different causes of thrombocytopenia via a set of diagnostic laboratory tests.[3]

To understand the process of antibody-mediated thrombocytopenia, it is important to first understand the physiologic production and clearance of platelets which should be in balance to maintain hemostasis with stable platelet numbers. Delineation of the physiologic processes determining the platelet number will aid our understanding of the pathophysiology of thrombocytopenia and contribute to identification of potential therapeutic and compensatory interventions that could be applied in patients suffering from such disorders.

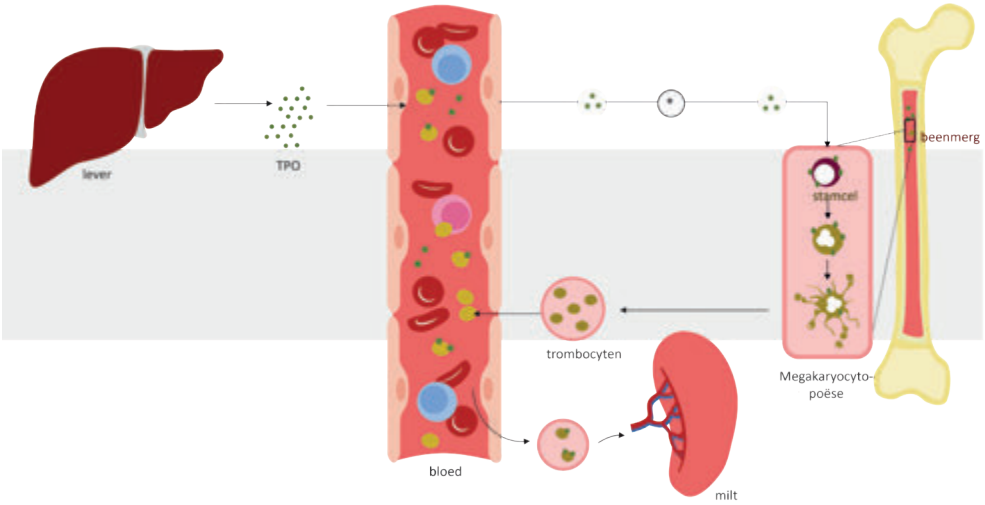
Platelet production

Platelets are enucleated cell-fragments originating from megakaryocytes (MK) in their turn derived from hematopoietic stem cells (HSC) located in the bone marrow. Each MK fragments into 1000-3000 platelets.[6,7] An adult person, weighing 70 kg, has approximately 5.6 L of whole blood with between 150 and 400 x 109 platelets/L. Therefore, the total platelet number in an adult person varies between 840 and 2,240 billion. With a lifespan of approximately ten days,

this means that every single day between 84 and 224 billion platelets need to be replaced.
Hence the production of blood platelets from hematopoietic stem cells, is a mind-blowing efficient process, Thrombopoietin, which is mainly produced in the liver, and the megakaryocyte-formation driving hematopoietic growth factor is the most important regulator of this process.[8]

Thrombopoietin
Thrombopoietin as the major regulatory growth factor for platelet production is produced mainly in the liver.[9–11] The existence of such a platelet growth inducing factor was already suspected in 1958 as injecting the serum of thrombocytopenic rats in normal rats resulted in thrombocytosis. It, however, lasted until 1994 before TPO was discovered for which first its receptor mpl needed to be identified.[12,13] In this respect, in 1990 research in the field of acute myeloproliferative syndrome led to the identification of a virus responsible for leukemia and the isolation of v-mpl, a mouse oncogene that is part of the genome of the myeloproliferative leukemia virus (MPLV). Two years later the human homolog gene c-mpl, coding a type 1 homodimeric cytokine receptor, mainly restricted to the megakaryocytic lineage of differentiation, was isolated. DNA sequencing showed a strong resemblance with genes encoding for receptors of hematopoietins including erythropoietin, interleukin (IL)-2 to -7, GM-CSF (granulocyte/monocyte colony stimulating factor) and G-CSF (granulocyte colony stimulating factor). This suggested that the c-mpl gene encoded for likewise a hematopoietin receptor. In 1994 this coincidental finding resulted in the cloning of the c-mpl (MPL) ligand thrombopoietin, which proved to be the major growth factor for platelet production.[9–11,14,15] It was initially thought that TPO production in the liver was not subjected to a proper feed-back mechanism, and that TPO binding to MPL, subsequent endocytosis by platelets and lowering the level of free circulating TPO was regulating the plasma TPO levels and thus platelet production (TPO-sink model). However, it is now also known that TPO production is (partly) driven by clearance of senescent, desialylated, platelets.16 As is described later this clearance, of an as yet unknown percentage of platelets, is mediated by the Ashwell-Morell receptor (AMR) on hepatocytes. Binding of these desialylated platelets to the AMR, in its turn activates Janus kinase 2 (JAK2) and Signal transducer and activator of transcription 3 (STAT3) resulting in upregulation of the TPO gene (THPO) transcription in hepatocytes.[8,17–20]

For thrombocytopenic patients, the simple ‘TPO sink’ model, more or less explained high levels of plasma TPO to discern platelet production failure from increased platelet degradation where TPO levels tend to be normal. In case of a decreased platelet production or in other words ‘’platelet mass’’, TPO cannot be scavenged by binding to the MPL receptors on megakaryocytes and platelets,



AMR Aswell-Morell receptor, TPO thrombopoietin

resulting in increased plasma TPO levels. In contrast, plasma TPO levels in ITP are normal or only slightly elevated for reasons not yet fully understood. [21]
It is, however, known that also platelet production in ITP is, to varying degrees, affected22. The relationship between the level of platelet production, the level of platelet clearance, the degradation route (spleen/liver) and any existing influences on the degree of TPO production remains a topic for research. This thesis shows our contributions to the use of TPO levels in predominantly discerning decreased production vs increased clearance and destruction disorders.

Our laboratory developed a sensitive TPO ELISA (Folman, 1997)[1] and studied the diagnostic value:

- In differentiating thrombocytopenia due to increased platelet destruction and decreased platelet production for the diagnostic work-up for thrombocytopenia in adults *(Chapter 2)*.
- For FNAIT, (analogue to hemolytic disease of the fetus and newborn (HDFN) for red blood cells) with maternal platelet alloantibodies causing fetal/ neonatal platelet destruction *(Chapter 3)*. If indeed plasma TPO levels are normal in neonates suffering from thrombocytopenia due to FNAIT, measuring neonatal TPO levels early post-partum could help with narrowing down the differential diagnosis.
- Building on the former study, we expanded our TPO measurements to more general neonatal thrombocytopenia this due to heterogeneity in the underlying pathology, such as congenital viral infections, bacterial infections, asphyxia, Trisomy 21 and amegakaryocytosis. We wondered whether

measuring neonatal TPO values could be used to recognize or rule out these different causes for thrombocytopenia *(Chapter 4)*.

Platelet clearance and destruction

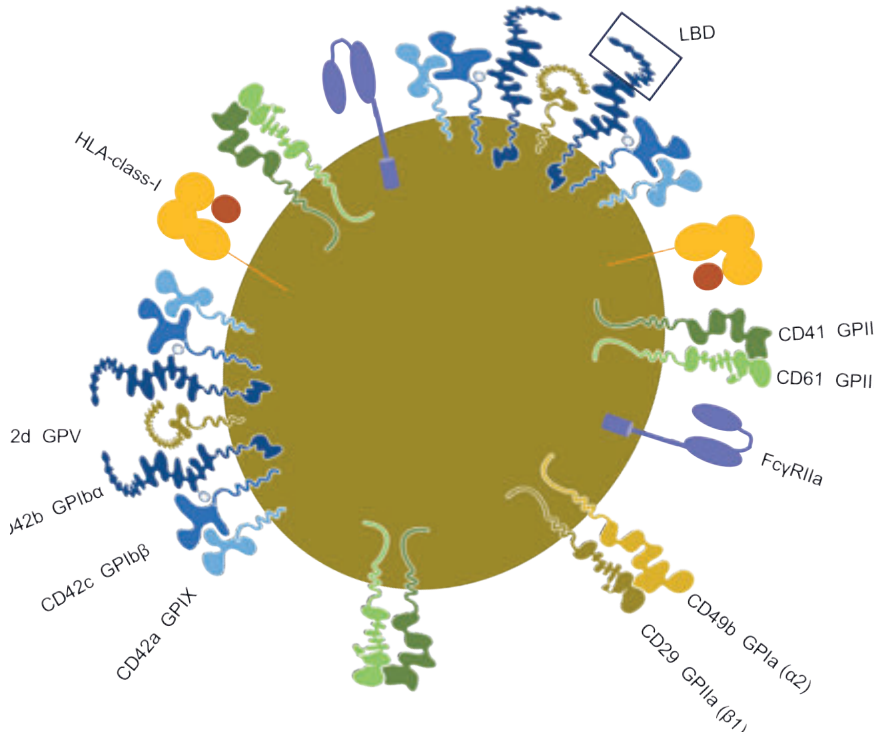
The life span of produced platelets is dependent on the rates of their clearance from the circulation and subsequent destruction in the reticuloendothelial system in spleen and liver where macrophages target platelets. The obligatory physiologic 'disappearance' of 'old platelets' in this respect is intriguing. It has been proposed that this process is regulated by both "programmed' internal signals and changes of membrane-bound glycoproteins leading to external signals. The regulated process of cell death occurs via the pathway of apoptosis. Apoptosis is a genetically programmed cell death where an aging cell at the end of its life cycle shrinks, and its remaining fragments are phagocytosed without any inflammatory reaction. For platelets it has been shown that containment of the pro-apoptotic molecules Bak and Bax by the anti-apoptotic Bcl-2 protein family member Bcl-xl is a major regulator for survival[23,24] Initially, a 'molecular clock' model was considered due to a combination of consumption and the gradual inability to produce Bcl-xl in ageing platelets.[24–26] However, Josefsson et al. (2013) showed that both aged and young platelets contain the same amount of Bcl-xl, which indicates that further research into this so-called ''clock'' model is needed.[27] Apart from the internal apoptotic signals, also changes of membrane structures are likely indicating the senescence of the platelets and resulting in their increased clearance.

The possibility of platelet autoantibodies in ITP patients also causing changes of membrane structures, e.g. desialylation of glycoproteins, resulting in platelet clearance and possibly also apoptosis (see also below 'Desialylation by platelet autoantibodies') has been suggested, but is still uncertain and needs further investigation.[28]

Some of the, also for this thesis, important platelet membrane structures are (Figure 2):

- the for activation and higher binding affinity for its ligands calcium dependent integrin complex glycoprotein (GP)IIb/IIIa (αIIbβ3) being the platelet activating transmembrane receptor for fibrinogen, is necessary for platelet aggregation and adhesion to endothelial surface. Complete deficiency of the normally 80,000 copies of GPIIb/IIIa per platelet, due to a genetic defect, is known as Glanzmann thrombasthenia, a severe bleeding disorder.[29,30] Most of the known human platelet antigens (HPA), i.e. 31 of 41 are located on GPIIb/IIIa. Nine are located on GPIIb and 22 on GPIIIa (including HPA-1a)(see https://versiti.org).
- the transmembrane symmetric GPIX-Ibβ-Ibα-V-Ibα-Ibβ-IX complex, of

Figure 2: Some (for this thesis) key structures expressed on platelets



LBD ligand binding domain, HLA human leucocyte antigen, CD cluster of differentiation

which the highly glycosylated (i.e. approximately 60% of the total platelet membrane glycosylation) Ibα is the major receptor von Willebrand factor mediating the platelet binding to injured vascular wall. Complete absence of the normally 12,500 copies of GPIb/IX/V per platelet is known as the genetic severe bleeding disorder Bernard Soulier Syndrome.[30] Four HPAs are located on the GPIb/IX complex, HPA-2a and -2b on GPIbα, one low frequency antigen on each GPIbβ and GPIX. Like GPIIb/IIIa,

- Glycoprotein Ia/IIa (α2β1), a receptor for collagen type I and IV, with (varies per individual) 800-2000 copies per platelet. Four HPA's are located on GPIa, including HPA-5a and -5b (see https://versiti.org).
- FcγRIIa (CD32A), a type I transmembrane protein, is located on neutrophils, macrophages, dendritic cells and on platelets. Binding of the Fc-tail of IgG can play an important role in platelet activation, adhesion and aggregation, such as also described in heparin induced platelet activation (HIT) and vaccine induced thrombotic thrombocytopenia (VITT).[31,32]

- Human leucocyte antigen (HLA)-class-I being one of two major histocompatibility complex (MHC) molecules which are expressed on all nucleated cells are also expressed on platelets.

In the last decade, studies have indicated an important role of glycan modifications or loss on the surface of platelets. It has now become clear that loss of terminal sialic acid from the platelet surface and subsequently the exposure of the underlying β-galactose can occur. This desialylation is attributed to both exogenous, i.e. pathogen-derived, and endogenous platelet-derived neuraminidase.[8,17–20,33,34] The exposed β-galactose is recognized by the C-type family lectin Ashwell-Morell receptor (also known as the asialoglycoprotein receptor) on hepatocytes and Kupffer cells, resulting in the clearance of senescent platelets.19 A central role for this AMR platelet degradation pathway is attributed to GPIbα, as 70% of the total platelet surface sialic acid is located on GPIbα, especially on the ligand binding domain (LBD).[8]

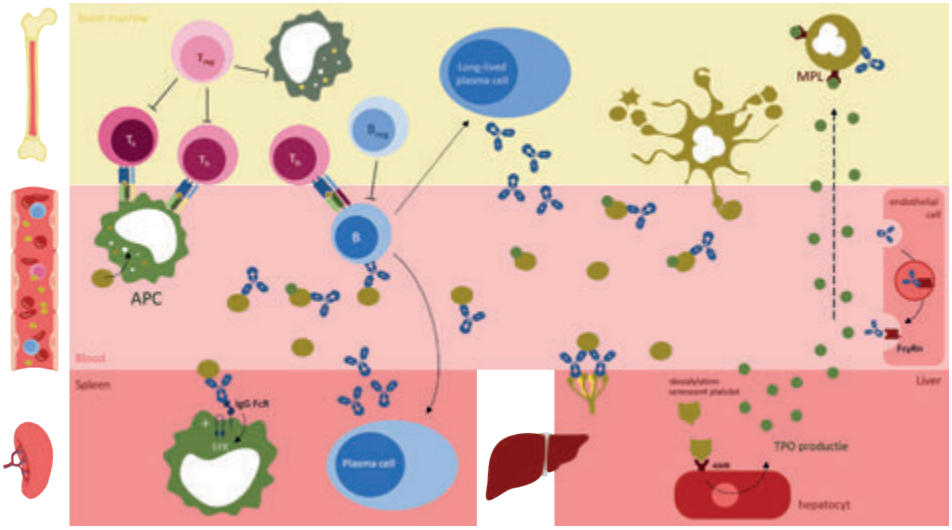
Autoantibody mediated platelet clearance and destruction.
ITP is an isolated thrombocytopenia and results from increased platelet clearance and probably also some decrease in platelet production. The underlying cause is not yet fully understood although increased platelet destruction via the reticulo-endothelial system in the spleen and liver as a result of platelet autoantibody binding is considered to be the main causative mechanism. [35–38] Next to the autoantibody mediated increased platelet clearance, there is now sufficient evidence showing an accompanying impairment of platelet production. It is conceivable that the impairment of platelet precursor cells also expressing the glycoproteins with the preferred binding sites for autoantibodies, is caused by autoantibody binding. However, there also appears to be role for cytotoxic T cells in ITP both in the destruction of platelets and in the impairment of megakaryocytes.[35,38–40] In 1735, Paul Gottlieb Werlhof, a German physician and poet, described an adult girl with ‘a sudden severe hemorrhage from the nose’ and ‘spots in the neck and on the arms, partly black and partly violaceus or purple’. This condition, named Werlhof’s disease, was seen as the first ITP case documented in writing.[41] Despite the fact that at that time the microscope was already invented, it took until 1865 before Max Schulze (German microscopic anatomist, 1825-1874) showed that platelets are a normal component of blood, after which in 1882 Giullio Bizzozero (Italian physician and medical researcher, 1846-1901) found them to be ‘necessary for blood clotting’.[41,42] With these, Werlhof’s disease was found to be associated with low platelet numbers and renamed idiopathic thrombocytopenic purpura (ITP). The experiment in 1950 of William Harrington (American hematologist, 1923-1992) and colleagues, injecting themselves with plasma from ITP patients, showed that a factor in the plasma indeed caused the platelet destruction.[43] This factor was later suggested

to most likely be an antibody, as described by Shulman and colleagues in 1967.[44]

In recent years some pieces of the puzzle seem to have fallen into place pointing into the direction of a failing immune regulatory system in ITP patients. T and B cells are continuously produced in the thymus organ and the bone marrow, respectively, after which positive selection (survival) or negative selection (programmed cell death) takes place. The positive selection of the T cells occurs through interaction with MHC molecules on thymus cells and the formation of functional T cell receptors. The positive selection of B cells depends on whether or not they can form functional B cell receptors. Negative selection for both T and B cells takes place when they respond (strongly) to self-antigens. Unfortunately, some auto-reactive T and B cells can escape negative selection (thymic escape). Platelet proteins are constantly presented by antigen presenting cells to the immune system which, as these proteins are recognized by the immune system as 'self' proteins, does not lead to activation and proliferation of B and/or T cells. Activation of the immune system is furthermore contained by regulatory cells, e.g. dendritic cells, myeloid derived suppressor cells, regulatory T cells. However, for reasons as yet unclear, this immune regulatory system fails in ITP patients. This results in an increase in CD4+ Thelper (Th) cells activating cytokines, including interleukin (IL)2, IL10 and interferon-Y (IFN-Y), and an increase in certain pro-inflammatory Th cells, like Th1 and Th17, leading to the development of autoantibody producing plasma cells from B cells.[35,45]
The production of platelet autoantibodies is believed to be one of the central features of ITP. [35,38] In contrast to ITP in childhood where the autoantibodies are mostly of the IgM class [46], autoantibodies in adult ITP patients are almost always of IgG class, sometimes (in combination with) antibodies of the IgM class and rarely of the IgA class.[47] These antibodies bind to platelet glycoproteins, preferably GPIIb/IIIa and GP Ib/IX[48–50] and subsequently induce thrombocytopenia through various mechanisms, i.e.:

- Antibody-dependent cellular phagocytosis, i.e. binding of the Fc-tail of the platelet-bound autoantibodies by Fc-gamma-receptors (FcγR) Ia or IIIa expressed on splenic macrophages.
- Antibody mediated platelet destruction can also result from complement activation causing binding of C3b to the complement receptor 1 (CR1).[51,52]
- Antibody-induced translocation of neuraminidase-I to the platelet surface causing de-sialylation of GPIbα and (Fc-independent) platelet destruction via the AMR. [34,53,54]
- Binding of autoantibodies to MK causes functional impairment and possibly apoptotic-like morphological changes and decreased platelet production.[22,55–57]

Figure 3: Different mechanisms involved in ITP



Immune thrombocytopenia (ITP) is now known to be a condition in which different pathological mechanisms can be involved. Billions of platelets are each day produced in the bone marrow by megakaryocytes (MK), a process driven by thrombopoietin (TPO) produced in the liver, binding to the MPL receptors on MK precursor cells .

Normally platelets live approximately ten days after which they are removed from the circulation in the spleen and liver. Glycoprotein de-sialylation causes recognition and binding by the Ashwell Morell receptor (AMR) in the liver and subsequent platelet destruction. This process is now known to influence TPO production (see text).

Continuously platelets are presented by antigen-presenting-cells (APC) to the immune system, but in the physiological situation, immune activation is prevented by regulatory mechanisms. For some reason this regulatory system, consisting ofTreg cells (Treg), Dendritic Cells (DC) and possibly also Myeloid Derived Suppressor cells (not depicted), fails in ITP patients. As a result the immune system is no longer contained and proliferation of T and B cells occurs. Cytotoxic T cells (Tc) are able to directly destroy platelets. B cells give rise to plasma cells, producing huge amounts of antibodies. Not only in the spleen, but also long-lived plasma cells in the BM produce these antibodies.

Opsonization of platelets causes destruction via the Fcgamma receptors in the spleen. At the same time it is thought that complement activation, at least for a percentage of patients causes platelet destruction in the liver. Furthermore, it is shown that antibody binding can cause de-sialylation of platelet glycoproteins activating the AMR (Fe-receptor independent) destruction route. It is also important to know that IgG is reentered via the FcRn receptors on endothelial cells, prolonging the antibody half-life.

Furthermore, next to autoantibodies causing thrombocytopenia, depending on the precise binding site of the antibodies they can also cause a functional defect. Acquired Glanzmann thrombasthenia, a condition in which autoantibodies block the fibrinogen binding site on GPIIb/IIIa, resulting in reduced platelet aggregation and a primary coagulation defect despite normal or near normal platelet counts, is already known for decades.[58,59] We showed in different cases that the reason for platelet bound autoantibodies blocking the GPIIb/IIIa functional site but

not causing thrombocytopenia can be explained because the autoantibodies were either of the IgG2- or IgG4-subclass binding only slightly or not at all to the FcγR on macrophages and therefore do not cause platelet degradation in spleen or liver, or were of the IgG1- or IgG3-subclass capable of binding to the FcγR, but splenectomy prevented clearing of the opsonized platelets. 60 In those cases splenectomy results in a rise in platelet counts, but unfortunately without solving the hemorrhagic diathesis because the autoantibodies block the fibrinogen binding site on GPIIb/IIIa. It is therefore conceivable that in a percentage of ITP patients, especially if they have strong GPIIb/IIIa reactive autoantibodies, thrombocytopenia is accompanied by a platelet function defect, which may be one of the reasons for varying bleeding tendencies in patients with similar platelet counts. We indeed found such an effect, measuring platelet aggregation with a novel flow cytometry test of platelet aggregation in which a 10 to 25 times lower platelet number can be used than necessary for a standard platelet aggregation test.[61,62]

Most often ITP occurs spontaneously (primary ITP) and except for thrombocytopenia, other blood cell lineages show counts in the normal range. Immune thrombocytopenia, is still diagnosed by exclusion of other causes for thrombocytopenia, including a thorough anamnesis, physical examination and basic laboratory diagnostics expanded with virus serology, immunoglobulin levels and Helicobacter Pylori test (See Dutch ITP guideline 2020).[63,64]

The treatment of ITP has changed in recent years. With intravenous immunoglobulins (IVIG) and high dose corticosteroids as still the most effective acute treatments in case of deep thrombocytopenia associated bleeding, until about 15 years ago also a more uniform choice was made to suppress the immune system using corticosteroids followed by splenectomy and thrombopoietin receptor agonist (TPO-RA) or rituximab (anti-CD20) only for non-reactive patients, there is now, with the number of available medications increasing rapidly, the possibility for a more individual approach.[35,65] Taking into account ITP disease severity (e.g. the increased bleeding tendency and low platelet counts) and the condition of the patient, if no response to corticosteroids is obtained, 2nd line treatment can start with TPO RA via weekly injections or orally to increase platelet production, rituximab for destruction of B cells and short-lived plasma cells with, or even a spleen tyrosine kinase (SYK) inhibitor (fostamatinib) blocking the SYK-mediated immune receptor signaling in macrophages, neutrophils, natural killer cells and B cells. TPO-RA is often the first choice following corticosteroid and IVIG treatment, with a durable response rate of 60-90%. TPO-RA, however, can have side effects (also depending on which TPO-RA is used) as headaches, diarrhea, upper respiratory tract infections and hepatotoxicity. Rituximab with no major serious side effects apart from some acute injection related or allergy mediated

ones, and fostamatinib causing diarrhea, hypertension and/or nausea in 20-30%, have durable response rates of approximately 40% (25% after five years) and between 18 and 40%, respectively.[35,64–66] Splenectomy, removing the major site of platelet clearance, destruction and autoantibody production, with a durable response rate of approximately 70%, is now preferred not to be performed anywhere close to diagnosis and will generally be postponed until at least 1 to 2 years after diagnosis, depending on the response to the aforementioned treatment and/or personal preference of the patient. Growing insights into the persistence of splenic and bone marrow long-lived plasma cells after rituximab treatment and splenectomy are now directing towards therapeutic options for more thorough B cell and plasma cell clearance. First communications about new therapies, e.g. combinations of anti-B cell activating factor (BAFF) , rituximab or (autologous) T cells engineered to target B cell and plasma cell antigens via chimeric antigen receptors (CARs), are now published.[67–69] Furthermore, random control studies into the effect of medication with other targets, such as neonatal Fc-receptor (FcRn) inhibitors, SYK and Bruton kinase inhibitors or complement inhibitors, are ongoing.

Unfortunately, it is not yet possible to predict the response to treatment for ITP and the treatment based on an individualized patient approach therefore remains a matter of trial and error.

Desialylation by platelet autoantibodies

As written above, it was shown that desialylation of GPIbα on senescent platelets triggers removal from circulation by the AMR expressed on hepatocytes and Kupffer cells in the liver.[8,17,19,34] Subsequently, it was demonstrated in mouse models that desialylation of GPIbα also occurred after binding of moab specific for the ligand binding domain of GPIbα, causing platelet destruction via the AMR.34 Thereafter, several studies were published with possible effects of the binding of GP specific autoantibodies on the desialylation and subsequent clearance of platelets via the Fc-independent AMR route.[53,54] Having tested many samples for the presence of GP specific antibodies in combination with the plasma TPO levels, we also studied the presence of a possible correlation.

The search for a reliable platelet autoantibody detection assay

For years, diagnosing ITP was done by ruling out other causes for thrombocytopenia. Not only because there was no good diagnostic method available, but also because ITP was regarded as an unambiguous disorder, i.e. an autoimmune disorder resulting in the production of autoantibodies and the breakdown of platelets, for which treatment with corticosteroids and splenectomy seemed the correct and only (available) choice.

New developments, including the discovery of the hematopoietic growth hormone thrombopoietin in 1994[13,15,70], with subsequently the availability of the TPO-RA for the treatment of ITP and the exponentially increasing understanding of immune-mediated platelet destruction, has sparked a renewed interest in ITP worldwide. Partly due to these new developments, the ITP support association UK was established in 1995 and in 2003 the Dutch ministry of Health, Welfare and Sport (VWS) gave the green light for subsidizing the establishment of the Dutch ITP patient association (ITP-PV). By establishing these ITP patient associations, it also became more feasible to start ITP registries and collect clinical and quality of life data. This allowed very valuable information to be collected, showing that diagnosis and management of ITP were based on observation and clinical experience more than on scientific evidence. It was shown that, when using diagnosis by exclusion of other causes, a significant percentage of thrombocytopenic patients were incorrectly diagnosed as patients suffering from ITP.[71] Due to these developments, the demand for a reliable diagnostic assay has intensified.

As can be read in chapter 8, the earliest (phase I) methods for the detection (i.e. circumstantial evidence for the presence) of antibodies, such as platelet aggregation tests, serotonin release and complement fixation techniques used the functional properties of blood platelets for indirect evidence of antibody binding, but lacked sensitivity and specificity and therefore, were not suitable for use as a diagnostic test. In the 1970s the availability of radiolabelled or immune fluorescence labelled anti-human immunoglobulins of the IgG, IgM or IgA class led to the development of methods (phase II) e.g. radio immune assay (RIA), platelet immunofluorescence test (PIFT) and mixed passive haemagglutination assay (MPHA), capable of detecting platelet antibodies.[72,73] With these assays, detection of antibody binding on donor platelets incubated with serum from the patient (indirect method) or directly on patient platelets (direct method) became possible. However, their sensitivity and specificity for the detection of platelet autoantibodies were still insufficient.

Incubation of patient platelets with fluorescent-labeled anti-IgG in the direct (i.e. measurement of patient platelet-bound autoantibodies) platelet immunofluorescence test (PIFT) detects platelet autoantibodies in 70% of ITP patients, with a low specificity of approximately 50%-60%. Nonspecific antibody binding, for example, by immune complexes binding via the platelet-IgG-Fcγ receptor type IIa causes false-positive test results in many non-ITP patients.[49,74]
Despite a high specificity of the glycoprotein-specific antibody detection methods (e.g. MAIPA) for detecting GPIIb/IIIa and GPIb/IX and GPIa/IIa reactive alloantibodies, for some reason the sensitivity for detecting autoantibodies directed against these platelet glycoproteins was relatively low (40-60%).[21,49,75,76]

This low sensitivity can only for a small percentage be explained if in a percentage of ITP patients the destruction of platelets is (largely) caused by cytotoxic T cells. We therefore set out to perform a series of experiments to improve platelet autoantibody detection. After optimization of a direct glycoprotein-specific assay, we used this method to investigate the effect on platelet autoantibody levels of new treatments and to gain more insight into the immune dysregulation underlying ITP.

The studies presented in this thesis:

- Revisited and optimized the MAIPA for detection of platelet-autoantibodies *(Chapter 5)*. Moreover, different aspects of autoantibody binding, e.g. the limit of detection for determining a cut-off level and the preferred glycoprotein specificity were investigated. By thus developing a reliable test, we hypothesized that it could become possible to implement it in our diagnostic work-up for ITP and to map the relationship between the detection of autoantibodies and the response to treatments. It could furthermore become possible to consider another underlying (immunological) cause for thrombocytopenic patients without demonstrable antibodies. For example, it could be conceivable that for a percentage of ITP patients, and especially those without demonstrable autoantibodies, platelet destruction by cytotoxic T cells plays a greater role.
- The presence of autoantibodies in serial samples from ITP patients treated with rituximab. Rituximab is a chimeric monoclonal antibody against the protein CD20 which is mainly present on B-lymphocytes. We studied the presence and course of autoantibody levels for a cohort of rituximab treated ITP patients *(Chapter 6)*.
- We studied the relationship between the glycoprotein specificity of the autoantibodies and the TPO levels, to see if indeed GPIb-specific autoantibodies increase platelet destruction via the Ashwell-Morell receptor resulting in increased TPO production *(Chapter 7)*.
- We analyzed the presence or absence of autoantibodies and the glycoprotein specificity of these antibodies in a cohort of patients with suspected vaccine-induced thrombotic thrombocytopenia (VITT) *(Chapter 9)*. A small percentage of vaccinations, e.g. with hepatitis A, varicella and measles-mumps-rubella (MMR) vaccines, results in the development of ITP. The general idea is that virus vaccine components and virus-induced molecular mimicry are causing this immune disturbance, but the exact underlying mechanism is still unknown. In the Netherlands, we were appointed as central laboratory for performing VITT diagnostics. We therefore received material from a cohort of patients suspected for VITT after vaccination with one of the SARS-coV-2 vaccines and were able to study the presence and specificity of platelet autoantibodies using the optimized glycoprotein-specific autoantibody

detection assay. Being interested if immunization against vaccine and/or viral components were triggering/increasing platelet autoantibody production, we compared the glycoprotein specificity data with data generated in the pre-covid/vaccination period.

Final remark

Over the years, we have aimed to improve diagnostics for patients with thrombocytopenia. We have studied the diagnostic value of measuring plasma TPO levels in adults and children with thrombocytopenia, and we have optimized the detection of platelet autoantibodies to provide an additional tool for the diagnosis of ITP, for research into underlying immunological causes and for optimizing treatment. With our attempts we tried to find normal plasma TPO level values, both for children and adults and to categorize the TPO levels for specific conditions causing thrombocytopenia. We studied both whole platelets and glycoprotein specific techniques to use the optimized autoantibody detection method for diagnostics, research and clinical trials.

References

1. Folman CC, von dem Borne AE, Rensink IH, et al. Sensitive measurement of thrombopoietin by a monoclonal antibody based sandwich enzyme-linked immunosorbent assay. Thromb Haemost. 1997;78(4):1262-1267. https://www.ncbi.nlm.nih.gov/pubmed/9364995
2. van der Meijden PEJ, Heemskerk JWM. Platelet biology and functions: new concepts and clinical perspectives. Nat Rev Cardiol. 2019;16(3):166-179. doi:10.1038/s41569-018-0110-0
3. Smock KJ, Perkins SL. Thrombocytopenia: an update. Int J Lab Hematol. 2014;36(3):269-278. doi:10.1111/ijlh.12214
4. VanDruff TA. Management of Select Thrombocytopenias. AACN Adv Crit Care. 2019;30(2):165-180. doi:10.4037/aacnacc2019186
5. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood. 2009;113(11):2386-2393. doi:10.1182/blood-2008-07-162503
6. Machlus KR, Italiano JE. The incredible journey: From megakaryocyte development to platelet formation. Journal of Cell Biology. 2013;201(6):785-796. doi:10.1083/jcb.201304054
7. Asquith NL, Carminita E, Camacho V, et al. The bone marrow is the primary site of thrombopoiesis. Blood. 2024;143(3):272-278. doi:10.1182/blood.2023020895
8. Grozovsky R, Giannini S, Falet H, Hoffmeister KM. Regulating billions of blood platelets: glycans and beyond. Blood. 2015;126(16):1877-1884. doi:10.1182/blood-2015-01-569129
9. Kaushansky K. Historical review: megakaryopoiesis and thrombopoiesis. Blood. 2008;111(3):981-986. doi:10.1182/blood-2007-05-088500
10. Kaushansky K. Thrombopoiesis. Semin Hematol. 2015;52(1):4-11. doi:10.1053/j.seminhematol.2014.10.003
11. Kaushansky K. Determinants of platelet number and regulation of thrombopoiesis. Hematology Am Soc Hematol Educ Program. Published online 2009:147-152. doi:10.1182/asheducation-2009.1.147
12. Wendling F, Maraskovsky E, Debili N, et al. cMpl ligand is a humoral regulator of megakaryocytopoiesis. Nature. 1994;369(6481):571-574. doi:10.1038/369571a0
13. Lok S, Kaushansky K, Holly RD, et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature. 1994;369(6481):565-568. doi:10.1038/369565a0
14. Hitchcock IS, Hafer M, Sangkhae V, Tucker JA. The thrombopoietin receptor: revisiting the master regulator of platelet production. Platelets. 2021;32(6):770-778. doi:10.1080/09537104.2021.1925102
15. Hitchcock IS, Kaushansky K. Thrombopoietin from beginning to end. Br J Haematol. 2014;165(2):259-268. doi:10.1111/bjh.12772
16. Grozovsky R, Begonja AJ, Liu K, et al. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. Nat Med. 2015;21(1):47-54. doi:10.1038/nm.3770
17. Hoffmeister KM. The role of lectins and glycans in platelet clearance. J Thromb Haemost. 2011;9 Suppl 1:35-43. doi:10.1111/j.1538-7836.2011.04276.x
18. Rumjantseva V, Hoffmeister KM. Novel and unexpected clearance mechanisms for cold platelets. Transfus Apher Sci. 2010;42(1):63-70. doi:10.1016/j.transci.2009.10.008

19. Hoffmeister KM, Falet H. Platelet clearance by the hepatic Ashwell-Morrell receptor: mechanisms and biological significance. Thromb Res. 2016;141 Suppl 2:S68-72. doi:10.1016/S0049-3848(16)30370-X
20. Li R, Hoffmeister KM, Falet H. Glycans and the platelet life cycle. Platelets. 2016;27(6):505-511. doi:10.3109/09537104.2016.1171304
21. Porcelijn L, Folman CC, Bossers B, et al. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. Thromb Haemost. 1998;79(6). doi:10.1055/s-0037-1615023
22. Houwerzijl EJ, Blom NR, van der Want JJ, Vellenga E, de Wolf JT. Megakaryocytic dysfunction in myelodysplastic syndromes and idiopathic thrombocytopenic purpura is in part due to different forms of cell death. Leukemia. 2006;20(11):1937-1942. doi:10.1038/sj.leu.2404385
23. Allan HE, Vadgama A, Armstrong PC, Warner TD. What can we learn from senescent platelets, their transcriptomes and proteomes? Platelets. 2023;34(1). doi:10.1080/09537104.2023.2200838
24. An O, Deppermann C. Platelet lifespan and mechanisms for clearance. Curr Opin Hematol. 2024;31(1):6-15. doi:10.1097/MOH.0000000000000792
25. Josefsson EC, Vainchenker W, James C. Regulation of Platelet Production and Life Span: Role of Bcl-xL and Potential Implications for Human Platelet Diseases. Int J Mol Sci. 2020;21(20). doi:10.3390/ijms21207591
26. Mason KD, Carpinelli MR, Fletcher JI, et al. Programmed anuclear cell death delimits platelet life span. Cell. 2007;128(6):1173-1186. doi:10.1016/j.cell.2007.01.037
27. Josefsson Dowling M.R. Leboi M. Kile B.T. EC. The regulation of platelet life span. In: Michelson A, ed. Platelets. 3rd ed. Elsevier Inc; 2013:51-65.
28. Zheng SS, Ahmadi Z, Leung HHL, et al. Antiplatelet antibody predicts platelet desialylation and apoptosis in immune thrombocytopenia. Haematologica. 2022;107(9):2195-2205. doi:10.3324/haematol.2021.279751
29. Krause KA, Graham BC. Glanzmann Thrombasthenia.; 2024.
30. Nurden AT, Nurden P. Inherited thrombocytopenias: history, advances and perspectives. Haematologica. 2020;105(8):2004-2019. doi:10.3324/haematol.2019.233197
31. Patel P, Michael J V., Naik UP, McKenzie SE. Platelet FcγRIIA in immunity and thrombosis: Adaptive immunothrombosis. Journal of Thrombosis and Haemostasis. 2021;19(5):1149-1160. doi:10.1111/jth.15265
32. Nagelkerke SQ, Kuijpers TW. Immunomodulation by IVIg and the Role of Fc-Gamma Receptors: Classic Mechanisms of Action after all? Front Immunol. 2015;5. doi:10.3389/fimmu.2014.00674
33. Grozovsky R, Giannini S, Falet H, Hoffmeister KM. Novel mechanisms of platelet clearance and thrombopoietin regulation. Curr Opin Hematol. 2015;22(5):445-451. doi:10.1097/MOH.0000000000000170
34. Li J, van der Wal DE, Zhu G, et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. Nat Commun. 2015;6:7737. doi:10.1038/ncomms8737
35. Provan D, Semple JW. Recent advances in the mechanisms and treatment of immune thrombocytopenia. EBioMedicine. 2022;76:103820. doi:10.1016/j.ebiom.2022.103820
36. Semple JW, Provan D. The immunopathogenesis of immune thrombocytopenia: T cells still take center-stage. Curr Opin Hematol. 2012;19(5):357-362. doi:10.1097/MOH.0b013e3283567541
37. Zufferey A, Kapur R, Semple JW. Pathogenesis and Therapeutic Mechanisms in Immune Thrombocytopenia (ITP). J Clin Med. 2017;6(2). doi:10.3390/jcm6020016

38. Semple JW, Rebetz J, Maouia A, Kapur R. An update on the pathophysiology of immune thrombocytopenia. Curr Opin Hematol. 2020;27(6):423-429. doi:10.1097/MOH.0000000000000612

39. Olsson B, Andersson PO, Jernas M, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. Nat Med. 2003;9(9):1123-1124. doi:10.1038/nm921

40. Malik A, Sayed AA, Han P, et al. The role of CD8+ T cell clones in immune thrombocytopenia. Blood. Published online February 7, 2023. doi:10.1182/blood.2022018380

41. Stasi R, Newland AC. ITP: a historical perspective. Br J Haematol. 2011;153(4):437-450. doi:10.1111/j.1365-2141.2010.08562.x

42. Brewer DB. Max Schultze (1865), G. Bizzozero (1882) and the discovery of the platelet. Br J Haematol. 2006;133(3):251-258. doi:10.1111/j.1365-2141.2006.06036.x

43. Harrington WJ, Minnich V, Hollingsworth JW, Moore C V. Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. J Lab Clin Med. 1951;38(1):1-10. https://www.ncbi.nlm.nih.gov/pubmed/14850832

44. Shulman NR, Marder VJ, Weinrach RS. SIMILARITIES BETWEEN KNOWN ANTIPLATELET ANTIBODIES AND THE FACTOR RESPONSIBLE FOR THROMBOCYTOPENIA IN IDIOPATHIC PURPURA. PHYSIOLOGIC, SEROLOGIC AND ISOTOPIC STUDIES. Ann N Y Acad Sci. 1965;124(2):499-542. doi:10.1111/j.1749-6632.1965.tb18984.x

45. Semple JW. Immunobiology of T helper cells and antigen-presenting cells in autoimmune thrombocytopenic purpura (ITP). Acta Paediatr Suppl. 1998;424:41-45. https://www.ncbi.nlm.nih.gov/pubmed/9736217

46. Schmidt DE, Heitink-Polle KMJ, Porcelijn L, et al. Anti-platelet antibodies in childhood immune thrombocytopenia: Prevalence and prognostic implications. Journal of Thrombosis and Haemostasis. 2020;18(5). doi:10.1111/jth.14762

47. von dem Borne AEGKr, Helmerhorst FM, van Leeuwen EF, Pegels HG, von Riesz E, Engelfriet CP. Autoimmune Thrombocytopenia: Detection of Platelet Autoantibodies with the Suspension Immunofluorescence Test. Br J Haematol. 1980;45(2):319-327. doi:10.1111/j.1365-2141.1980.tb07151.x

48. Porcelijn L, Huiskes E, Oldert G, et al. Detection of platelet autoantibodies revisited to identify immune thrombocytopenia. Blood. 2015;126(23):1142.

49. Porcelijn L, Schmidt DE, Oldert G, et al. Evolution and Utility of Antiplatelet Autoantibody Testing in Patients with Immune Thrombocytopenia. Transfus Med Rev. 2020;34(4). doi:10.1016/j.tmrv.2020.09.003

50. Porcelijn L, Huiskes E, Oldert G, Schipperus M, Zwaginga JJ, de Haas M. Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art. Br J Haematol. 2018;182(3). doi:10.1111/bjh.15404

51. Najaoui A, Bakchoul T, Stoy J, et al. Autoantibody-mediated complement activation on platelets is a common finding in patients with immune thrombocytopenic purpura (ITP). Eur J Haematol. 2012;88(2):167-174. doi:10.1111/j.1600-0609.2011.01718.x

52. Hauch TW, Rosse WF. Platelet-bound complement (C3) in immune thrombocytopenia. Blood. 1977;50(6):1129-1136.

53. Quach ME, Dragovich MA, Chen W, et al. Fc-independent immune thrombocytopenia via mechanomolecular signaling in platelets. Blood. 2018;131(7):787-796. doi:10.1182/blood-2017-05-784975

54. Wang Y, Chen W, Zhang W, et al. Desialylation of <i>O</i>-glycans on glycoprotein Ibα drives receptor signaling and platelet clearance. Haematologica. 2020;106(1):220-229. doi:10.3324/haematol.2019.240440
55. Grodzielski M, Goette NP, Glembotsky AC, et al. Multiple concomitant mechanisms contribute to low platelet count in patients with immune thrombocytopenia. Sci Rep. 2019;9(1):2208. doi:10.1038/s41598-018-38086-1
56. Chang M, Nakagawa PA, Williams SA, et al. Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro. Blood. 2003;102(3):887-895. doi:10.1182/blood-2002-05-1475
57. McMillan R, Wang L, Tomer A, Nichol J, Pistillo J. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. Blood. 2004;103(4):1364-1369. doi:10.1182/blood-2003-08-2672
58. Niessner H, Clemetson K, Panzer S, Mueller-Eckhardt C, Santoso S, Bettelheim P. Acquired thrombasthenia due to GPIIb/IIIa-specific platelet autoantibodies. Blood. 1986;68(2):571-576. doi:10.1182/blood.V68.2.571.571
59. Nurden AT. Acquired Glanzmann thrombasthenia: From antibodies to anti-platelet drugs. Blood Rev. 2019;36:10-22. doi:10.1016/j.blre.2019.03.004
60. Porcelijn L, Huiskes E, Maatman R, De Kreuk A, De Haas M. Acquired Glanzmann's thrombasthenia caused by glycoprotein IIb/IIIa autoantibodies of the immunoglobulin G1 (IgG1), IgG2 or IgG4 subclass: A study in six cases. Vox Sang. 2008;95(4). doi:10.1111/j.1423-0410.2008.01093.x
61. van Bladel ER, Laarhoven AG, van der Heijden LB, et al. Functional platelet defects in children with severe chronic ITP as tested with 2 novel assays applicable for low platelet counts. Blood. 2014;123(10):1556-1563. doi:10.1182/blood-2013-08-519686
62. De Cuyper IM, Meinders M, van de Vijver E, et al. A novel flow cytometry–based platelet aggregation assay. Blood. 2013;121(10):e70-e80. doi:10.1182/blood-2012-06-437723
63. Neunert C, Terrell DR, Arnold DM, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia. Blood Adv. 2019;3(23):3829-3866. doi:10.1182/bloodadvances.2019000966
64. Schipperus M, Nelson V, Amini S. Immuuntrombocytopenie (ITP): hoofdpunten uit de richtlijn van 2020 met aanbevelingen voor diagnostiek en behandeling. Nederlands tijdschrift voor Hematologie. 2021;18:20-28.
65. Kim DS. Recent advances in treatments of adult immune thrombocytopenia. Blood Res. 2022;57(S1):S112-S119. doi:10.5045/br.2022.2022038
66. DeSouza S, Angelini D. Updated guidelines for immune thrombocytopenic purpura: Expanded management options. Cleve Clin J Med. 2021;88(12):664-668. doi:10.3949/ccjm.88a.20201
67. Leandro MJ, De La Torre I. Translational Mini-Review Series on B Cell-Directed Therapies: The pathogenic role of B cells in autoantibody-associated autoimmune diseases – lessons from B cell-depletion therapy. Clin Exp Immunol. 2009;157(2):191-197. doi:10.1111/j.1365-2249.2009.03978.x
68. Roeser A, Lazarus AH, Mahévas M. B cells and antibodies in refractory immune thrombocytopenia. Br J Haematol. 2023;203(1):43-53. doi:10.1111/bjh.18773
69. Schett G, Mackensen A, Mougiakakos D. CAR T-cell therapy in autoimmune diseases. The Lancet. 2023;402(10416):2034-2044. doi:10.1016/S0140-6736(23)01126-1
70. Kaushansky K, Lok S, Holly RD, et al. Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature. 1994;369(6481):568-571. doi:10.1038/369568a0

71. Arnold DM, Nazy I, Clare R, et al. Misdiagnosis of primary immune thrombocytopenia and frequency of bleeding: lessons from the McMaster ITP Registry. Blood Adv. 2017;1(25):2414-2420. doi:10.1182/bloodadvances.2017010942

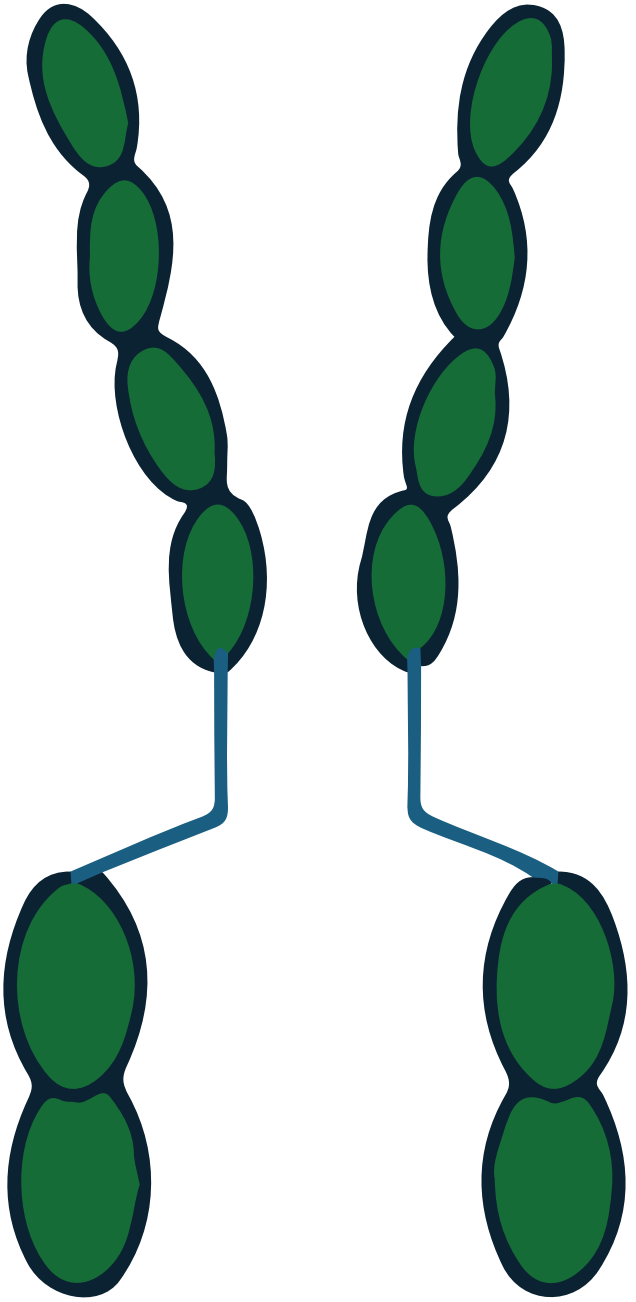
72. von dem Borne AE, Verheugt FW, Oosterhof F, von Riesz E, de la Riviere AB, Engelfriet CP. A simple immunofluorescence test for the detection of platelet antibodies. Br J Haematol. 1978;39(2):195-207. doi:10.1111/j.1365-2141.1978.tb01089.x

73. Shibata Y, Juji T, Nishizawa Y, Sakamoto H, Ozawa N. Detection of platelet antibodies by a newly developed mixed agglutination with platelets. Vox Sang. 1981;41(1):25-31. doi:10.1111/j.1423-0410.1981.tb01007.x

74. Kelton JG, Powers PJ, Carter CJ. A prospective study of the usefulness of the measurement of platelet-associated IgG for the diagnosis of idiopathic thrombocytopenic purpura. Blood. 1982;60(4):1050-1053. https://www.ncbi.nlm.nih.gov/pubmed/6889450

75. Allen DL, Chapman J, Phillips PK, Ouwehand WH. Sensitivity of the platelet immunofluorescence test (PIFT) and the MAIPA assay for the detection of platelet-reactive alloantibodies: a report on two U.K. National Platelet Workshop exercises. Transfus Med. 1994;4(2):157-164. doi:10.1111/j.1365-3148.1994.tb00257.x

76. Vrbensky JR, Moore JE, Arnold DM, Smith JW, Kelton JG, Nazy I. The sensitivity and specificity of platelet autoantibody testing in immune thrombocytopenia: a systematic review and meta-analysis of a diagnostic test. J Thromb Haemost. 2019;17(5):787-794. doi:10.1111/jth.14419



02

CHAPTER 2

The diagnostic value of trombopoietin level measurements in thrombocytopenia

Porcelijn L, Folman CC, Bossers B, Huiskes E, Overbeeke MA, v d Schoot CE, de Haas M, van dem Borne AE. The diagnostic value of thrombopoietin *level* measurements in thrombocytopenia. Thromb Haemost. 1998 Jun;79(6):1101-5.

The Diagnostic Value of Thrombopoietin level Measurements in Thrombocytopenia

Leendert Porcelijn[1], Claudia C. Folman[1], Masja de Haas[1], Elly Huiskes[1], Bernadette Bossers[1], Marijke A.M. Overbeeke[1], C. Ellen v.d. Schoot[1] and Albert E.G. Kr. v.d. Borne.[1,2]

[1]Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam
[2]Department of Hematology, Academic Medical Centre, Amsterdam

Summary
It has been reported that blood trombopoietin (TPO) levels can discriminate between thrombocytopenia due to increased platelet destruction and decreased platelet production. With our TPO ELISA and a glycocalicin ELISA we analysed a large group of patients in detail and could confirm and amplify the above notion in detail.

TPO levels were determined in plasma from 178 clinically and serologically well-defined thrombocytopenic patients: 72 patients with idiopathic autoimmune thrombocytopenia (AITP), 29 patients with secondary AITP, 5 patients with amegakaryocytic thrombocytopenia and 72 patients who suffered from various diseases (46 in whom megakaryocyte deficiency was not and 26 in whom it was expected). In addition, we measured the level of glycocalicin as a marker of total body mass of platelets.

In all patients with primary AITP and secondary AITP, TPO levels were within the normal range or in some (n=7) cases only slightly increased. The level of glycocalicin was not significantly different from that of the controls (n=95). The patients with amegakaryocytic thrombocytopenia had strongly elevated TPO levels and significantly decreased glycocalicin levels. Similarly, among the 72 thrombocytopenic patients with various disorders, elevated TPO levels were only found in patients in whom platelet production was depressed. The mean level of glycocalicin in these patients was decreased compared to that in controls and patients with AITP, but was not as low as in patients with amegakaryocytic thrombocytopenia.

In conclusion, all patients with depressed platelet production had elevated levels of circulating TPO, whereas the TPO levels in patients with an immune-mediated thrombocytopenia were mostly within the normal range. Therefore, measurement of plasma TPO levels provides valuable diagnostic information for the analysis of thrombocytopenia in general..
Moreover, treatment with TPO may be an option in AITP.

Introduction

Recent studies indicate that the measurement of the serum level of thrombopoietin (TPO) might be useful to discriminate between patients with thrombocytopenia due to increased platelet destruction, and those with a deficient platelet production.1-3 TPO levels were found to be not or only mildly increased in patients with autoimmune thrombocytopenia, drug-induced immune thrombocytopenia, post transfusion purpura and X-linked hereditary thrombocytopenia (a variant of Wiskott-Aldrich syndrome).1-3 In patients with bone-marrow hypoplasia TPO levels were significantly increased.1-3 However, it should be emphasized that in all these studies only sera were analysed. We found serum TPO levels in normal individuals to be 3.5 times higher than in plasma, due to the release of TPO from platelets during coagulation.4 Moreover, in the three published studies the number of analysed patients was small and the patient groups were not always clinically and serologically well defined.

To evaluate the value of plasma TPO levels in the differential diagnosis of thrombocytopenia, we analysed a large group of 178 patients in detail. Clinical data were obtained, TPO serum and plasma levels determined and serological tests were performed. Furthermore as a measure of total platelet mass glycocalicin levels were determined.5,6

Our results show that only in patients with a suppressed platelet production plasma TPO levels were found to be significantly increased. Therefore, the measurement of TPO levels is an important diagnostic tool for the evaluation of thrombocytopenia. Moreover, it could help in selecting those patients who might benefit from TPO therapy.

Materials and methods

Patient samples

EDTA-anticoagulated blood and serum samples from patients suspected of having autoimmune thrombocytopenia were sent to our laboratory for diagnostic evaluation. Informed consent and clinical data were obtained via the referring physicians by a questionnaire and/or an interview on the telephone. In this way, clinical data were collected from 217 of 377 analysed patients with various forms of thrombocytopenia. Of these 217 patients, 23 pregnant women (mostly with mild thrombocytopenia) were excluded. Gestational thrombocytopenia, thrombocytopenia associated with pregnancy-induced hypertension and the HELLP syndrome account for most of these cases and differentiation from AITP in pregnancy is difficult.7,8 Only patients with a platelet count of less than 100 x 109/L were included. All together 178 patients were analysed. The male/female

ratio was 0.76, the mean age was 55 ± 21 years (age range from 4 to 91 years). Based on the clinical data, four groups of thrombocytopenic patients were distinguished. Patients with autoimmune thrombocytopenic purpura (AITP) (n=72) were defined, in accordance with the recommendation of the American Society of Hematology (ASH)11, by their medical history, physical examination, complete blood count and examination of the peripheral blood smear. Secondary AITP patients (n=29) were defined as patients with isolated thrombocytopenia and an autoimmune disorder frequently associated with autoimmune thrombocytopenia, such as SLE, RA or autoimmune thyroiditis. In 51 AITP and secondary AITP patients bone marrow aspirates were taken and evaluated by the referring physicians. The bone-marrow was normocellular with normal or increased numbers of megakaryocytes was obtained in all patients, which is in agreement with the diagnosis of AITP.

Patients with amegakaryocytic thrombocytopenia (n=5) were AITP patients (as defined by the ASH) but with a severely decreased number of megakaryocytes in the bone marrow.

All other thrombocytopenic patients, suffering from a variety of diseases (table 1), were classified as the miscellaneous group (n=72). Based on clinical data the patients in the miscellaneous group were divided in patients without (A) and with (B) megakaryocyte deficiency (Table 1)

Table 1 Miscellaneous group

miscellaneous group A			miscellaneous group B
Neoplastic diseases (n=34)			
CLL		4	4 (myelosuppressive drugs)
ALL		1	1 (myelosuppressive drugs)
NHL		3	5 (myelosuppressive drugs)
Myelodysplasia*		1	1 (aplasia)
Breast cancer			3 (myelosuppressive drugs)
Lung neoplasms			1 (myelosuppressive drugs)
Intestinal tumors			2 (myelosuppressive drugs)
Prostatic cancer		1	
Melanoma		1	
Urinary tract tumor		1	
Primary tumor unknown		1	
Neoplasms of the brain		1	1 (myelosuppressive drugs)
Multiple myeloma		1	
Waldenström's macroglobul.			1 (infiltration)
Infections (n=11)			
viral (n=9)			
	HIV	2	2 (myelosuppressive drugs)
	EBV	2	
	HCV	1	
	CMV	1	
	unknown**	1	
bacterial (n=2)			
Borrelia		1	
Urinary tract infection		1	
Drug induced (n=4)			
peripheral (n=2)			
	Salazopyrin	1	
	Fraxiparin	1	
myelotoxic (n=2)			
	Imuran		1 (myelosuppressive drugs)
	Methotrexate		1 (myelosuppressive drugs)
Liver diseases		7	
Cardio-vascular diseases		5	
Renal disorders		2	
Diabetes mellitus		2	
Aplastic anemia			1 (aplasia)
Bone marrow transplantation			2 (aplasia)
Alpha-thalassemia		1	
Pernicious anemia/hypersplenism/ portal hypertension		1	
diffuse intravascular coagulation		1	

* *one patient with a normal number of megakaryocytes in the bone marrow*

** *suspected for viral infection*

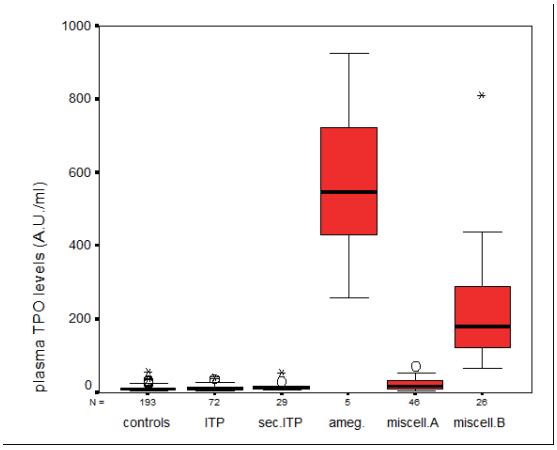


Fig. I Plasma TPO levels in different patiënt groups with thiombocytopenia. AITP, autoimmune thiombocytopenia; SAITP, secondary autoimmune thrombocytopenia; Ameg., amegakaryocytic thiombocytopenia; Miscell.A,`miscellaneous thrombocytopenic patients without expected megakaryocyte deficiency; Miscell.B, miscellaneous thrombocytopenic patients with expected megakaryocyte deficiency

Serological analysis

The direct platelet immunofluorescense test (PIFT), indirect eluate PIFT and direct monoclonal antibody immobilization of platelet antigens assay (MAIPA) were performed as described by von dem Borne et al9 and Kiefel et al10, respectively. The monoclonal antibodies (MoAb) used in the MAIPA were CLBthromb/1 (CD41, anti-GPIIb), MB45 (CD42a, anti-GPIX), SW16 (CD42d, anti-GPV), 10G11 (CD49b, anti-GPIa/IIa) or P58 (CD36, anti-GPIV)). All MoAb were from our institute.
Enough platelets could not be isolated in all cases to perform a MAIPA with all the listed MoAbs. In 90 of 178 (33 of 72 AITP, 16 of 29 SAITP and 41 of 72 miscellaneous) cases the MAIPA was performed.

TPO ELISA

A solid phase sandwich ELISA for the measurement of plasma TPO concentrations was performed as previously described.4 Briefly, a mixture of two non-crossreactive MoAb was coated on a microtiter plate. Plates were blocked and washed, after which samples were incubated together with a third biotinylated MoAb. A streptavidin horseradish peroxidase conjugate and a signal amplification system were used for the final colorimetric reaction. A pool of EDTA-anticoagulated plasma derived from thrombocytopenic patients with a high TPO level, was used as a standard. The first dilution of this standard was arbitrarily set at 100 A.U. Normal TPO levels, as determined in a population of 193 healthy individuals, ranged from 4 to 32 A.U. (2.5th- 97.5th percentile). Serum Tpo levels were on average 3.4 times higher.

Glycocalicin ELISA

MoAb MB45 (CLB, Amsterdam, The Netherlands) was coated overnight on a 96 wells microtiter plate (Nunc Immunoplate Maxisorp, Rockslide Denmark) at a concentration of 2 µg/ml in 100 µl 0.1M carbonate buffer pH9.6. Plates were washed with PBS/0.02% Tween (v/v) and remaining binding sites were blocked for 30 minutes with 150 µl PBS containing 2% pasteurized cows' milk. Subsequently, plates were washed 5 times and a 50 µl sample (or standard) diluted in High Performance Elisabuffer (CLB, Amsterdam, The Netherlands) was incubated for 2 hours together with 50 µl biotinylated MoAb MB15 (CLB, Amsterdam, The Netherlands) (1lg/ml). Again, plates were washed and incubated for 30 minutes with 100 µl streptavidin polyhorseradish peroxidase (1:10.000; CLB, Amsterdam, The Netherlands) in PBS with 2% pasteurized cows' milk. A colorimetric reaction was obtained by addition of 100 µl subtrate TMB (0.1mg/ml) in substrate buffer (0.11M NaAc pH5.5 with 0.003% H2O2) after plates were washed. After 15 minutes the reaction was stopped with 100 µl H2SO4. The absorbance at 450 nm was measured in a Titertek multiscan Elisa-reader (Flow laboratory, Rockville, MD). All incubations were performed at RT under shaking conditions. Supernatant of a platelet concentrate was used as a standard. Concentrations of glycocalicin (GC) were expressed in Arbitrary Units. Normal plasma GC values as determined in 95 healthy individuals were between 144-444 A.U./ml (mean ± twice the std.).

Statistical analysis

Statistical analysis was performed in SPSS for Windows, release 6.1.3 (SPSS Inc.). For comparison of groups the Mann-Whitney U - Wilcoxon Rank Sum W Test was used. The correlation between two variables was calculated with Spearman correlation coefficients.

Results

TPO and glycocalicin levels

Plasma TPO levels in AITP and SAITP patients were found to be within the normal range in most cases (89%) (13 ± 10 A.U., mean ± std., range 2 A.U to 54 A.U.) (fig.1). Four patients in the AITP group and three patiens in the SAITP group had a slightly increased TPO level (36, 39, 42, 41 A.U./ml and 54, 53, 53 A.U./ml, respectively), whereas in four patients in the AITP group the TPO level was lower than 4 A.U./ml (one 1 A.U./ml and three 3 A.U./ml). As shown in figure 1, the mean level of TPO was slightly higher in the group of AITP patients as well as the SAITP group, as compared to the controls. This difference was statistically significant (p=0.03 and p=0.01, respectively). There was no correlation between the platelet count and the plasma TPO level in either AITP or SAITP. Serum TPO levels ranged from 6 A.U. to 81 A.U.(28 ± 14, mean±std.). The serum/plasma ratio in these two groups of patients was 2 ± 0.9 and 2 ± 0.8 (mean ± std.),

respectively, which is significantly lower than the ratio found in controls (3 ± 0.6, mean ± std.)($p<0.001$). There was no correlation between either the serum TPO level or the serum/plasma ratio and the platelet number.

As shown in figure 1, all five patients, classified as amegakaryocytic thrombocytopenia, had strongly elevated TPO levels (range 258-927 A.U./ml). Furthermore in all patients in the miscellaneous B group whose medical data indicated that a decreased hematopoiesis was the cause of their thrombocytopenia, TPO levels were clearly increased (231 ± 160 A.U./ml, mean ± std, range 66-811 A.U./ml, n=26)(fig.1). These patients either had a malignant infiltration of the bone marrow and/or were receiving myelotoxic therapy (n=22) or suffered from bone marrow aplasia (n=4, one aplastic anemia, two post transplant bone-marrow failure and one myelodysplasia). In contrast the TPO levels of most patients included in the miscellaneous group A, were within the normal range, although the mean TPO level in this group was somewhat higher compared to that in controls (21 ± 16 A.U/ml, mean ± std, range 2-72 A.U/ml).

Figure 2 shows for the patients in the miscellaneous group that analysis of the number of megakaryocytes in bone marrow aspirates, which was performed in 36 of the 72 patients, correlated with the TPO level. In all, but one, decreased numbers of megakaryocytes were counted in the bone-marrow aspirate.

Most AITP and SAITP patients had normal levels of plasma glycocalicin (Fig.3). The level of glycocalicin in the plasma of patients was not significantly different from that in controls. In the amegakaryocytic thrombocytopenia patients the glycocalicin levels were clearly decreased (fig.3). Also in the miscellaneous group with megakaryocyte deficiency (group B), glycocalicin levels were significantly decreased ($p=0.042$). In the miscellaneous group without megakaryocyte deficiency (group A) the glycocalicin levels were mildly increased ($p=0.046$) (fig.3).

Serological analysis

Autoantibodies were detected by the direct PIFT in 66 of the 101 (65%) AITP and SAITP patients. This was not different in the two AITP groups: 46 of 72 (64%) AITP patients were positive versus 20 of 29 (69%) SAITP patients. The MAIPA was positive in 39% of the patients suffering from AITP. In all but one of the AITP patients with a positive MAIPA the PIFT was also positive. The autoantibodies were directed against the GP IIb/IIIa complex (n=5), the GP Ib/IX complex (n=2), GP V (n=6) and combinations of these three GP complexes (n=6) (table 2). In 35% of the patients of the miscellaneous group a positive result with the PIFT was obtained and only in five of 41 (12%) miscellaneous patients a positive MAIPA was found (Table 2).

There was no correlation between the presence or absence of detectable platelet autoantibodies in either the PIFT or the MAIPA, and the TPO level (data not shown).

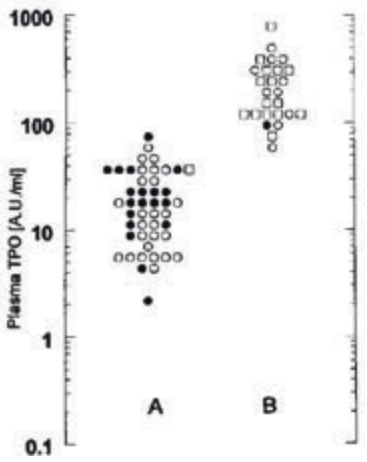


Fig. 2 Plasma TP0 levels in patients fmming the miscellaneous group. A) without expected megaka1yocyte deficiency (n = 46), B) with expected megaka1yocyte deficiency (n = 26); 0, BM aspiration not pe1f01med; •, nor mal or increased megakaiyocyte number in BM aspirate; D, decreased mega karyocyte number in BM aspirate

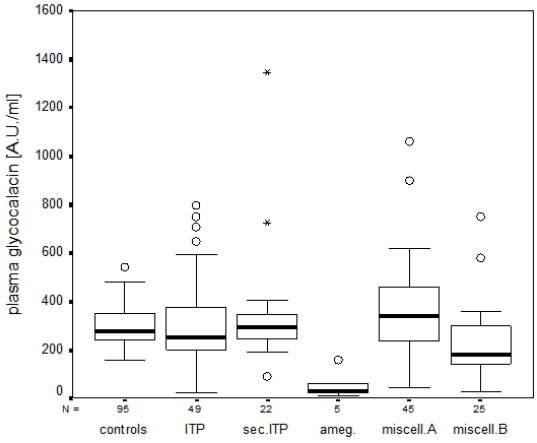


Fig. 3 Plasma glycocalicin levels in different thrombocytopenic patient groups. AITP, autoimmune thrombocytopenia; SAITP, secondary autoimmune thrombocytopenia; Ameg., amegakaryocytic thrombocytopenia; Miscell.A, miscellaneous thrombocytopenic patients without expected megaka1yocyte deficiency; Miscell.B, miscellaneous thrombocytopenic patients with expected megakatyocyte deficiency

Table 2: Direct MAIPA results

Performed*	AITP 31/72	SAITP 16/29	Miscellaneous 26/72
GPIIb/IIIa	4	1	
GPIb/IX	1	1	1
GPV	1	5	1
GPIa/IIa			1
Combination**	6		2
Negative	19/31 (61%)	9/16 (56%)	21/26 (81%)
Total positive	12/31 (39%)	7/16 (44%)	5/26(19%)

* at least the MAIPA performed for GPIIb/IIIa, GPIb/IX and GPV
** all combinations of GPIIb/IIIa, GPIb/IX and GPV

Discussion

In this study, we confirm and extend the results of earlier studies1-3, that TPO levels in AITP patients are normal or only mildly increased, while they are strongly increased in patients with suppressed megakaryocytopoiesis. In contrast to other published studies, we measured TPO levels in EDTA-anticoagulated plasma samples in stead of in serum, because we previously found that serum TPO levels are 3.4 times higher than plasma levels due to TPO release from the platelets during clotting.4 In our opinion, plasma levels are therefore a more accurate measure for circulating TPO than serum TPO levels.

TPO is the major regulator of platelet production and is normally produced mainly by the liver and the kidney.12-14 Early hematopoietic progenitor cells, all cells of the megakaryocyte lineage and platelets express the receptor for TPO, MPL.15 Binding of plasma TPO by circulating platelets has been proposed to be the main regulatory mechanism of plasma TPO level.16-18 This conclusion was based on static levels of TPO-encoding mRNA in the liver and kidney of mice made thrombocytopenic.16,17 However, recent data showed that some upregulation of TPO-encoding mRNA in bone-marrow stromal cells may occur in thrombocytopenic mice and humans.13,14 TPO produced locally, in the bone-marrow environment, may largely account for the increased numbers of megakaryocytes found in 27 of the 51 analyzed patients with AITP in this study (data not shown).

Although thrombocytopenia may induce an elevated production of TPO in the bone marrow, this TPO does not seem to be capable of enhancing platelet

production in AITP patients. Circulating TPO presumably is trapped by the platelets and/or megakaryocytes and is subsequently destroyed in these cells or in the spleen.18 The decreased serum/plasma TPO ratio's in the AITP groups could be explained by the low platelet counts which results in a lower release of TPO upon coagulation.

It has been agreed that the lifespan of the platelets in AITP patients is shortened. However, mostly platelet production seems to be within normal limits and, platelet production is increased or decreased in only a small percentage of AITP patients.19-21 This is in accordance with the finding that glycocalicin levels in AITP patients are not significantly different from those in controls, because glycocalicin levels seem to reflect the total body mass of platelets5,6 In our study, only in a small percentage of AITP cases (both from the primary and the secondary group), an increased (11 of 71 (15%)) or decreased (5 of 71 (7%)) glycocalicin level was found. All other AITP patients had normal glycocalicin levels and thus a normal platelet production. Severely decreased glycocalicin levels were measured in plasma from patients with amegakaryocytic thrombocytopenia only. The glycocalicin levels were also decreased, although to a lesser extent, in plasma of patients suffering from diseases accompanied by a suppressed megakaryocytopoiesis (miscellaneous group B). Thus, our study confirms the value of glycocalicin measurement as a marker for total platelet mass. However, the levels in individual patients were found to be too widely spread. This makes plasma glycocalicin measurement less useful for diagnostic purposes.

The absence of elevation of circulating TPO levels in immune-mediated thrombocytopenia, can be considered to be a relative endogenous TPO deficiency, and therefore may be of clinical importance. It might indicate that in AITP treatment with TPO is relevant. Hematopoietic growth factors are already used in the treatment of autoimmune mediated blood cell destruction. Granulocyte Colony Stimulating Factor has been successfully used to obtain normal neutrophil counts in several cases of autoimmune neutropenia.22-28 These patients showed an increase in the number of neutrophils and a decrease in the titer of neutrophil-specific autoantibodies, most probably by endogenous consumption of the autoantibodies.22-28 The same mechanism might apply in AITP.

The diagnosis of AITP remains a clinical diagnosis. The suggested diagnostic evaluation of AITP, based principally on medical history, physical examination and examination of the peripheral blood is confirmed by the exclusion of other causes of thrombocytopenia.11 According to the American Society of Hematology guidline bone marrow evaluation is not found to be necessary.

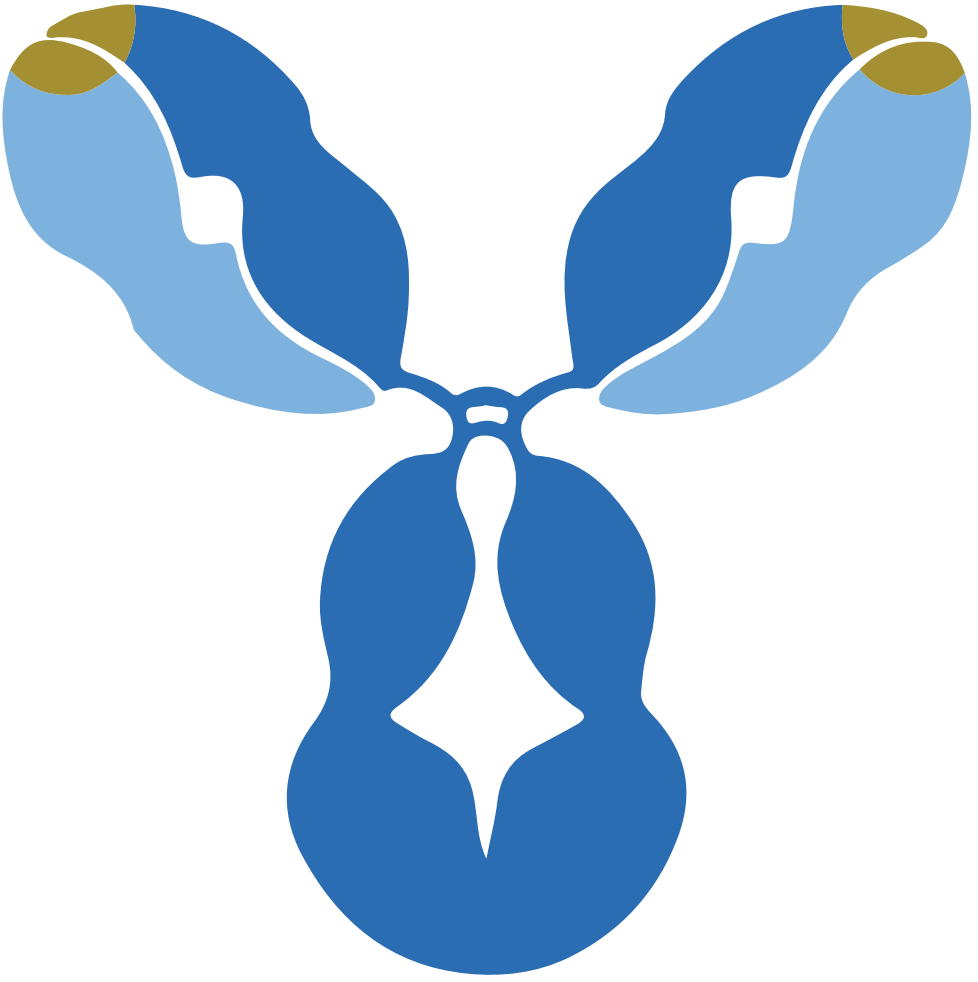
However, cases of amegakaryocytic thrombocytopenia would be missed in this way. We show here that measurement of the TPO level may give additional information in the screening of thrombocytopenic patients, to exclude amegakaryocytosis and a depressed megakaryocyte/platelet formation as the cause of the thrombocytopenia. The sensitivity of serological tests was found to be quite low. Platelet-bound antibodies were detected with the PIFT in 63%, and with the MAIPA in only 39% of clinically well-defined AITP patients. In all cases of AITP we found normal or only mildly increased TPO levels, whereas all amegakaryocytic thrombocytopenia patients and patients suspected of having megakaryocyte deficiency showed strongly increased TPO levels.
In conclusion, our study confirms that measurement of TPO levels is an important diagnostic tool for the evaluation of thrombocytopenic patients. The low TPO levels in AITP patients indicate that AITP patients might benefit from the administration of TPO.

Acknowledgement
We want to thank Christine Kramer for her technical assistance.

References

1. Tahara T, Usuki K, Sato H, et al. A sensitive sandwich ELISA for measuring thrombopoietin in human serum: serum thrombopoietin levels in healthy volunteers and in patients with haemopoietic disorders. Br J Haematol 1996; 93: 783-788.
2. Emmons RV, Reid DM, Cohen RL, et al. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. Blood 1996; 87: 4068-4071.
3. Kosugi S, Kurata Y, Tomiyama Y, et al. Circulating thrombopoietin level in chronic immune thrombocytopenic purpura. Br J Haematol 1996; 93: 704-706.
4. Folman CC, von dem Borne AEGK, Rensink IHJAM, et al. Sensitive measurement of thrombopoietin by a monoclonal antibody based sandwich enzyme-linked immunosorbent assay. Thromb Haem. 1997 (in press).
5. Steinberg MH, Kelton JG, Coller BS. Plasma glycocalicin: an aid in the classification of thrombocytopenic disorders. N Engl J Med 1987; 317: 1037-1042.
6. Beer JH, Büchi L, Steiner B. Glycocalicin: a new assay-the normals plasma levels and its potential usefulness in selected diseases. Blood 1994; 83: 691-702.
7. Burrows RF, Kelton JG. Fetal thrombocytopenia and its relation to maternal thrombocytopenia. N Engl J Med 1993; 329: 1463-1466.
8. Kobayashi M, Laver JH, Kato T, Miyazaki H, Ogawa M. Thrombopoietin supports proliferation of human primitive hematopoietic cells in synergy with steel factor and/or interleukin-3. Blood 1996; 88: 429-436.
9. von dem Borne AEGK, Verheugt FWA, Oosterhof F, von Riesz E, Brutel de la Riviere A, Engelfriet CP. A simple immunofluorescence test for the detection of platelet antibodies. Br J Haematol 1978; 39: 195-207.
10. Kiefel V, Santoso S, Weisheit M. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): A new tool for the identification of platelet reactive antibodies. Blood 1987; 70: 1722-1726.
11. George JN, Woolf SH, Raskob GE. thrombocytopenic purpura: A practice guideline developed by explicit methods for the American Society of Hematology. Blood 1996; 88: 3-40.
12. Gurney AL, Kuang WJ, Xie MH, Malloy BE, Eaton DL, de Sauvage FJ. Genomic structure, chromosomal localization, and conserved alternative splice forms of thrombopoietin. Blood 1995; 85: 981-988.
13. Nagahisa H, Nagata Y, Ohnuki T. Bone marrow stromal cells produce thrombopoietin and stimulate megakaryocyte growth and maturation but suppress proplatelet formation. Blood 1996; 87: 1309-1316.
14. McCarty JM, Sprugel KH, Fox NE, Sabath DE, Kaushansky K. Murine thrombopoietin mRNA levels are modulated by platelet count. Blood 1995; 86: 3668-3675.
15. Debili N, Wendling F, Cosman D, Titeux M, Florindo C, Dusanter-Fourt I. The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. Blood 1995; 85: 391-401.
16. Stoffel R, Wiestner A, Skoda RC. Thrombopoietin in thrombocytopenic mice: evidence against regulation at the mRNA level and for a direct regulatory role of platelets. Blood 1996; 87: 567-573.
17. Fielder PJ, Gurney AL, Stefanich E. Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets. Blood 1996; 87: 2154-2161.

18. Kuter DJ, Rosenberg RD. The reciprocal relationship of thrombopoietin (c-Mpl ligand) to changes in the platelet mass during Busulfan-induced thrombocytopenia in the rabbit. Blood 1995; 85: 2720-2730.
19. Heyns AP, Badenhorst PN, Lotter MG, Pieters H, Wessels P, Kotze HF. Platelet turnover and kinetics in immune thrombocytopenic purpura: Results with autologous 111In-labeled platlets and homologous 51Cr-labeled platelets differ. Blood 1986; 67: 86-92.
20. Ballem PJ, Segal GM, Stratton JR, Gernsheimer T, Adamson JW, Slichter SJ. Mechanisms of thrombocytopenia in chronic autoimmune thrombocytopenic purpura. J Clin Invest 1987; 80: 33-40.
21. Siegel RS, Coleman RE, Kurlander R, Rosse WF. Platelet turnover: An important factor in predicting response to splenectomy in autoimmune thrombocytopenic purpura. Blood 1984; 64: 873
22. Ganser A, Ottmann OG, Erdmann H, Schulz G, Hoelzer D. The effect of recombinant human granulocyte-macrophage colony-stimulating factor on neutropenia and related morbidity in chronic severe neutropenia. Ann Intern Med 1989; 111: 887-892.
23. Klumpp TR, Herman JH, Macdonald JS, Schnell MK, Mullaney M, Mangan KF. Autoimmune neutropenia following peripheral blood stem cell transplantation. Am J Hematology 1992; 41: 215-217.
24. Stroncek DF, Shapiro RS, Filipovich AH, Plachta LB, Clay ME. Prolonged neutropenia resulting from antibodies to neutrophil-specific antigen NB1 following marrow transplantation. Transfusion 1993; 33: 158-163.
25. Takahashi K, Taniguchi S, Akashi K. Human recombinant granulocyte colony-stimulating factor for the treatment of autoimmune neutropenia. Acta Haematol 1991; 86: 95-98.
26. Taniguchi S, Shibuya T, Harada M, Niho Y. Decreased levels of myeloid progenitor cells associated with long-term administration of recombinant human granulocyte colony-stimulating factor in patients with autoimmune neutropenia. Br J Haematology 1993; 83: 384-387.
27. Kuijpers TW, de Haas M, de Groot CJ, von dem Borne AEGK, Weening RS. The use of rh-G-CSF in chronic autoimmune neutropenia: reversal of autoimmune phenomena, a case history. Br J Haematology 1996; 94: 464-469.
28. Vlasveld LT, de Haas M, Ermens AAM, Porcelijn L, van Marion-Kievit JA, von dem Borne AEGK. G-CSF-induced decrease of the anti-granulocyte autoantibody levels in a patient with autoimmune granulocytopenia. Ann Haem 1997 (in press).



03

CHAPTER 3

Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia.

Porcelijn L, Polman CC, de Haas M, Kanhai HH, Murphy MP, von dem Borne AE, Bussel JB. Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia. pediatr Res. 2002 Jul;52(1):105-8.

Fetal and Neonatal Thrombopoietin Levels in Alloimmune Thrombocytopenia

L.Porcelijn[1], C.C. Folman[1], M. de Haas[1], H.H.H. Kanhai[2], M.F. Murphy[3], A.E.G. Kr. vd Borne[4], J.B. Bussel[5]

[1] CLB, Sanquin Blood Supply Foundation, Amsterdam. [2]Leiden University Medical Center [3]John Radcliffe Hospital, Oxford [4] Academical Medical Center, Amsterdam. [5]Weill Medical College of Cornell University, New York.

Abbreviations

EDTA	Ethylenediaminetetra-acetate
HDN	Haemolytic disease of the newborn
HPA	Human platelet antigens
ITP	Idiopathic thrombocytopenic purpura
IVIg	Intravenous immunoglobulins
NAIT	Neonatal alloimmune thrombocytopenia
Tpo	Thrombopoietin

Summary

Thrombopoietin (Tpo) is the main hematopoietic growth factor for platelet production. Plasma Tpo levels in autoimmune thrombocytopenic (ITP) patients are normal or slightly elevated. Although thrombocytopenia exists, Tpo levels are not increased because the produced megakaryocytes and platelets can bind circulating Tpo, thereby normalizing Tpo levels. In this report plasma samples from fetuses and neonates with neonatal alloimmune thrombocytopenia (NAIT), a different form of immune thrombocytopenia, were measured. Umbilical cord samples of 50 fetuses before treatment because of severe thrombocytopenia, 51 fetuses after treatment and peripheral blood samples of 21 untreated newborns with NAIT were analyzed. As controls, plasma Tpo levels were determined in 21 umbilical cord samples of 14 non-thrombocytopenic fetuses with hemolytic disease (HDN) due to red blood cell alloimmunization and in umbilical cord samples of 51 healthy newborns. The values were also compared with the plasma Tpo levels in 193 healthy adults.

Mean Tpo levels from the groups of fetuses and neonates including both NAIT and control plasma were slightly but significantly elevated compared to levels in healthy adults. Tpo levels in NAIT samples were not significantly different from the levels in HDN samples or in samples from healthy newborns. Thus, like in ITP, in NAIT patients normal Tpo levels are present.

Keywords: thrombopoietin, Neonatal alloimmune thrombocytopenia, NAIT

Introduction

Thrombopoietin is the main haematopoietic growth factor for the megakaryocytic lineage and therefore for platelet production. In adults Tpo is mainly produced by the liver and kidney.1 The concentration of Tpo in blood has been found to largely depend on the number of megakaryocytes in the bone marrow and platelets in the peripheral blood. The plasma level of Tpo is regulated by binding of Tpo to its receptor (mpl) expressed by platelets and megakaryocytes. 2-6 After binding, Tpo is eventually destroyed together with the platelets. We and others have shown the diagnostic value of measurement of plasma Tpo levels for discrimination between thrombocytopenia caused by megakaryocyte and platelet production failure (highly elevated Tpo levels) and thrombocytopenia caused by elevated platelet destruction observed in autoimmune thrombocytopenia (normal or only slightly elevated Tpo levels).7-11 In the latter disorder, megakaryocyte numbers in the marrow are normal or increased as is platelet production. Because sufficient numbers of platelets are produced, even though their destruction may be rapid, Tpo levels are comparable to that in healthy individuals.

NAIT occurs in about 1 in 1000 births and results from maternal alloimmunization against platelet antigens present on fetal platelets but absent on maternal platelets. Human Platelet Antigen 1a (HPA-1a) is the most frequently offending antigen, accounting for approximately 85% of cases of severe thrombocytopenia. Because of severe thrombocytopenia in utero, in up to 10% of cases intracerebral haemorrhage occurs, often leading to severe neurological sequelae or death.12-14 As in autoimmune thrombocytopenia, in NAIT, fetal platelets are destroyed as a result of antibody binding. Furthermore, it is suggested that reduced megakaryocyte platelet production, or even megakaryocyte destruction, resulting from anti-HPA-1a antibody binding might also contribute to the thrombocytopenia in NAIT patients.15-17 In the current study, Tpo levels were measured in 129 fetal and neonatal NAIT plasma samples and 72 fetal and neonatal control plasma samples. We found Tpo levels in fetuses and neonates with NAIT to be not significantly different from the levels in fetal and neonatal controls.

Materials and methods

Patients

The study was performed with approval of all the involved institutes and all measurements were performed after informed consent.

Plasma Tpo levels were measured in samples from fetuses with NAIT (n=113), full term newborns with NAIT (n=16) and in nonthrombocytopenic fetuses and neonates, i.e. 21 first or subsequent umbilical cord samples of 14 fetuses with HDN and 51 umbilical cord plasma samples of healthy, full term newborns (Table 1). The samples were collected in New York (Weill Medical College), Leiden

(Leiden University Medical Center) and Oxford (John Radcliffe Hospital). Tpo measurement was performed in the Central Laboratory of the Blood Transfusion Service, Sanquin Diagnostics, Amsterdam. All NAIT cases included in this study involved HPA-1a alloantibodies. Sixteen neonatal samples and 50 umbilical cord samples of 50 fetuses were from NAIT patients who were not treated, Sixty-three umbilical cord samples of 51 fetuses were from NAIT patients who received treatment in utero via administration to their mothers; 40 of these 63 samples were from 34 fetuses who were also included in the untreated group. Treatment consisted of IVIg (1 gram/Kg/wk) with or without corticosteroids (prednisone 1 mg/Kg/day or dexamethason 1.5 mg/d) administered to the mother during pregnancy and/or intra-uterine platelet transfusions administered to the fetus, after umbilical cord sampling. Samples were drawn, at 24±3 (mean±std.) wk of pregnancy for the untreated fetal group, 33±4 wk of pregnancy for the treated fetal group 28±4 wk of pregnancy for the HDN group, at d 1-7 post partum for the neonatal NAIT group and directly post partum for the neonatal control group. If treatment was necessary and consisted of intrauterine platelet transfusions, samples were drawn before transfusions were given (this was also true for the patients included in the untreated fetal NAIT group). Fetal and neonatal blood samples were aspirated in syringes with EDTA or heparin. Platelet counts were performed with a coulter counter (Beckman Coulter, Inc, Fullerton, CA, U.S.A.). Methods used for aspiration of samples and for intra-uterine platelet transfusions were comparable in the different institutes and are described earlier.18,19 No problems occurred that could be related to the bloodsampling.

Plasma Tpo levels of 193 healthy adults to use as controls were measured previously in another study.20

Tpo ELISA

A solid phase sandwich ELISA for measurement of plasma Tpo concentrations was performed as previously described.20 Normal Tpo levels, as determined in a population of 193 healthy individuals, were 11±8 A.U./ml (range 4-32 A.U., 2.5th- 97.5th percentile). One AU equals 9 pg of recombinant Tpo (Research Diagnostics Inc. Flanders NJ USA)

Statistical analysis

Statistical analysis was performed in SPSS for Windows, release 6.1.3 (SPSS Inc., Chicago, IL, U.S.A.).

For comparison of groups the Kruskal Wallis ANOVA and the Mann-Whitney U/ Wilcoxon Rank Sum W Test was used. The correlation between two variables was calculated with Spearman correlation coefficients.

Results

The platelet count was 68±28 x 109/L (mean ± std) in the full term, untreated newborn NAIT group, 37± 34 x 109/L in the untreated fetal NAIT group, 76±61 x 109/L in the treated fetal NAIT group and 190±53 x 109/L in the nonthrombocytopenic hemolytic disease group (Table 1). Platelet counts were not determined in the healthy neonates but they had no signs or symptoms of bleeding. Further study will be necessary to analyze the influence of treatment on the fetal platelet numbers, but although the mean platelet numbers in the treated fetal group is significant higher ($p=0.003$, Table 1) than in the untreated group, the statistical analysis of platelet numbers in fetuses (n=40) included in both the untreated and treated fetal NAIT groups did not show a significant difference ($p=0.06$). Plasma Tpo levels were 21±13 A.U./ml (range 9-47) in the neonatal NAIT samples (n=16), 26±18 A.U./ml (range 4-89) in the untreated fetal NAIT samples (n=50), 26±17 A.U./ml (range 6-97) in the treated fetal NAIT samples (n=63), 27±24 (range 2-93) in the samples from healthy neonates (n=51) and 18±8 A.U./ml (range 5-34) in the fetal HDN samples(n=21).

Post hoc testing showed that plasma Tpo levels in the samples of the NAIT neonates, the untreated and the treated NAIT fetuses were comparable with Tpo levels in the samples of the non-thrombocytopenic HDN controls ($p=0.09$, 0.03 and 0.8, respectively) and the healthy neonates ($p=0.8$, 0.5 and 0.3, respectively) (Fig. 1). The plasma Tpo levels in both the fetal HDN and the healthy newborn controls were slightly, though significantly ($p<0.001$) higher than plasma Tpo levels in healthy adults. The same held true when comparing the Tpo values of the NAIT neonates and the treated and untreated NAIT fetuses with the Tpo levels in healthy adults ($p<0.001$). No difference in Tpo plasma levels were detected comparing plasma samples from fetuses with NAIT before and after treatment. No statistically significant correlation was detected between platelet numbers and Tpo levels, either in the total NAIT group (Fig. 2) or in the separate groups or between Tpo levels and age at the time of blood sampling. Furthermore, the anticoagulant used during blood collection or the institute at which blood was drawn did not influence the test results (data not shown).

Table 1: Patient characteristics

	Number of samples	Number of patients	Gestational age (weeks) (mean ± std.)	Platelet count (x 10^9/L) (mean ± std.)	Plasma Tpo levels (mean ± std.) (A.U./ml) (pg/ml)	
untreated fetal NAIT	50	50	24 ± 3	37 ± 34	26 ± 18	234±162
Treated fetal NAIT	63	51	33 ± 4	76 ± 61	26 ± 17	234±153
full term newborns NAIT	16	16	1-7 days p.p.	68 ± 28	21 ± 13	189±117
Control fetuses (HDN)	21	14	28 ± 4	190 ± 53	18 ± 8	162±72
Healthy full term newborns	51	51	directly p.p.	n.t.	27 ± 24	243±216
Healthy adults	193	193	38±11*	150-450	11 ± 8	99±72

p.p.: post partum; n.t.: not tested; *age [yrs]

Figure 1. Box plots of Tpo levels in NAIT and Controls. Box plots represent the interquartile range that contains 50% of the values. The whiskers are lines that extend ffom the box to the highest and lowest values, excluding outliers. A line across the box indicates the median. Tpo values of all separate groups were elevated compared with the adult Controls; p < 0.001.

Figure 2. Platelet counts w plasma Tpo levels in NAIT. Platelet counts and plasma Tpo levels are not correlated. Tpo levels are comparable in the different groups (p > 0.1), with or without the two outliers. The two outliers cannot be explained by differences in clinical data, treatment, or sampling procedures. Perhaps Tpo release ffom platelets occurred in vitro as a result of some unwanted platelet clotting in the EDTA samples.

Discussion

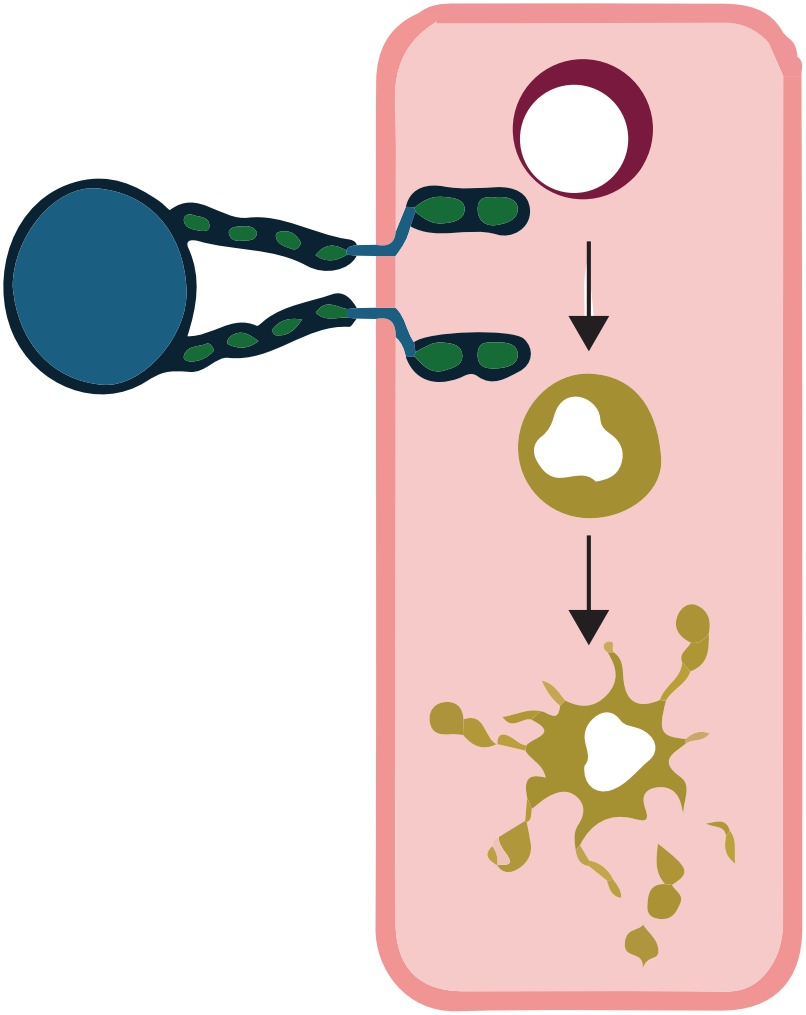
Screening for NAIT is not routinely performed and treatment of the initial case in a family mostly consists of post-natal treatment with intravenous gammaglobulin (IVIg) and/or platelet (negative for the antigen involved) transfusions. In subsequent pregnancies treatment of the mother with IVIg and corticosteroids and of the fetus with intrauterine platelet transfusions are possible and often employed. Trials are in progress to clarify the relative merits of each form of treatment although increasingly avoidance of fetal sampling is desired if feasible. We and others detected normal or only slightly elevated Tpo levels in ITP patients.7-10 We now find that Tpo levels in NAIT fetuses are also normal or only slightly elevated compared to levels in non-thrombocytopenic age-matched controls. Thus, as in ITP, in alloimmune mediated thrombocytopenia it appears that normal Tpo removal occurs resulting in normal levels being maintained in the circulation. This indicates that there is predominantly normal platelet production in fetuses and newborns with NAIT. It seems to contradict the hypothesis that the anti-HPA-1a antibodies would bind to glycoprotein IIb/IIIa on megakaryocytes and either inhibit thrombocytopoiesis or destroy them outright.15-17 We, like others21,22, found slightly but significantly higher Tpo levels in fetal/neonatal plasma compared to plasma from adults (Fig. 1). For the slightly elevated Tpo levels in fetuses/neonates (also with normal platelet counts) several explanations are possible - e.g. an increased production of Tpo considering that in fetuses and neonates the spleen may also contribute to Tpo production23; less expression of Tpo receptors on megakaryocytes in neonates, as described by Kuwaki et al24, resulting in slower removal; or an increased constitutive production of Tpo, inasmuch as the rate of growth of the fetus as well as the neonate is remarkably high, and, therefore, there is a continuous requirement for blood elements for neovasculature. Further study is necessary to explain the higher Tpo levels in neonates compared to adult levels.

The down regulation of Tpo plasma levels by platelets appears to prevent an increase in Tpo levels in all cases in which thrombocytopenia is due to increased platelet destruction. Thus, the fact that, as in ITP patients, Tpo levels in NAIT patients are not or only marginally increased may have important therapeutic implications. Injection of Tpo, or Tpo-mimicking peptide, could likely be applied to increase platelet production and platelet count. This approach has been shown to be effective in chimpanzees with HIV-ITP25 and in a few patients.26

References

1. Kaushansky K 1998 Thrombopoietin. N Engl J Med 339: 746-54.
2. Debili N, Wendling F, Cosman D, Titeux M, Florindo C, Dusanter-Fourt I, Schooley K, Methia N, Charon M, Nador R et al 1995 The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. Blood 85: 391-401.
3. Broudy VC, Lin NL, SAbath DF, Papayannopoulou T, Kaushansky K 1997 Human platelets display high affinity receptors for thrombopoietin. Blood 89: 1896-904.
4. Folman CC, de Jong SCM, de Haas M, von dem Borne AEGKr 2001 Analysis of the kinetics of thrombopoietin uptake during platelet transfusion. Transfusion 41: 517-21.
5. Fielder PJ, Hass P, Nagel M, Stefanich E, Widmer R, Bennett GL, Keller GA, de Sauvage FJ, Eaton D 1997 Human platelets as a model for the binding and degradation of thrombopoietin. Blood 89: 2782-8.
6. Li J, Xia Y, Kuter DJ 1999 Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: binding, internalization, stability and pharmacokinetics. Br J Haematol 106: 345-56.
7. Tahara T, Usuki K, Sato H, Ohashi H, Morita H, Tsumura H, Matsumoto A, Miyazaki H, Urabe A, Kato T 1996 A sensitive ELISA for measuring thrombopoietin in human serum: serum thrombopoietin levels in healthy volunteers and in patients with haematopoietic disorders. Br J Haematol 93: 783-8.
8. Emmons RVB, Reid DM, Cohen RL, Meng G, Young NS, Dunbar CE, Shulman NR 1996 Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. Blood 87: 4068-71.
9. Kosugi S, Kurata Y, Tomiyama Y, Tahara T, Kato T, Tadokoro S, Shiraga M, Honda S, Kanakura Y, Matsuzawa Y 1996 Circulating thrombopoietin level in chronic immune thrombocytopenic purpura. Br J Haematol 93: 704-6.
10. Porcelijn L, Folman CC, Bossers B, Huiskes E, Overbeeke MA, vd Schoot CE, de Haas M, von dem Borne AE 1998 The diagnostic value of thrombopoietin levels measurements in thrombocytopenia. Thromb Haemost 79: 1101-5.
11. Cohen-Solal K, Vitrat N, Titeux M, Vainchenker W, Wendling F 1999 High-level expression of Mpl in platelets and megakaryocytes is independent of thrombopoietin. Blood 93: 2859-66.
12. Mueller-Eckhardt C, Kiefel V, Grubert A, Kroll H, Weisheit M, Schmidt S, Mueller-Eckhardt G, Santoso S 1989 348 cases of suspected neonatal alloimmune thrombocytopenia. Lancet 1: 363-6.
13. Williamson LM, Hackett G, Rennie J, Palmer CR, Maciver C, Hadfield R, Hughes D, Jobson S, Ouwehand WH 1998 The natural history of fetomaternal alloimmunization to the platelet-specific antigen HPA-1a (PlA1, Zwa) as determined by antenatal screening. Blood 92: 2280-7.
14. von dem Borne AEGK, van Leeuwen EF, von Riesz LE, van Boxtel CJ, Engelfriet CP 1981 Neonatal alloimmune thrombocytopenia: detection and characterization of the responsible antibodies by the platelet immunofluorescence test. Blood 57: 649-56.
15. Warwick RM, Vaughan J, Murray N, Lubenko A, Roberts I 1994 In vitro culture of colony forming unit-megakaryocyte (CFU-MK) in fetal alloimmune thrombocytopenia. Br J Haematol 88: 874-7.
16. Bizzaro N and Dianese G 1988 Neonatal alloimmune amegakaryocytosis. Case report. Vox Sang 54: 112-4.
17. Tsukada T, Ueyama J, Miyakoshi S, Morinaga S, Mutoh Y, Yamaguchi H, Matsuya S 1990 [Diagnostic significance of the number of bone marrow megakaryocytes in cases with decreased platelet production evidenced by platelet kinetic study]. Rinsho Ketsueki 31: 903-10.

18. Bussel JB, Zabusky MR, Berkowitz RL, McFarland JG: Fetal alloimmune thrombocytopenia. N.Engl.J.Med. 337:22, 1997
19. Kanhai HHH, Porcelijn L, van Zoeren D, Klumper F, Vietor H, Meerman RH, Brand A: Antenatal care in pregnancies at risk of alloimmune thrombocytopenia: report of 19 cases in 16 families. Eur J Obstet Gynecol Reprod Biol 68:67, 1996
20. Folman CC, von dem Borne AEGKr, Rensink IHJAM, Gerritsen W, van der Schoot CE, de Haas M, Aarden L 1997 Sensitive measurements of thrombopoietin by a monoclonal antibody based sandwich enzyme-linked immunosorbent assay. Thromb Haemost 78: 1262-97.
21. Sola MC, Juul SE, Meng YG, Garg S, Sims P, Calhoun DA, Dame JB, Christensen D 1999 Thrombopoietin (Tpo) in the fetus and neonate: Tpo concentrations in preterm and term neonates, and organ distribution of Tpo and its receptor (c-mpl) during human fetal development. Early Hum Dev 53: 239-50.
22. Ishiguro A, Nakahata T, Matsubara K, Hayashi Y, Kato T, Suzuki Y, Shimbo T 1999 Age-related changes in thrombopoietin in children: reference interval for serum thrombopoietin levels. Br J Haematol 106: 884-8.
23. Wolber EM, Dame C, Fahnenstich H, Hofmann D et al 1999 Expression of the thrombopoietin gene in human fetal and neonatal tissues. Blood 94: 97-105.
24. Kuwaki T, Hagiwara T, Yuki C, Kodama I, Kato T, Miyazaki H 1998 Quantitative analysis of thrombopoietin receptors on human megakaryocytes. FEBS lett 427: 46-50.
25. Harker LA, Marzec UM, Novembre F, Sundell IB, Waller EK, Karpatkin S, McClure HM, Kelly AB, Stead RB 1998 Treatment of thrombocytopenia in chimpanzees infected with human immunodeficiency virus by pegylated recombinant human megakaryocyte growth and development factor. Blood 91: 4427-33.
26. Harker LA 1999 Physiology and clinical applications of platelet growth factors. Curr Opin Hematol 6: 127-34.



04

CHAPTER 4

Plasma thrombopoietin levels as additional tool in clinical management of thrombocytopenic neonates.

Porcelijn L, Huiskes E, Onderwater-Van Den Hoogen L, Polman CC, Zwaginga JJ, De Haas M. Plasma thrombopoietin levels as additional tooi in clinical management of thrombocytopenic neonates. Platelets. 2020;31(1):62-67.

Plasma thrombopoietin levels as additional tool in clinical management of thrombocytopenic neonates.

Leendert Porcelijn MD[1], Elly Huiskes[1], Laura Onderwater[1], Claudia C Folman PhD[1], Jaap Jan Zwaginga MD PhD[2,3], Masja de Haas MD PhD[1,2,3]

[1]Sanquin Diagnostic Services, Department of Immunohematology Diagnostic Services Amsterdam, The Netherlands, [2] Department of Immuno-hematology and Blood Transfusion, Leiden University Medical Center, the Netherlands, [3]Center for Clinical Transfusion Research, Sanquin Research, Leiden and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands.

Abbreviations:

AU	arbitrairy units
AMR	Ashwell Morell receptor
CMV	cytomegalo virus
c-mpl	myeloproliferative leukemia protein
ELISA	enzyme-linked immunosorbent assay
FNAIT	fetal/neonatal alloimmune thrombocytopenia
HSV	herpes simplex virus
ITP	immune thrombocytopenia
IVIg	intravenous immunoglobulin
ROC	receiver operating characteristics
Tpo	thrombopoietin

Key words: Thrombopoietin, Neonates, Thrombocytopenia, Diagnostics

Abstract

Plasma thrombopoietin (Tpo) levels distinguish thrombocytopenia resulting from increased platelet destruction or decreased platelet production. We investigated whether measuring plasma Tpo levels in thrombocytopenic newborns is of diagnostic value to establish the underlying mechanism of thrombocytopenia.
Tpo levels were measured with in-house developed ELISA in samples referred to our center because of thrombocytopenia noticed in the first 10 days of life. Clinical data was collected.

Plasma Tpo levels < 128 AU/ml were found in the majority (92%) of 121 newborns with immune mediated thrombocytopenia (n=104) and thrombocytopenia due to bacterial infections (n=7), increased plasma Tpo levels (≥ 128 AU/ml) were found in thrombocytopenic newborns with severe asphyxia (n=24). Highly increased plasma Tpo levels (> 200 AU/ml) in thrombocytopenic neonates with

congenital viral infections (n=22) or amegakaryocytosis (n=6). A plasma Tpo level < 128 AU/ml excludes (negative predictive value 96%, 95% CI 90-99%) severe asphyxia, congenital viral infections and amegakaryocytosis as the cause for thrombocytopenia in newborns.

Increased plasma Tpo levels indicate that thrombocytopenia in newborns, as a result of various non-immune disorders, is often caused by (temporary) bone marrow suppression/failure. Measurement of plasma Tpo levels provides the clinician with an additional tool to decide on the differential diagnosis, the necessity for subsequent diagnostics and treatment in neonates with thrombocytopenia.

Introduction

In the neonatal intensive care unit (NICU), thrombocytopenia is one of the most common haematological problems.1-3 The most significant risk of severe thrombocytopenia is intracranial hemorrhage, causing neurological impairment or even death. Early recognition of the underlying pathology guides appropriate treatment and is essential to predict the clinical course. However, the main aetiologies and patterns of thrombocytopenia in neonates are highly variable and difficult to diagnose. 1-3 Therefore, we decided to include plasma Tpo level measurements in our routine diagnostic laboratory work-up for suspected fetal and neonatal alloimmune thrombocytopenia (FNAIT).

Regulation of platelet production strongly depends on thrombopoietin (Tpo) levels. Tpo, mainly produced in the liver, binds to the c-mpl-receptors on CD34+ stem cells, megakaryocytes and platelets.4,5 A sufficient megakaryocyte and platelet mass will passively eliminate free Tpo from the circulation.4-10 Furthermore, Groznovsky et al. recently showed active regulation of Tpo levels via the hepatic expression of Tpo mRNA and protein regulation induced by the binding of desialylated (senescent) platelets to the hepatic Ashwell-Morell receptor (AMR).11 In previous studies, we showed that plasma Tpo levels are useful to discriminate thrombocytopenia caused by megakaryocyte and platelet production failure (highly elevated Tpo levels) from thrombocytopenia caused by elevated platelet destruction as in immune thrombocytopenia (ITP) and FNAIT (normal or only slightly elevated Tpo levels).12-13 We have now analyzed the collected data to determine which underlying causes of neonatal thrombocytopenia show elevated plasma Tpo levels and investigated whether plasma Tpo levels can indeed help the clinician in early recognition of the underlying cause for the neonatal thrombocytopenia.

Patients and Methods

Between 1998 and 2013, plasma Tpo levels were measured in samples from 798 thrombocytopenic newborns, for whom blood samples were sent to our platelet and leucocyte serology reference laboratory. Clinical data, sent to our laboratory by the treating physicians as part of the diagnostic laboratory request, included the neonatal platelet count, gestational age at birth, birth weight, 1-min and 5-min Apgar scores, data on the presence of laboratory and clinical signs for perinatal asphyxia, bacterial or viral infections or other early neonatal causes for thrombocytopenia, neonatal treatment and course in neonatal platelet count. All thrombocytopenic (platelet count < 120 x 109/L) neonates for whom sufficient clinical information was available and material was sent to our laboratory within 10 days after birth, were included in the study.

We compared the results of the neonatal plasma Tpo levels with a control group of healthy full-term neonates (n=51, plasma Tpo levels measured in a previous study13), and we used a plasma Tpo cutoff level of 97 AU, being the highest level in a previously described FNAIT cohort for comparison of laboratory findings and therapeutic interventions between neonates with low or high plasma Tpo results.13

A solid-phase sandwich ELISA for measurement of plasma Tpo concentrations was performed in real time with each patient studied as previously described.14 One AU equals 9 pg of recombinant Tpo (Research Diagnostics, Flanders, NJ, USA).

Statistical analysis was performed with GraphPad Prism, version 6.04 (GraphPad Software, San Diego, California, USA). For comparison of continuous values the Mann-Whitney U test was used. For comparison of categorical variables the Pearson's chi-square test or Fisher exact probability test was used.

Results

For 585 of 798 referred neonatal samples, plasma Tpo levels were measured within 10 days post-partum. Sufficient clinical and laboratory data could be obtained for 303 thrombocytopenic neonates (supplemental Figure 1).

In our series of 303 included thrombocytopenic neonates, 137 neonates showed plasma Tpo levels ≤ 97 AU/mL and 166 showed plasma Tpo levels > 97 AU/mL.

Parity, gestational age, birth weight, platelet counts, haemoglobin level, leucocyte counts and day of blood collection, were not significantly different for neonates with plasma Tpo levels ≤ 97 AU/mL and those with levels > 97 AU/mL (Table 1). Only the one and five minute Apgar scores were significantly lower in the 'high Tpo' group ($p < 0.001$ and $p=0.004$, respectively). Neonatal treatment differed only regarding the use of immunoglobulines (IVIg). The latter was prescribed for 18 (13%) neonates with low plasma Tpo levels compared to 6 (4%) in the 'increased Tpo' group ($p=0.002$). The majority of IVIg-treated neonates were categorized as suffering from FNAIT or maternal ITP (Table 2).

Fetal/neonatal alloimmune thrombocytopenia was diagnosed as the main cause for the thrombocytopenia in 88 neonates. Twenty-eight of 88 (32%) FNAIT cases showed plasma Tpo levels > 97 AU/mL (Figure 1). A significant difference ($p<0.0001$) was seen with the healthy controls (Table 3).

Plasma Tpo levels did not significantly differ between the 'maternal ITP' cases (n=16), diagnosed according to the guidelines of the American Society of Hematology, and the FNAIT cases ($p=0.479$).

Acquired bacterial infection was clinically diagnosed for seven neonates based on predictive maternal problems (e.g. prolonged time of membranes rupture), neonatal behaviour (e.g. quiet, nutritional problems), physical examination (fever, hypotonia), laboratory parameters (including cell count, leucocyte differentiation, CRP), and response on antibiotics treatment and by excluding other causes for thrombocytopenia. For none of the children a positive culture was obtained and at the time of blood collection, between three and seven day's post-partum, none of the children suffered from disseminated intravascular coagulation. Plasma Tpo levels were slightly increased ($p=0.01$) compared to healthy controls but comparable ($p=0.638$) with plasma Tpo levels in FNAIT. Plasma Tpo levels in low birth weight, i.e. small for gestational age (SGA, birth weight below the 10th percentile for gestational age, n=33) and premature (born <37 weeks gestational age, n=62) thrombocytopenic neonates were significantly higher compared with both healthy controls and FNAIT cases. Furthermore, a significant inversed correlation ($p=0.016$) was detected between birth weight and plasma Tpo levels.

Table 1: Comparison between the 'low' plasma Tpo and 'high' plasma Tpo group.

	Neonatal plasma Tpo level < 97 AU/mL	Neonatal plasma Tpo level ≥ 97 AU/mL	p value*
	n=137	n=166	
Parity			
Nullipara	55 (40%)	66 (40%)	p=1.0
Gestational age			
< 32 weeks	3 (1%)	9 (6%)	p=0.151
32-36 weeks	17 (12%)	36 (22%)	p=0.034
37-42 weeks	117 (86%)	118 (71%)	p=0.020
> 42 weeks	0 (0%)	3 (2%)	p=0.07
Range	24-42	25-43	
Neonatal birth weight (g)			
Median	3058	2827	p=0.004
Range	760-4800	650-5500	
AS 1 min			
Median	8	6	p<0.001
Range	1-10	1-10	
AS 5 min			
Median	9	8	p<0.001
Range	5-10	2-10	
Sample for Tpo on day			
Median	4.0	3.9	p=0.679
Range	0-10	0-10	
Platelet count (x 10^9/L)			
Median	44	36	p=0.513
Range	3-119	1-118	
Leucocyte count (x 10^9/L)			
Median	16.1	15.8	p=0.05
Range	3.9-34.1	3.9-36.9	
Hb (mmol/L)			
Median	11.6	11.2	p=0.045
Range	6.5-15.2	4.4-15.5	

**continuous variables are tested with Students t-test or Mann-Whitney U test. Categorical variables are tested with the Pearson chi-square test. Std=standard deviation*

Table 2: Neonatal therapy

	n/137<97AU/mL	n/166 ≥97AU/mL	p value*
Random platelet transfusion	38 (28%)	52 (31%)	p=0.498*
HPA-1a negative platelet transfusion	25 (18%)	23 (14%)	p=0.296*
IVIg treatment	18 (13%)	6 (4%)	P=0.002*
Antibiotics	10 (7%)	18 (11%)	p=0.290*
RBC transfusion	0 (0%)	4 (2%)	p=0.129†
RBC exchange transfusion	1 (1%)	2 (1%)	p=1.0†
No treatment	72 (53%)	78 (47%)	p=0.335*

**Pearson chi-square test, †Fisher exact probability test, HPA=human platelet antigen, IVIg=intravenous immunoglobuline, RBC=red blood cell*

Table 3. Platelet count and plasma Tpo level parameters

	n	Platelet count (x 10^9/L) Median (Range)	Plasma Tpo (AU/mL) Median (Range)	Mann-Whitney U test	
				Tpo levels compared to healthy controls p=	Tpo levels compared to FNAIT p=
Healthy full-term neonates*	51	Not tested	17 (2-93)		<0.0001
FNAIT	88	13 (5-117)	60 (5-565)	<0.0001	
ITP	16	19 (8-103)	60 (17-250)	<0.0001	0.479
Prematurity	62	40 (2-116)	113 (5-916)	<0.0001	<0.0001
Small for gestational age	33	50 (5-118)	143 (15-600)	<0.0001	<0.0001
Asphyxia	24	45 (2-110)	220 (16-772)	<0.0001	<0.0001
Cong viral infections	22	30 (7-97)	314 (118-1194)	<0.0001	<0.0001
Trisomy 21	19	50 (15-84)	107 (16-303)	<0.0001	<0.0001
Bacterial infections	7	50 (12-119)	52 (23-104)	0.01	0.638
Amegakaryocytosis	6	20 (2-43)	745 (298-1560)	<0.0001	<0.0001

**Plasma Tpo levels measured in a previous study[13]*

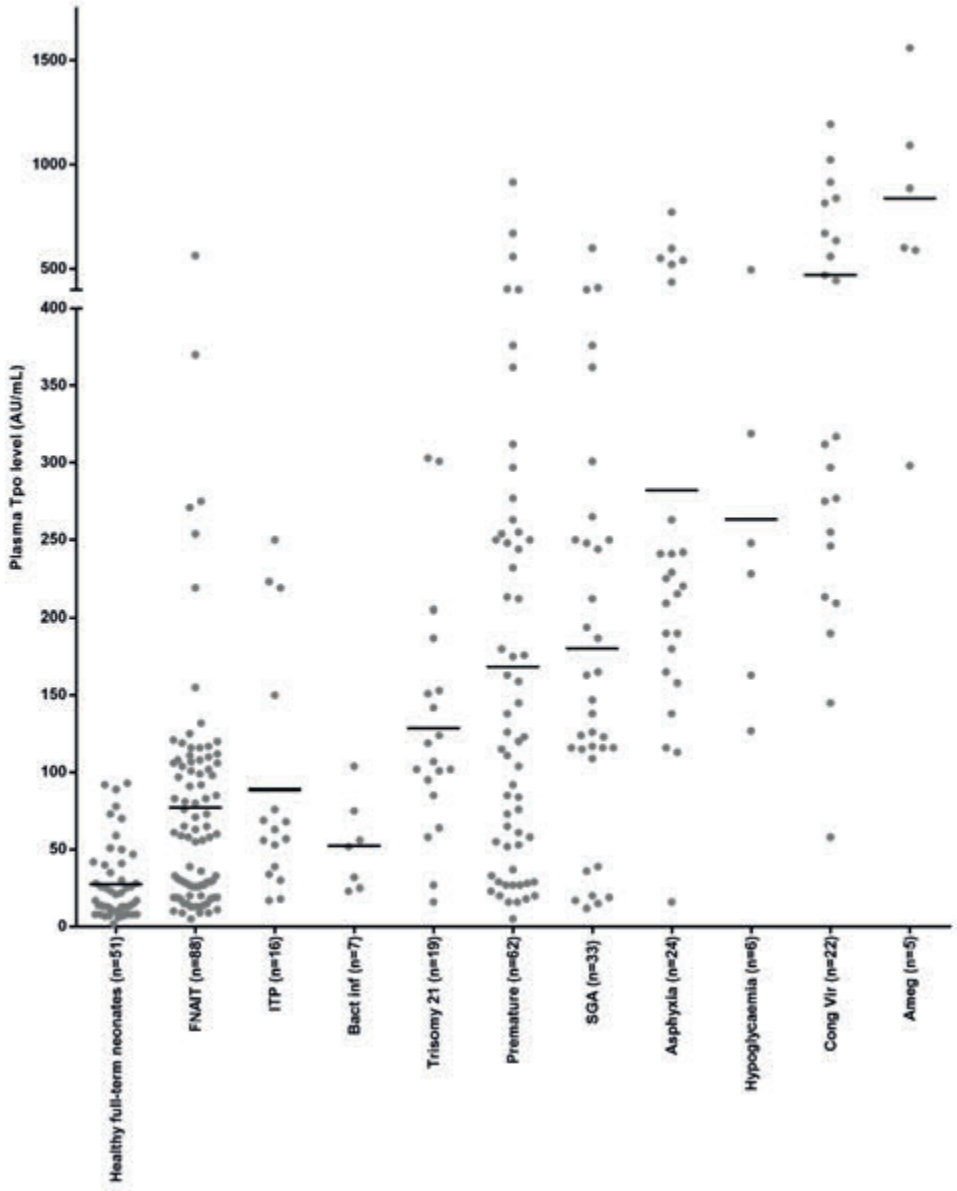


Figure 1. Plasma Tpo values for the various neonatal disorders.

However, most cases were combined with other pathology, and for that reason, the precise contribution of SGA and prematurity cannot be identified properly.
In our series, congenital viral infections (n=22), next to amegakaryocytosis, showed the highest plasma Tpo levels. All, but one, (95%) neonates suffering from (suspected) congenital viral infections showed highly elevated Tpo levels. Maternal and neonatal viral serology, viral cultures and/or viral PCR tests showed; 14 recent cytomegalovirus (CMV) infections including one twin pregnancy, one herpes simplex virus (HSV) infection and one parvovirus infection. For five neonates, congenital viral infections were supported by hepatosplenomegaly, abnormal blood counts, haemolytic anaemia, elevated transaminases and elevated serum bilirubin levels, but viral diagnostics were not or only partly performed. One neonate, born after 36 weeks of gestation, birth weight 2840 grams, platelet count 23 x 109/L and plasma Tpo level of 58 AU/mL, did not show any signs of infection, however, viral serology showed CMV antibodies of the IgG and IgM class. As no other cause for the thrombocytopenia could be detected, congenital CMV infection was accepted as most probable cause. The proven or suspected viral infections were combined with prematurity in 8 neonates (including the twin neonates), leaving 14 neonates without multifactorial causes for the elevated plasma Tpo levels.

Increased plasma Tpo levels were measured in all but one neonates with asphyxia (n=24), as diagnosed by Apgar scores (< 4 after 1 minute), cord blood pH (≤ 7.05) and/or base deficit and necessary resuscitative interventions. One neonate, born after emergency caesarean section was carried out for foetal distress at 37 weeks gestational age, with Apgar scores 3 and 8 after 1 and 5 minutes, cord blood pH 7.05 and platelet count 66 x 109/L at day four post-partum, showed a plasma Tpo level of 16 AU/mL.

As shown previously,14 highly elevated Tpo levels (>200 AU/mL) were found in the group with thrombocytopenia due to bone marrow failure (n=6); i.e. bone marrow biopsy showed a substantially reduced number or absence of megakaryocytes in all these six cases. Furthermore, in these cases mutations in the coding-regions of the c-mpl gene, encoding for the Tpo-receptor, were shown, leading to an amino-acid substitution or to a premature stop-codon.
Although, neonatal hypoglycaemia can be due to various clinical underlying causes and on itself is not associated with thrombocytopenia, significantly elevated plasma Tpo levels were measured in six neonatal hypoglycaemia (plasma glucose levels < 30 mg/dL) cases. Two of those neonates were born with congenital cardiac defects of whom one was also premature and SGA (36 weeks, 1515 grams) and the other premature (36 weeks, 2940 gram), two with extreme birth weights of 4400 and 5500 grams as a result of maternal pregnancy diabetes, one after 43 weeks pregnancy by caesarean section and one neonate

suffering from convulsions e.c.i.
Trisomy 21, was diagnosed for 19 neonates. In this group, platelet counts and plasma Tpo levels showed a significant inversed correlation (p=0.001, data not shown). Neonates with platelet counts <50 x 109/L, all produced high plasma Tpo levels.
Miscellaneous neonatal pathology was seen for 35 cases, i.e. foetal/neonatal intracranial haemorrhage e.c.i. (n=10), red blood cell antagonisms (n=5), maternal pre-eclampsia (n=12) and rare syndromes (n=8). Elevated or highly elevated plasma Tpo levels were produced for all syndromal cases, i.e. hygroma colli, Jacobson syndrome, Langerhans cell histiocytoma, thrombocytopenia absent radius, Noonan syndrome, Fragile X syndrome, with the exception of a neonate suffering from Turner's syndrome and a mild thrombocytopenia (platelet count 88 x 109/L).
Despite extensive clinical and laboratory investigation, no clear explanation for the thrombocytopenia could be detected for 33 neonates, with plasma Tpo levels ranging from 15 to 1015 AU/mL (mean 194 AU/mL, Std 265 AU/mL).

Discussion
The neonatal samples were drawn between day one and ten post-partum. Although platelet counts for all neonates included in this study were below 120 x 109/L, it might be that with increasing platelet counts in the first day's post-partum, possibly due to treatment, plasma Tpo levels inversely decreased. However, despite this uncertainty, the difference between the Tpo levels in immune and non-immune mediated thrombocytopenia is clear and does not seem to be dependent on which day the samples were drawn. Plasma Tpo levels in the foetus and newborns with thrombocytopenia depend on the production of Tpo in the more or less mature liver and the scavenging of Tpo by binding to the c-mpl receptors, expressed on platelets, platelet precursors and most early haematopoietic progenitor cells.15,16 Tpo levels can be decreased by increased numbers of blast cells expressing c-mpl binding free Tpo, which may occur in myeloproliferative disorders in newborns with Trisomy 21. In the series of newborns with Trisomy 21 (n=19) no transient myeloproliferative disorders were reported. As also described by Matsubara et al. 2010, 17 we detected for newborns with Trisomy 21, a significant inversed correlation (p=0.001) between platelet counts and Tpo levels. This indicates again that the concentration of free circulating Tpo in blood depends largely on the number of megakaryocytes in the bone marrow and platelets in the peripheral blood, suggesting a temporary impaired platelet production.

Confirming previous studies we observed normal or slightly elevated plasma Tpo levels for thrombocytopenic neonates with antibody mediated foetal/neonatal platelet destruction disorders, as seen for FNAIT.13 It is intriguing that

6/104 (6%) neonates with a primary diagnosis FNAIT or maternal ITP showed highly (>200 AU/mL) increased plasma Tpo levels. It may well be that in spite of extensive investigations and the lack of accompanying clinical signs, an additional mechanism for the thrombocytopenia was missed. Another explanation could be that these neonates temporarily suffered from exhausted platelet production due to limited ability of neonatal megakaryocytes to increase their size in response to increased platelet demand, but unfortunately, no bone marrow investigations were performed.18,19 Antibody-mediated platelet destruction occurs mainly in the spleen, but it is recently suggested that glycoprotein Ib/IX specific (auto-) antibodies can cause glycoprotein desialylation, resulting in an alternative route for platelet destruction by binding to the Ashwell Morell Receptor, leading to increased Tpo production.11 We did not detect GPIb specific (auto-)antibodies in the 'FNAIT' or 'ITP' neonates with increased Tpo levels, and therefore, this cannot explain our findings.

Normal plasma Tpo levels were detected in the seven suspected (for none of these neonates a positive blood culture was detected) bacterial infection cases indicating an increased platelet destruction. This notion is supported by Oygür et al. 2001,20 who found that the principal mechanism responsible for thrombocytopenia in bacterial (or fungal) infections in neonates seemed to be accelerated platelet destruction, probably secondary to endothelial damage with subsequent platelet adhesion and aggregation. Alternatively, bacterial infections may lead to platelet lysis or removal by the reticuloendothelial system.

Elevated to highly elevated plasma Tpo levels were detected in the majority of neonates diagnosed with congenital viral infections, severe asphyxia and amegakaryocytosis (Table 3, Figure 1). The increased plasma Tpo levels can be the result of disruption of the myeloproliferative leukemia protein (c-mpl) receptor function due to mutations in the c-MPL gene, resulting in congenital amegakaryocytosis, impaired megakaryocytopoiesis due to immaturity of the bone marrow function, progenitor and/or non-progenitor bone marrow cell damage possibly caused by active viral infections (CMV, Parvo, Herpes, HIV, Rubella) or oxygen deprivation and as a result of increased cortisol levels after stress causing reduced c-mpl expression on the cell membranes.18,19,21,22,23
If used in a diagnostic setting to distinguish immune mediated neonatal thrombocytopenia (FNAIT and ITP) from (temporary) platelet production failure (asphyxia, congenital viral infection and amegakaryocytosis), the area under the curve (AUC) is 0.957 (95% CI 0.922-0.991). The receiver operating characteristic (ROC)-curve shows an optimal sensitivity of 95% (95% CI 83.1-99.4) and specificity of 91% (95% CI 82.9-96) combination at a plasma Tpo level of 128 AU/mL
(Figure 2).

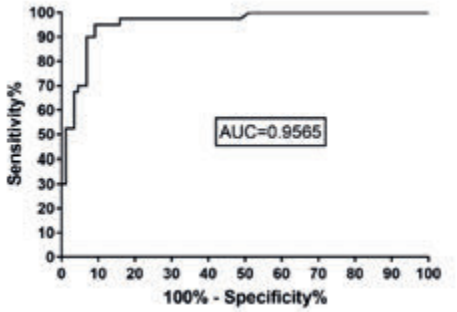


Figure 2. ROC-curve plasma Tpo levels for thrombocytopenic neonates due to FNAIT (zz = 77) and ITP (zz = 14) compared with congenital viral infections (zz = 14), asphyxia (zz = 24) and amegakaryocytosis (zz = 6) (only cases without multifactorial causes are included).The optimal sensitivity of 95% (95% Cl 83.1-99.4) and specificity of 91% (95% Cl 82.9-96) combination was shown at a plasma Tpo level of 128 AU/mL.

6/104 (6%) neonates with a primary diagnosis FNAIT or maternal ITP showed highly (>200 AU/mL) increased plasma Tpo levels. It may well be that in spite of extensive investigations and the lack of accompanying clinical signs, an additional mechanism for the thrombocytopenia was missed. Another explanation could be that these neonates temporarily suffered from exhausted platelet production due to limited ability of neonatal megakaryocytes to increase their size in response to increased platelet demand, but unfortunately, no bone marrow investigations were performed.18,19 Antibody-mediated platelet destruction occurs mainly in the spleen, but it is recently suggested that glycoprotein Ib/IX specific (auto-) antibodies can cause glycoprotein desialylation, resulting in an alternative route for platelet destruction by binding to the Ashwell Morell Receptor, leading to increased Tpo production.11 We did not detect GPIb specific (auto-)antibodies in the 'FNAIT' or 'ITP' neonates with increased Tpo levels, and therefore, this cannot explain our findings.

Normal plasma Tpo levels were detected in the seven suspected (for none of these neonates a positive blood culture was detected) bacterial infection cases indicating an increased platelet destruction. This notion is supported by Oygür et al. 2001,20 who found that the principal mechanism responsible for thrombocytopenia in bacterial (or fungal) infections in neonates seemed to be accelerated platelet destruction, probably secondary to endothelial damage with subsequent platelet adhesion and aggregation. Alternatively, bacterial infections may lead to platelet lysis or removal by the reticuloendothelial system.

Elevated to highly elevated plasma Tpo levels were detected in the majority

of neonates diagnosed with congenital viral infections, severe asphyxia and amegakaryocytosis (Table 3, Figure 1). The increased plasma Tpo levels can be the result of disruption of the myeloproliferative leukemia protein (c-mpl) receptor function due to mutations in the c-MPL gene, resulting in congenital amegakaryocytosis, impaired megakaryocytopoiesis due to immaturity of the bone marrow function, progenitor and/or non-progenitor bone marrow cell damage possibly caused by active viral infections (CMV, Parvo, Herpes, HIV, Rubella) or oxygen deprivation and as a result of increased cortisol levels after stress causing reduced c-mpl expression on the cell membranes.18,19,21,22,23
If used in a diagnostic setting to distinguish immune mediated neonatal thrombocytopenia (FNAIT and ITP) from (temporary) platelet production failure (asphyxia, congenital viral infection and amegakaryocytosis), the area under the curve (AUC) is 0.957 (95% CI 0.922-0.991). The receiver operating characteristic (ROC)-curve shows an optimal sensitivity of 95% (95% CI 83.1-99.4) and specificity of 91% (95% CI 82.9-96) combination at a plasma Tpo level of 128 AU/mL
(Figure 2).

In our series, only 4 (8%) of 52 neonates categorized as suffering from severe asphyxia, congenital viral infections or amegakaryocytosis showed a plasma Tpo level < 128 AU/mL. On the other hand, only 12 (12%) of 104 neonates suffering from FNAIT and maternal ITP and bacterial infections showed plasma Tpo levels ≥ 128 AU/mL (Figure 3). Subsequently, we can use plasma Tpo levels as an additional diagnostic tool, wherein the measuring of a neonatal plasma Tpo level < 128 AU/mL excludes (Negative Predictive Value 96%, 95%CI 90-99%) severe asphyxia, amegakaryocytosis and congenital viral infections as causes for neonatal thrombocytopenia. This can assist the treating physician in deciding on any subsequent treatments and/or the necessity for further diagnostic investigations (e.g. the necessity for diagnosing FNAIT). In this study we did not look at the neonatal mean platelet values (MPV) and immature platelet counts, which may also contribute to the elucidation of the underlying cause for thrombocytopenia. Further studies can focus on the comparison of these values and plasma Tpo levels.

We conclude that determination of plasma Tpo levels in thrombocytopenic newborns can be used as one of the diagnostic parameters to guide the differential diagnosis and most optimal treatment.

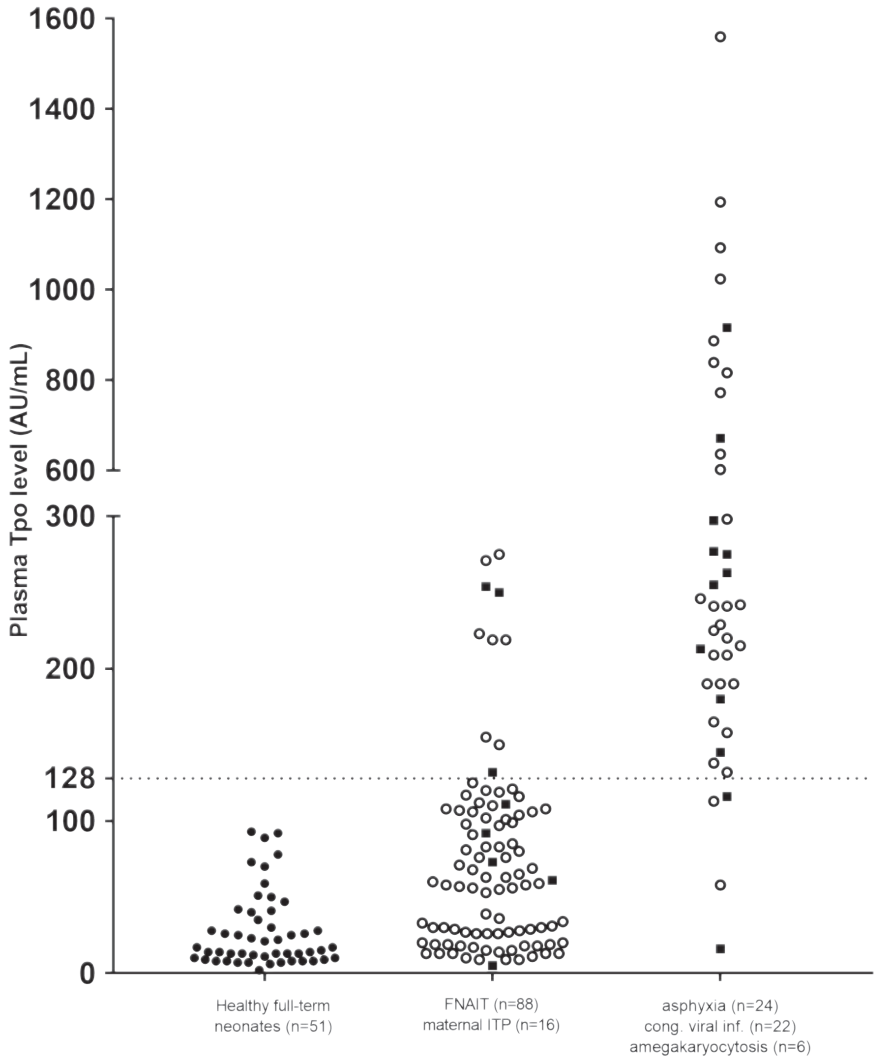


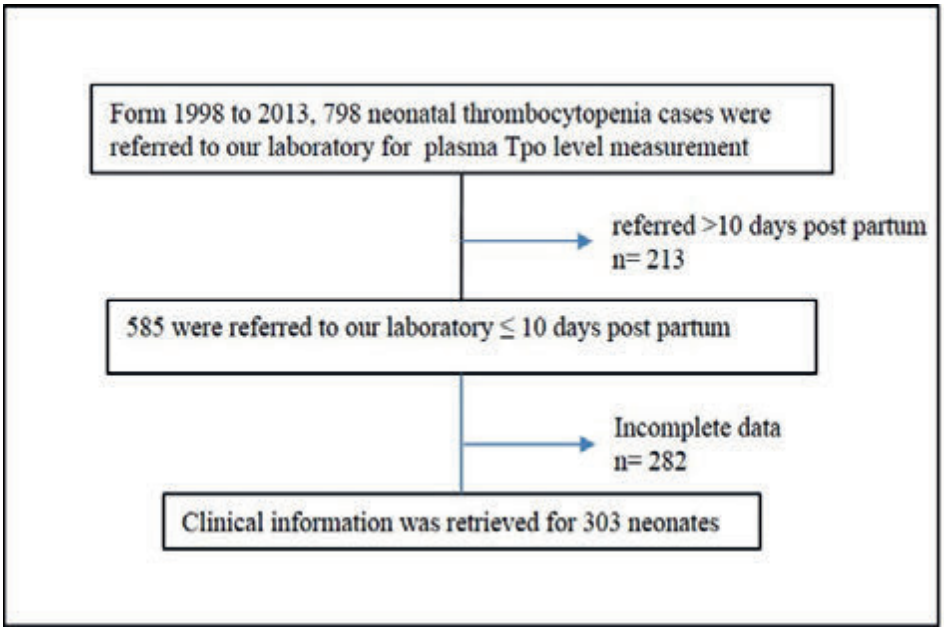
Figure 3. Tpo levels in neonates with different causes for thrombocytopenia.Open circles are used for those cases without secondary pathology (FNAIT n = 77, ITP n = 14, asphyxia n = 21, congenital viral infection n = 14, amegakaryocytosis n = 6).

References

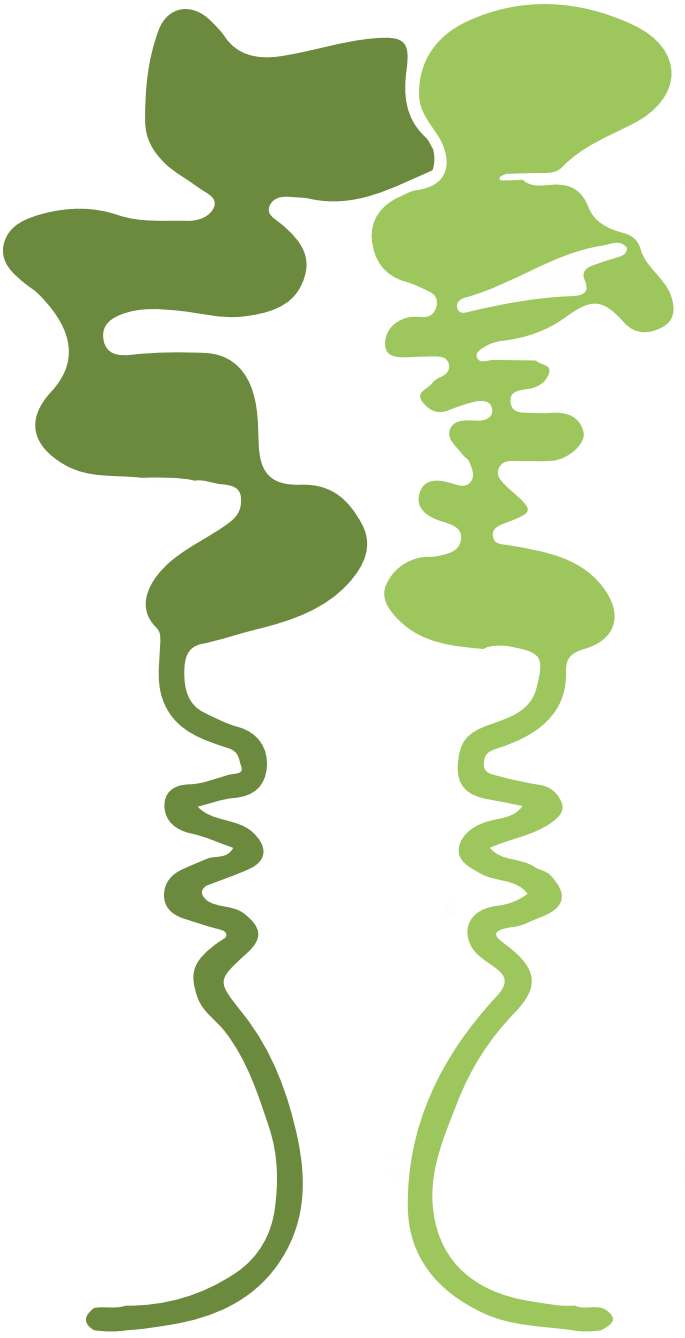
1. Del Vecchio A. Evaluation and management of thrombocytopenic neonates in the intensive care unit. Early Hum Dev. 2014;90: Suppl 2:S51-55
2. Baer VL, Lambert DK, Henry E, Christensen RD. Severe Thrombocytopenia in the NICU. Pediatrics 2009;124(6):e1095-1100
3. Hohlfeld P, Forestier F, Kaplan C, Tissot J.D, Daffos F. Foetal thrombocytopenia: a retrospective survey of 5,194 foetal blood samplings. Blood 1994;84:1851-1856
4. Kaushansky K. Thrombopoietin. N Engl J Med. 1998;339:746-754
5. Hitchcock IS, Kaushansky K. Thrombopoietin from beginning to end. Br J Haematol. 2014;165(2):259-268
6. Debili N, Wendling F, Cosman D, Titeux M, Florindo C, Dusanter-Fourt I, Schooley K, Methia N, Charon M, Nador R, et al. The Mpl receptor is expressed in the megakaryocytic lineage from late progenitors to platelets. Blood 1995;85:391-401
7. Broudy VC, Lin NL, Sabath DF, Papayannopoulou T, Kaushansky K. Human platelets display high affinity receptors for thrombopoietin. Blood 1997;89:1896-1904
8. Folman CC, de Jong SCM, de Haas M, von dem Borne AEGKr. Analysis of the kinetics of thrombopoietin uptake during platelet transfusion. Transfusion 2001;41: 517-521
9. Fielder PJ, Hass P, Nagel M, Stefanich E, Widmer R, Bennett GL, Keller GA, de Sauvage FJ, Eaton D. Human platelets as a model for the binding and degradation of thrombopoietin. Blood 1997;89:2782-2788
10. Li J, Xia Y, Kuter DJ. Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: binding, internalization, stability and pharmacokinetics. Br J Haematol. 1999;106:345-356
11. Grozovsky R, Begonja AJ, Liu K, Visner G, Hartwig JH, Falet H, Hoffmeister KM. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. Nat Med. 2015;21(1):47-54
12. Porcelijn L, Folman CC, Bossers B, Huiskes E, Overbeeke MA, v d Schoot CE, de Haas M, von dem Borne AE.The diagnostic value of thrombopoietin level measurements in thrombocytopenia. Thromb Haemost. 1998;79(6):1101-1105
13. Porcelijn L, Folman CC, de Haas M, Kanhai HH, Murphy MF, von dem Borne AE, Bussel JB. Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia. Pediatr Res. 2002;52(1):105-108
14. Folman CC, von dem Borne AE, Rensink IH, Gerritsen W, van der Schoot CE, de Haas M, Aarden L.Sensitive measurement of thrombopoietin by a monoclonal antibody based sandwich enzyme-linked immunosorbent assay. Thromb Haemost. 1997;78:1262-1267
15. Watts TL, Murray NA, Roberts IA. Thrombopoietin has a primary role in the regulation of platelet production in preterm babies. Pediatr Res. 1999;46:28–32
16. Sola MC, Calhoun DA, Hutson AD, Christensen RD. Plasma thrombopoietin concentrations in thrombocytopenic and non-thrombocytopenic patients in a neonatal intensive care unit. Br J Haematol. 1999;104:90–92
17. Matsubara K, Nigami H, Yura K, Inoue T, Isome K, Fukaya T. Serum thrombopoietin level and thrombocytopenia during the neonatal period in infants with Down's syndrome. J Perinatol. 2010;30(2):98-102

18. Saxonhouse MA, Rimsza LM, Stevens G, Jouei N, Christensen RD, Sola MC. Effects of hypoxia on megakaryocyte progenitors obtained from the umbilical cord blood of term and preterm neonates. Biol Neonate. 2006;89(2):104-108
19. Liu ZJ, Sola-Visner M. Neonatal and adult megakaryopoiesis. Curr Opin Hematol. 2011;18(5):330-337
20. Li J, van der Wal DE, Zhu G, Xu M, Yougbare I, Ma L, Vadasz B, Carrim N, Grozovsky R, Ruan M, et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. Nat Commun. 2015;6:7737
21. Oygür N, Tunga M, Mumcu Y, Yeşilipek A, Güra A, Coşkun M, Yeğin O. Thrombopoietin levels of thrombocytopenic term and preterm newborns with infection. Am J Perinatol. 2001;18:279-285
22. van den Oudenrijn S, Bruin M, Folman CC, Peters M, Faulkner LB, de Haas M, von dem Borne AE. Mutations in the thrombopoietin receptor, Mpl, in children with congenital amegakaryocytic thrombocytopenia. Br J Haematol. 2000;110(2):441-448
23. Ikeno K, Koike K, Takeshita A, Shinjo K, Higuchi T, Nakabayashi T, Akanuma S, Hizume K, Ishiguro A, Ogami K, et al. Stressful delivery influences circulating thrombopoietin (TPO) levels in newborns: possible role for cortisol in TPO-mpl binding. Early Hum Dev. 2000;58(3):225-235

Supplemental data:



Supplemental Figure 1: inclusion of cases



05

CHAPTER 5

Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art

Porcelijn L, Huiskes E, Oldert G, Schipperus M, Zwaginga JJ, de Haas M. Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art. Br J Haematol. 2018 Aug;182(3):423-426.

Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art.

Leendert Porcelijn[1], Elly Huiskes[1], Gonda Oldert[1], Martin Schipperus[2], Jaap Jan Zwaginga[3,4], Masja de Haas[1,3,4]

[1] Immunohematology Diagnostic Services, Sanquin Diagnostic Services, Amsterdam, the Netherlands [2] Department of Internal Medicine, HagaZiekenhuis, Den Haag, the Netherlands [3] Department of Immuno-hematology and Blood Transfusion, Leiden University Medical Center, the Netherlands [4] Center for Clinical Transfusion Research, Sanquin Research, Leiden and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands.

Summary

Immune Thrombocytopenia (ITP) is diagnosed by exclusion of other causes for thrombocytopenia. To prevent misdiagnosis, reliable detection of platelet autoantibodies would support the clinical diagnosis. We optimized our diagnostic algorithm for suspected ITP using the direct monoclonal antibody immobilization of platelet antigens (MAIPA) assay. In this test, the presence of platelet autoantibodies on the glycoproteins (GP) IIb/IIIa, Ib/IX and V bound on the patient platelets, is evaluated. The direct MAIPA showed to be a valuable technique for the detection of platelet autoantibodies and can possibly become a guide for optimizing therapy towards a more personalized treatment of ITP.

Platelet autoantibodies are regarded to be the major underlying cause of immune thrombocytopenia (ITP), although a role for cytotoxic T cells is also described (Cines et al, 2014). Screening for platelet autoantibodies however, is not part of the recommended diagnostic and therapeutic work up (Neunert et al, 2011). The latter is due to so far low sensitivity (60-70%) and specificity (≤ 60%) of the different types of platelet autoantibody tests (Hagenström et al, 1983; Helmerhorst et al, 1983) and ITP is therefore diagnosed by exclusion of other causes for thrombocytopenia (Neunert et al, 2011). To prevent misdiagnosis, reliable detection of platelet autoantibodies however, would be of great value for the clinical diagnosis. In this respect, we re-evaluated our diagnostic algorithm for suspected ITP using the direct monoclonal antibody immobilization of platelet antigens assay (MAIPA).

Platelet autoantibodies in ITP are predominantly directed against the platelet glycoproteins (GP) IIb/IIIa (CD41/61; fibrinogen receptor), GPIb/IX (CD42c/CD42a) or GPV (CD42d) (Joutsi & Kekomäki, 1997; McMillan, 2003). The presence of platelet antibodies directed against any of these targets can be investigated by ELISA-based GP specific assays, such as MAIPA and Luminex beads assays (Kiefel et al, 1987; Porcelijn et al, 2014). While the indirect MAIPA and commercially

available GP specific assays are known for their high sensitivity and specificity for identification of human platelet antigen (HPA)-specific allo-antibodies (Porcelijn et al, 2008), platelet autoantibodies in ITP serum or plasma are less easy detected (McMillan, 2003). Also the direct MAIPA, developed to directly detect platelet-bound antibodies, showed in previous studies a sensitivity for autoantibodies ranging from only 29 to 54% (Joutsi & Kekomäki, 1997; McMillan, 2003).
A more accurate detection of platelet autoantibodies in this respect would be of great value and for this reason, we validated detection of platelet autoantibodies by the direct MAIPA in known ITP and non-ITP mediated thrombocytopenic patients as well as non-thrombocytopenic controls. Subsequently, we tested the direct MAIPA for its discriminatory power between ITP and non-ITP patients in consecutively diagnostic samples sent to our laboratory.

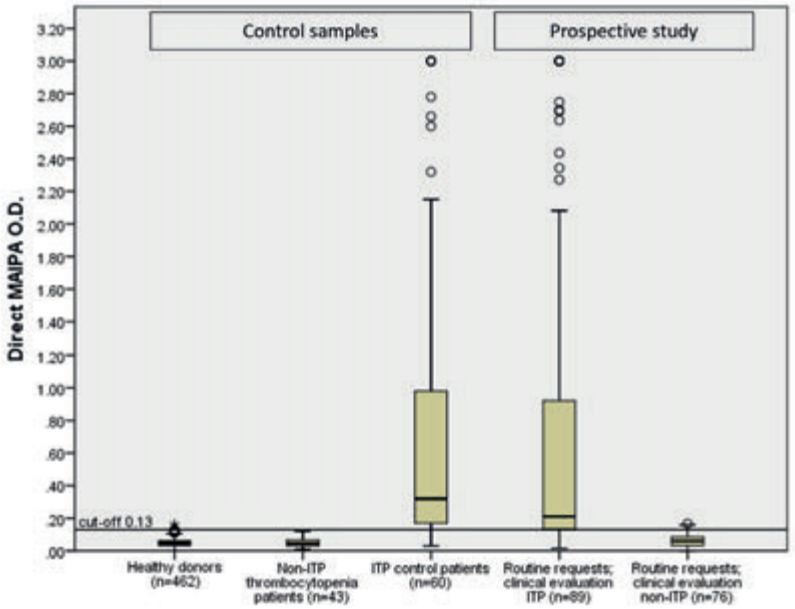
Healthy control and patient platelets, platelet eluates and sera were tested, within 24 hours after sampling, with the direct and indirect platelet immunofluorescence test (PIFT) (as described by von dem Borne et al, 1978). for the presence of platelet-associated and free circulating autoantibodies of the immunoglubolin (Ig)G- and IgM-class, and with the direct MAIPA (as described by Kiefel et al, 1987), for the presence of the IgG-class platelet-associated autoantibodies.
Statistical analyses were performed using SPSS 21 for Windows statistical package (SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered significant.
Healthy control (n=462) platelets, tested with the direct MAIPA, produced a range of normally distributed very low extinctions (Fig 1, between E=0.048 and E=0.052 (range 0.01 – 0.16 ± 0.023 – 0.026) for all five autoantibody targets GPIIb/IIIa (CD41/CD61), GPIb/IX (CD42c/CD42a), GPV (CD42d), GPIa/IIa (CD49b/CD29) and GPIV (CD36)). With the calculated cut-off value E = 0.13 (mean + 3 x standard deviation (SD)) only one of the healthy controls showed a positive (O.D. 0.137) direct MAIPA result for only CD41/61 (GPIIb/IIIa) (specificity of 99.8%: Fig 1, Table I).

Six of the 462 healthy controls showed positive results in the direct PIFT by the presence of antibodies of the IgM-class.
None of the non-immune thrombocytopenic patients (n=43, Supplemental Table Ia) showed a positive direct MAIPA result, whereas 16 of these 43 (37%) non-immune thrombocytopenia samples were positive, both with direct PIFT and eluate PIFT (Table I).

Known ITP patients (n=60) were diagnosed - in accordance with the recommendations of the American Society of Hematology (Neunert et al, 2011) - by means of the medical history, physical examination, complete blood count, the peripheral blood smear, platelet counts between 10 and 50 x 109/L, normal or slightly increased plasma thrombopoietin levels (as described by Folman et

al, 1997) and did not receive treatment for at least 3 months. The direct MAIPA produced positive reactions for 51/60 (85%) of these known ITP patients (Fig 1, Table I). The PIFT (direct + eluate) produced positive reactions in only 39 (65%) of these samples. Most autoantibodies were directed against GPIIb/IIIa, GPIb/IX and/or GPV. None of the samples had autoantibodies exclusively directed against GPIa/IIa and/or GPIV detected (Table SII).

For 178/204 (86%) thrombocytopenic patients suspected for ITP, the MAIPA was performed without knowledge of the clinical diagnosis. For 26 of these patients, all with a platelet count < 10 x 109/L, an insufficient number of platelets could be isolated. Clinical data was obtained for 165 of the remaining 178 tested patients (Figure S1). ITP was excluded based on the clinical data in 76 of these 165 patients (46%) with a mean platelet count of 104 x 109/L (range 9-386, SD 71) and a mean plasma Tpo level of 106 AU/ml (range 5-956, SD 166; Figure S2). In 25 of these patients, the platelet counts never fell below 100 x 109/L (Table SIB). Seventy-four of these 76 patients (97%) had a negative direct MAIPA result (Figure 1). Of the two patients with a positive direct MAIPA result, one



Direct MAIPA results for ITP patients and controls.
Direct MAIPA O.D. above 0.13 is considered positive. Control samples: historically well characterized ITP patients. Prospective study: requests for serological ITP diagnostics, after final clinical evaluation classified as ITP or non-ITP.

suffering from autoimmune haemolytic anaemia; showed GPV (CD42d)-bound platelet autoantibodies (O.D. 0.199), PIFT negative. The other patient, diagnosed with EDTA-dependent pseudothrombocytopenia (platelet counts in EDTA- and citrate-anticoagulated blood respectively, 60 and 109 x 109/L) showed GPV- and GPIb/IX-bound platelet autoantibodies (O.D. 0.176 and 0.186); the direct PIFT was weak positive for IgM only; the eluate PIFT was negative.

Table I. Test results for: healthy donors (n=462), ITP patients (n=60), non-ITP patients (n=43) and prospective requests for ITP diagnostics (n=165).

	Direct MAIPA positive Direct PIFT* positive n(%)	Direct MAIPA positive Direct PIFT* negative n (%)	Direct MAIPA negative Direct PIFT* positive n(%)	Direct MAIPA negative Direct PIFT* negative n(%)
Controls (n=545)				
Healthy donors (n=462)	0	1 (0.2%)	6† (1.3%)	455 (98.5%)
Non ITP (n=43)	0	0	16 (37%)	25 (58%)
ITP (n=60)	39 (65%)	12 (20%)	4 (7%)	5 (8%)
Routine requests (n=165)				
ITP (n=89)	60 (67%)	9 (10%)	2 (2%)	18 (20%)
Non-ITP (n=76)#	2 (3%)	1 (1%)	26 (34%)	47 62%)
Total group (n=268)				
ITP (n=149)	99 (66%)	21 (14%)	6 (4%)	23 (15%)
Non-ITP (n=119)#	2 (2%)	1 (1%)	44 (37%)	72 (61%)

ITP, immune thrombocytopenia; MAIPA, monoclonal antibody immobilization of platelet antigens assay; PIFT, platelet immunofluorescence test.
**PIFT= direct PIFT + eluate PIFT, †all six positive results were due to antibodies of the IgM class, # By clinical data analysis, ITP could be excluded for 76 of the 165 patients, initially suspected for ITP.*

In 89 of the 165 tested samples, the diagnosis of ITP was clinically made, the mean platelet count in this group was 45 x 109/l (range 8-171, SD 34.7). A mean plasma Tpo level of 38 AU/ml (range 4-381 AU/ml, SD 62 AU/ml; Figure S2)) was found. The direct MAIPA was positive for 69/89 (78%) of these patients (Figure 1, Table I).

Platelet associated antibodies of the IgG (and/or IgM) class were detected in the direct PIFT in 62/89 (70%) suspected ITP patients; for two of these, no antibodies were detected by the direct MAIPA.

Overall, the direct MAIPA correlated with the clinical diagnosis of ITP with a sensitivity of 81% (95% CI, 73-87%), and a specificity of 98% (95% CI, 94-100%). A positive predictive value of 98% (95% CI, 94-100%) for clinical ITP and a negative predictive value of 80% (95% CI, 72-86%) were obtained.

The direct MAIPA has two limitations. First, from approximately 16% of the routine ITP diagnostics referred samples insufficient patient platelets can be isolated to perform a direct MAIPA. Second, no autoantibodies are detected in approximately 20% patients suspected for ITP. Intriguingly, this lack of antibodies might still be considered as an immune dependent thrombocytopenia i.e. caused by T-cell autoimmunity. Additional research in these clinically typical ITP patients without detectable antibodies should reveal the nature of such thrombocytopenias.

Notwithstanding the limitations, advantages of the direct MAIPA assay are many-fold. Next to its value for enabling a much more reliable ITP diagnosis, the presence and further characterization of the glycoprotein specificity of platelet autoantibodies in the direct MAIPA assay may be correlated with the severity of bleeding symptoms and additionally lead to a more personalized ITP therapy. For instance, autoantibodies blocking the fibrinogen binding-site of GPIIb/IIIa were found associated with more severe bleeding in ITP (De Cuyper et al, 2013). Furthermore, platelet autoantibodies binding to platelet GPIb/IX, have been shown to induce desialylation of GPIb/IX and as such more prevalent destruction of the platelets by the Ashwell-Morell receptor of hepatocytes (Li et al, 2015). If so, such findings would make intravenous Ig treatment and splenectomy less likely effective. Third the inhibitory effect of platelet autoantibodies on compensatory thrombocytopoiesis might also depend on the glycoprotein specificity of the antibodies and less response of these patients to Tpo analogues (Iraqi et al, 2015). Finally, in our assays, the observed changes in antibody presence might steer the continuation, tapering or stopping of treatment. Our recently described association of antibody presence and lowering thereof after rituximab nicely underlines the value of our assays (Porcelijn et al, 2017).

We conclude that the direct MAIPA not only enables a more reliable diagnosis of ITP but may also help in the choice and continuation of therapy i.e. by monitoring the immune activity in ITP during long term TPO analogues. This in the end will be indispensable for more personalized treatment algorithms for ITP.

Authorship

L.P. conceptualized and designed the study, conducted the data analysis and statistical analysis, drafted the initial manuscript, and approved the final manuscript as submitted.

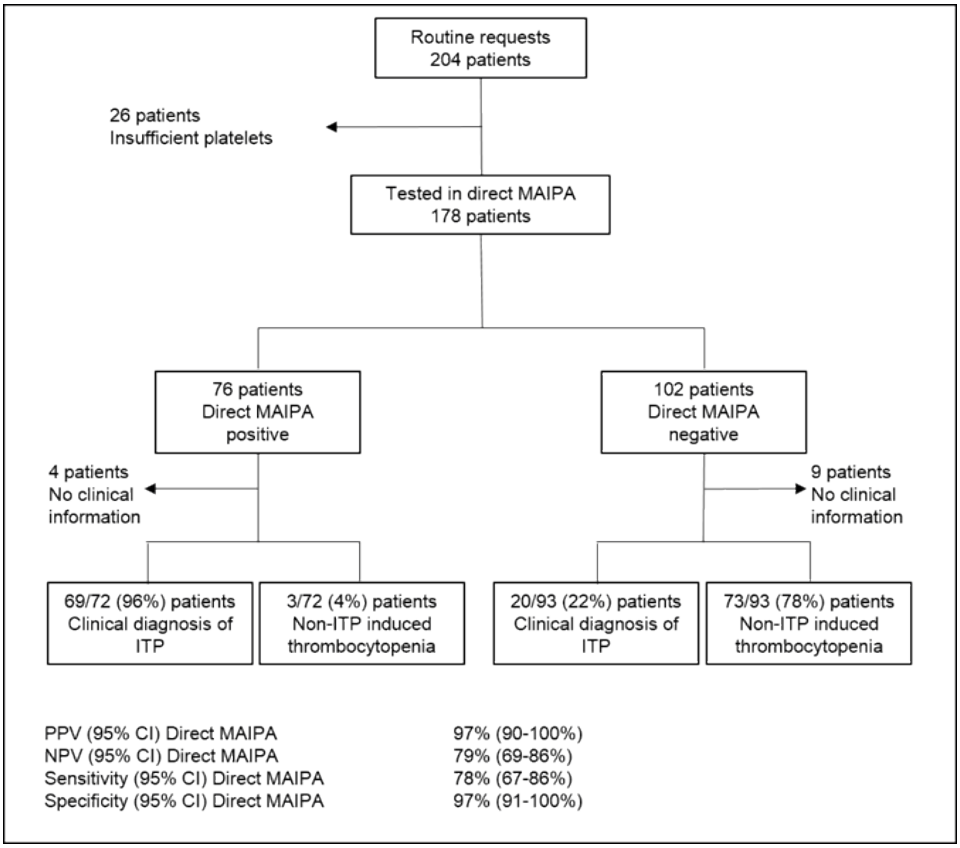
E.H. and G.O. coordinated and supervised data collection, critically reviewed the manuscript and approved the final manuscript as submitted.
M.S. reviewed and revised the manuscript and approved the final manuscript as submitted.

J.J.Z. and M.de H. supervised the study, conceptualized and co-drafted the initial manuscript, and approved the final manuscript as submitted.
None of the authors has a financial conflict of interest.

References

1. von dem Borne AE, Verheugt FW, Oosterhof F, von Riesz E, de la Rivière AB, Engelfriet CP. (1978) A simple immunofluorescence test for the detection of platelet antibodies. British Journal of Haematology, 39, 195–207.
2. Cines DB, Cuker A, Semple JW. (2014) Pathogenesis of immune thrombocytopenia. Presse Medicale, 43, 49-59.
3. De Cuyper IM, Meinders M, van de Vijver E, de Korte D, Porcelijn L, de Haas M, Eble JA, Seeger K, Rutella S, Pagliara D, Kuijpers TW, Verhoeven AJ, van den Berg TK, Gutiérrez L. (2013) A novel flow cytometry-based platelet aggregation assay. Blood, 121, 70-80.
4. Folman CC, von dem Borne AE, Rensink IH, Gerritsen W, van der Schoot CE, de Haas M, Aarden L. (1997) Sensitive measurement of thrombopoietin by a monoclonal antibody based sandwich enzyme-linked immunosorbent assay. Thrombosis and Haemostasis, 78, 1262-1267.
5. Hagenström H, Schlenke P, Hennig H, Kirchner H, Klüter H. (2000) Quantification of platelet-associated IgG for differential diagnosis of patients with thrombocytopenia. Thrombosis and Haemostasis, 84, 779-783.
6. Helmerhorst FM, Smeenk RJ, Hack CE, Engelfriet CP, von dem Borne AE. (1983) Interference of IgG, IgG aggregates and immune complexes in tests for platelet autoantibodies. British Journal of Haematology, 55, 533-545.
7. Iraqi M, Perdomo J, Yan F, Choi PY, Chong BH. (2015) Immune thrombocytopenia: antiplatelet autoantibodies inhibit proplatelet formation by megakaryocytes and impair platelet production in vitro. Haematologica, 100, 623-632.
8. Joutsi L, Kekomäki R. (1997) Comparison of the direct platelet immunofluorescence test (direct PIFT) with a modified direct monoclonal antibody-specific immobilization of platelet antigens (direct MAIPA) in detection of platelet-associated IgG. British Journal of Haematology, 96, 204-209.
9. Kiefel V, Santoso S, Weisheit M, Müeller-Eckhardt C. (1987) Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood, 70, 1722-1726.
10. Li J, van der Wal DE, Zhu G, Xu M, Yougbare I, Ma L, Vadasz B, Carrim N, Grozovsky R, Ruan M, Zhu L, Zeng Q, Tao L, Zhai ZM, Peng J, Hou M, Leytin V, Freedman J, Hoffmeister KM, Ni H. (2015) Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. Nature Communications, 6, 7737.
11. McMillan R. (2003) Antiplatelet antibodies in chronic adult immune thrombocytopenic purpura: assays and epitopes. Journal of Pediatric Hematology/Oncology, 25, Suppl 1, S57-61.
12. Neunert C, Lim W, Crowther M, Cohen A, Solberg L Jr, Crowther MA; American Society of Hematology. (2011) The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. Blood, 117, 4190-4207.
13. Porcelijn L, van Beers W, Gratama JW, van't Veer M, De Smet A, Sintnicolaas K. (2008) External quality assessment of platelet serology and human platelet antigen genotyping: a 10-year review. Transfusion, 48, 1699-1706.
14. Porcelijn L, Huiskes E, Comijs-van Osselen I, Chhatta A, Rathore V, Meyers M, de Haas M. (2014) A new bead-based human platelet antigen antibodies detection assay versus the monoclonal antibody immobilization of platelet antigens assay. Transfusion, 54, 1486-1492.
15. Porcelijn L, Huiskes E, Schipperus M, van der Holt B, de Haas M, Zwaginga JJ; Dutch HOVON 64 Study Group. (2017) Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients. Blood, 129, 3389-3391.

Supplemental data:
Detection of platelet autoantibodies revisited to identify immune thrombocytopenia.



Supplemental Figure 1: *correlation direct MAIPA results and clinical diagnosis*
PPV positive predictive value, NPV negative predictive value, CI confidence interval

Supplemental Table Ia. non-ITP thrombocytopenic patients (n=43)

non-ITP thrombocytopenia patients	n=43	Platelet counts x 10^9/L
hematological malignancies	n=17	ranging from 10-131
gestational thrombocytopenia	n=7	100, 107, 111, 119, 121, 124 and 130
viral infections	n=6	66, 83, 110, 120, 134 and 147
drug-induced thrombocytopenia	n=4	54, 77, 85 and 100
aplastic anemia	n=3	9, 87 and 100
hepato-splenomegalic pooling	n=3	50, 60 and 123
pseudothrombocytopenia	n=2	in EDTA and citrate; 50, 70 and both > 150, respectively
microangiopathy	n=1	20 x 10E9/L

Supplemental Table Ib. Routine ITP serology request patients, ITP excluded* (n=76)

Total	*n=76*	Mean platelet count 94 x 10^9/L, range 9-386, STD 50
hematological malignancies	n=24	ranging from 9-124
gestational thrombocytopenia	n=13	ranging from 69-134
drug-induced thrombocytopenia	n=9	ranging from 9-147
erroneous requests by administrative errors	n=9	all > 150
pseudothrombocytopenia	n=5	in EDTA and citrate; ranging from 33-102 and 109-231, respectively
aplastic anemia	n=5	64, 72, 88, 106 and 113
hepato-splenomegaly	n=4	45, 63, 71 and 73
bacterial sepsis	n=3	12, 24 and 31
anti-phospholipid syndrome	n=1	36
thrombotic thrombocytopenic purpura	n=1	99
congenital thrombocytopenia	n=1	12
post cardiac infarct	n=1	133

** By clinical and laboratory data analysis, ITP could be excluded for 76 of the 165 patients, initially suspected for ITP.*

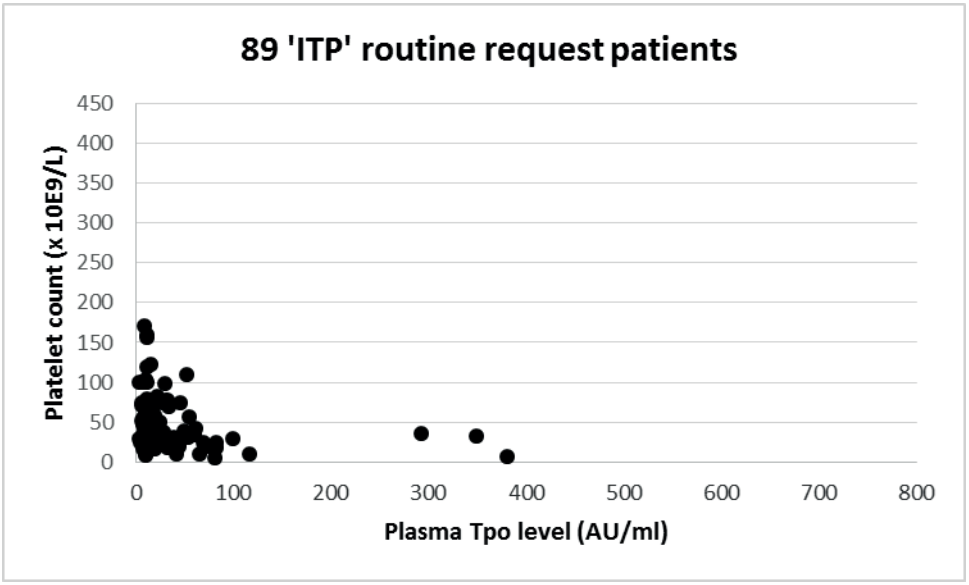
Supplemental Table IIa: Pattern of reactivity of platelet autoantibodies in ITP samples, as determined with the direct MAIPA.

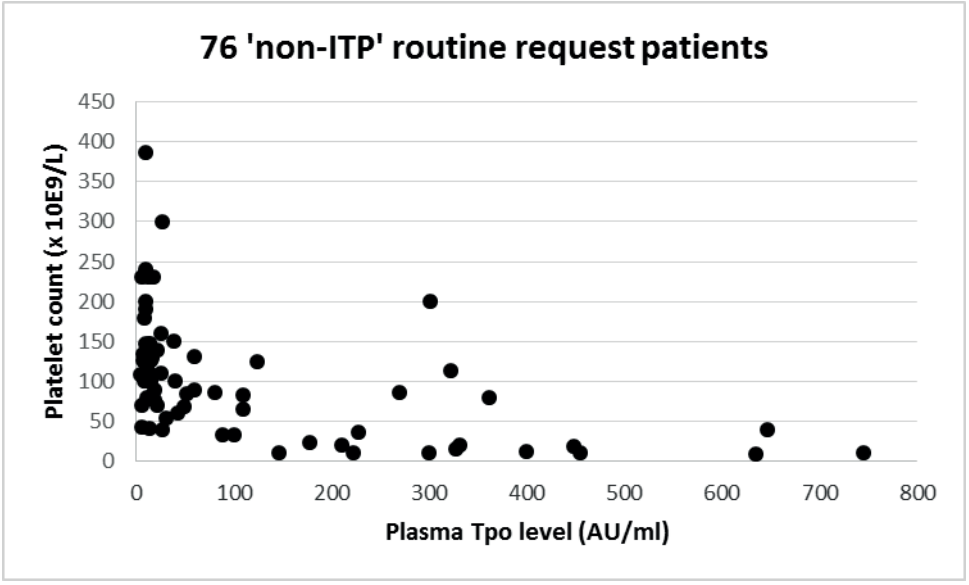
	GPIIb/IIIa+ GPIb/IX+ GPV	GPIIb/IIIa+ GPIb/IX	GPIIb/IIIa+ GPV	GPIb/IX+ GPV	GPIIb/IIIa	GPIb/IX	GPV	negative	total
Number of samples reactive with	18 (30%)	10 (16.7%)	6 (10%)	4 (6.7%)	6 (10%)	4 (6.7%)	3 (5%)	9 (15%)	60 (100%)

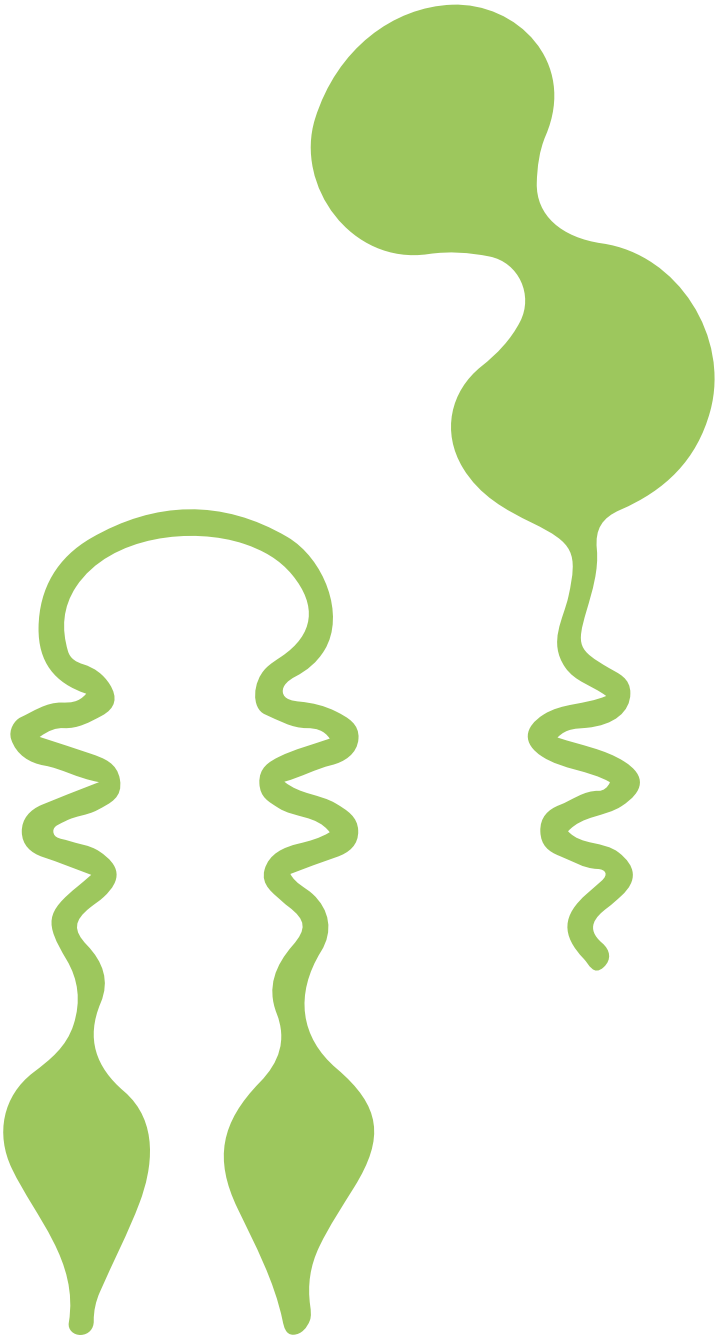
Supplemental Table IIb: Platelet autoantibody reactivity in ITP samples as tested for five platelet glycoproteins with the direct MAIPA.

	GPIIb/IIIa	GPIb/IX	GPV	GPIa/IIa	GPIV
Number of samples reactive with	40/60 (66.7%)	36/60 (60%)	31/60 (51.7%)	13/32 (40.6%)	7/26 (26.9%)

Supplemental Figure 2: *Platelet counts versus plasma Tpo levels for routine request patients*







06

CHAPTER 6

Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients

Porcelijn L, Huiskes E, Schipperus M, van der Holt B, de Haas M, Zwaginga JJ; Dutch HOVON 64 Study Group. Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients. Blood. 2017 Jun 22;129(25):3389-3391.

Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients

Leendert Porcelijn,[1] Elly Huiskes,[1] Martin Schipperus,[2] Bronno van der Holt,[3] Masja de Haas,[1,4-6] and Jaap Jan Zwaginga,[5,6] for the Dutch HOVON 64 Study Group

[1]Immunohematalogy Diagnostics, Sanquin Diagnostic Services, Amsterdam, The Netherlands; [2]Department of Internal Medicine, HagaZiekenhuis, Den Haag, The Netherlands; [3]Haemato Oncology Foundation for Adults in The Netherlands (HOVON) Data Center, Erasmus MC Gancer Institute—Clinical Trial Centre, Rotterdam, The Netherlands; [4]Sanquin Research and Landsteiner Laboratory, Academie Medical Centre, Universlty of Amsterdam, Amsterdam, The Netherlands; [5]Center for Clinical Transfusion Research, Sanquin Research, Leiden, The Netherlands; and [6]Department of Immuno-hematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

A complete list of the members of the Dutch HOVON 64 Study Group appears in the "Appendix."

Rituximab, a chimeric CD20 monoclonal antibody that causes depletion of B cells, shows a short term treatment efficacy in 40% to 60% of immune thrombocytopenia (ITP) patients.1,2 Our recently published multi-center randomized open label phase 2 trial comparing three rituximab dosing schemes showed 50% responses in 138 ITP patients.3 Unfortunately, the treatment efficacy of rituximab so far cannot be predicted. Here, however, we show absent levels of platelet-bound antibodies to be associated with refractoriness to rituximab. Assays on platelet bound antibodies may hence lead to a more personalized treatment approach for these patients.

ITP was diagnosed in accordance with the recommendation of the American Society of Hematology (ASH)4, by patient and family history, physical examination and laboratory investigations (e.g. normal WBC count and differentiation, normal RBC count, RBC indices and mean platelet volume, absence of HIV, HBV,HCV, Helicobacter Pylori, and antinuclear factor, antinuclear antibodies, antiphospholipid antibodies). Furthermore, low to normal thrombopoietin (Tpo) levels supported thrombocytopenia to be due to increased platelet destruction.5 Eligible patients were 18 years of age or older, with an ITP relapse or refractoriness (minimally two platelet counts < 30 x 109/L), at least 3 weeks after high-dose (≥ 1 mg/kg) corticosteroids and who were randomized between three rituximab dosing schemes, i.e. 2 or 4 once-weekly standard 375 mg/m2 doses and a twice-weekly 750 mg/m2 regimen.3 Complete (CR) good/partial (PR) and moderate (MR) response were defined as platelet counts of 150x109/L or more, 50x109/L

or more on 2 consecutive occasions and a platelet count over 30x109/L with at least twice the base-line count, respectively. The different dosages of rituximab did not lead to changes in response rate.3

Prior to starting the first rituximab dose and subsequently for ten weeks, weekly EDTA-anticoagulated blood sampling was requested to assess the presence of platelet-associated IgG and IgM autoantibodies by the direct platelet immunofluorescence test (PIFT) and the eluate-(indirect) PIFT, as described previously.6 In the current report, a positive 'PIFT' refers to the combination of a reactive (=1+ to 3+) direct as well as eluate PIFT. One or both tests with negative reactions were defined as a negative 'PIFT'. Additionally, in samples with sufficient platelet numbers, both the PIFT and a direct 'monoclonal antibody immobilization of platelet antigens' (MAIPA) assay were performed; the latter to detect IgG-class autoantibodies.7 All MoAbs, CLBthromb/1 (CD41, anti-GPIIb), MB45 (CD42a, anti-GPIX) and SW16 (CD42d, anti-GPV) were supplied by Sanquin Reagents (Amsterdam, the Netherlands).

For statistical analysis: correlation between continuous variables was calculated by Spearman's rank test, association between categorical valuables was calculated by the 2-tailed Fisher Exact probability test and 95% confidence intervals were calculated using Stata version 14.0 (StataCorp LP, Texas, USA).
Prerituximab treatment samples from 112 of 138 patients and follow-up samples during and after rituximab treatment from 80 patients were sent to our laboratory (Supplemental Figure 1).

In 99 of 112 patients samples (88%), the platelet count in prerituximab treatment samples was sufficient to enable platelet associated auto-antibody detection by PIFT. Of these, a representative 47 (47%) responded to rituximab (CR: n=17, PR: n=22 and MR: n=8, respectively) while 52 (53%) were nonresponders. For all tested patients (n=99) direct PIFT results corresponded with the eluate-PIFT results. Antibodies were present in 79 patients, of whom 43 resonded (54%) with 16 complete (21%). In contrast, the absence of antibodies in 20 patients was associated with 4 responses (20%) of which only 1 (5%) was complete (Table 1, p=0.006). In summary, undetectable platelet autoantibodies in the direct PIFT strongly predict absence of or less than complete response to rituximab (Table 1: negative predictive value 95.0% ;CI 73.2-99.25%, positive predictive value 20.3%; CI 17.7-23.1%).

Vice versa, 16 patients (94%) with a complete response to rituximab showed positive to very-strong-positive direct PIFT reactions (mean level 2+) whereas the percentage of non-responsive patients with a positive direct PIFT was much lower (69%; p=0.051). In patients with partial or moderate platelet responses, positive

PIFT occurred with similar frequency in 19 (86%) and 8 (100%) respectively.
Serial autoantibody testing was performed for 80 of the sampled patients (71%). Of these, the presence of platelet autoantibodies and corresponding platelet counts were evaluated “per response group” at baseline and at the time of the highest platelet counts that were reached within 10 weeks after start of rituximab treatment (Figure 1; supplemental Figure 2). Of 30 patients predominantly responding (10 CR, 10 PR, 3 MR), the platelet count also enabled the direct MAIPA. Both PIFT and MAIPA results in these patients appeared in full concordance, i.e. seven patients (4 NR and 3 PR) were negative with both PIFT and direct MAIPA and 23 (3 NR, 3 MR, 7 PR and 10 CR) positive with both PIFT and direct MAIPA.

CR in the Haemato Oncology Foundation for Adults in The Netherlands (HOVON) 64 Study was defined as a rise in platelet counts to 150x109/L or more. Nowadays, ITP is defined as platelet counts <100 x 109/L.8 Re-categorization of the response groups, using 100 x 109/L did not result in different response numbers.

	detectable platelet-associated antibodies at baseline		
	Positive (%)	Negative (%)	total
CR	16 (20)	1 (5)	17
PR	19 (24)	3 (15)	22
MR	8 (10)	0 (0)	8
NR	36 (46)	16 (80)	52
total	79	20	99

P=0.006

Anti-CD20 monoclonal antibodies deplete B cells for periods varying from months to more than one year. In this respect B cells are essential to present antigens to CD4+ T cells and moreover secrete cytokines activating macrophages, dendritic cells and immune-regulatory cells and thus an ongoing autoimmune response.9-11 Additionally, rituximab is reported to normalize both the abnormalities and dysfunction of the T cell compartment in ITP patients.9,12-14 However, depletion of pre-plasma cell B cells and with it decreased autoantibody production is regarded as most likely mechanism of action. But, so far not one of these explanations could be correlated to rituximab’s variable therapeutic effects.

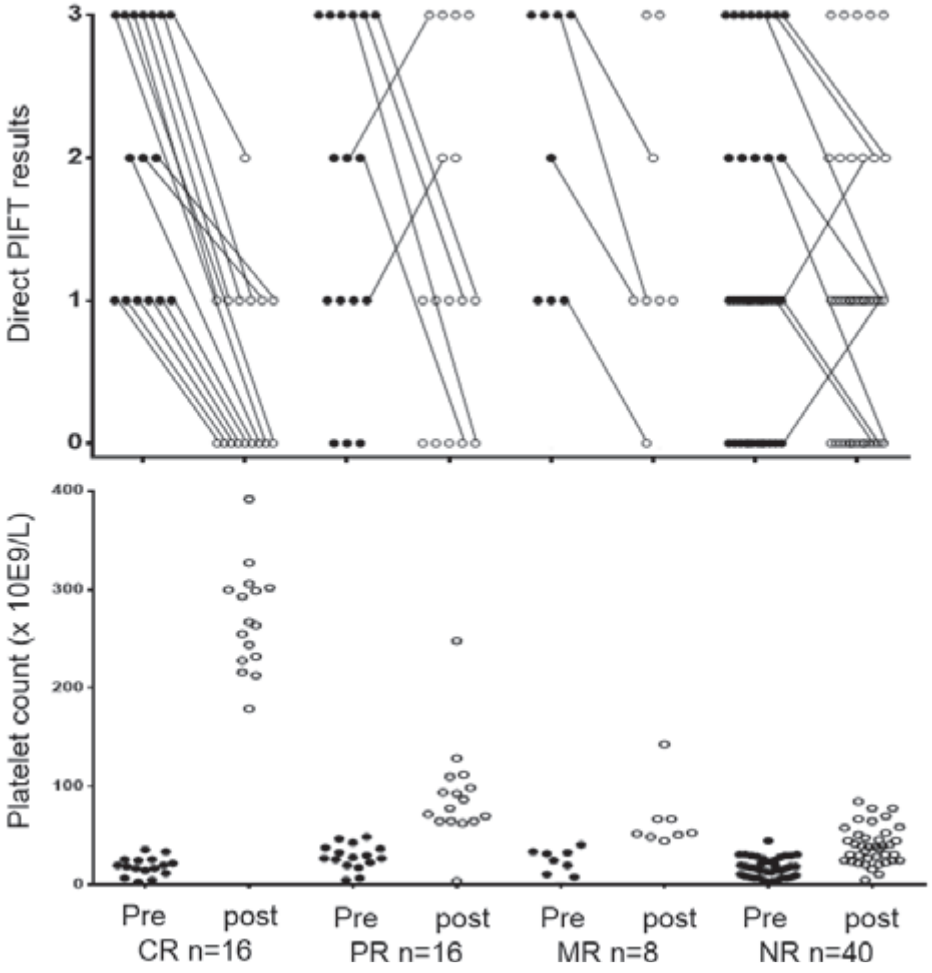


***Figure 1**. Direct PIFT results and platelet counts prior to initiation with rituximab and at the time of the highest platelet counts reached within 10 weeks after start of rituximab treatment for 80 serially tested ITP patients. X-axis for figure B also applies for figure A. A: CR=complete responders (n=16), PR=partial responders (n=16), MR=moderate responders (n=8), NR=non-responders (n=40). Direct PIFT scores before (pre) and at the time of the highest platelet count within ten weeks after (post) treatment with rituximab: 0=negative, 1=positive, 2=strong positive and 3=very strong positive. Changes in antibody detection results are indicated by means of connecting lines.*

B: Platelet counts before and the highest platelet count within ten weeks after treatment with rituximab.

Our results are in agreement with the latter mechanism while all serially tested CR patients (n=16) showed decreasing antibody results and 32 of 39 non-responsive patients have non-decreasing autoantibodies after or undetectable autoantibodies before rituximab treatment (Figure 1). A possible explanation for nondecreasing antibodies could be insufficient eradication of antibody producing plasma cells. Indeed long-lived plasma cells were detected in the spleen of ITP patients non responsive to rituximab treatment and it has been suggested that B cell depletion promotes the differentiation and settlement of these long-lived plasma cells in the spleen.15 Although nonresponsive patients without detectable platelet associated autoantibodies, fit the ASH criteria for ITP, these patients might represent a subgroup of ITP patients with possibly T-cell mediated platelet destruction. Indeed, Audia et al. in this respect showed the importance of activated splenic CD8+ T cells in ITP patients unresponsive to rituximab.16 Furthermore, CD8+ T cells were shown to contribute to the murine splenocyte's ability to induce thrombocytopenia; recently Arthur et al. showed CD8+ T cell mediated antibody-independent platelet clearance in a murine model for refractoriness to platelet transfusions.17, 18

Immune suppressive treatment in combination with Rituximab, as recently published, may improve the limited response for rituximab as single treatment.19 In this respect it will be interesting to study the effect of combination therapy in ITP patients with and without detectable platelet autoantibodies.

In conclusion, our results show absence of platelet-bound antibodies to be associated with a low response to rituximab. Additionally, response to rituximab appeared strongly associated with a decline in platelet-bound antibodies. Our findings importantly implicate that both antibody-negativity vs. strong antibody presence might enable a more individualized therapeutic approach in this group of ITP patients.

Appendix: study group members

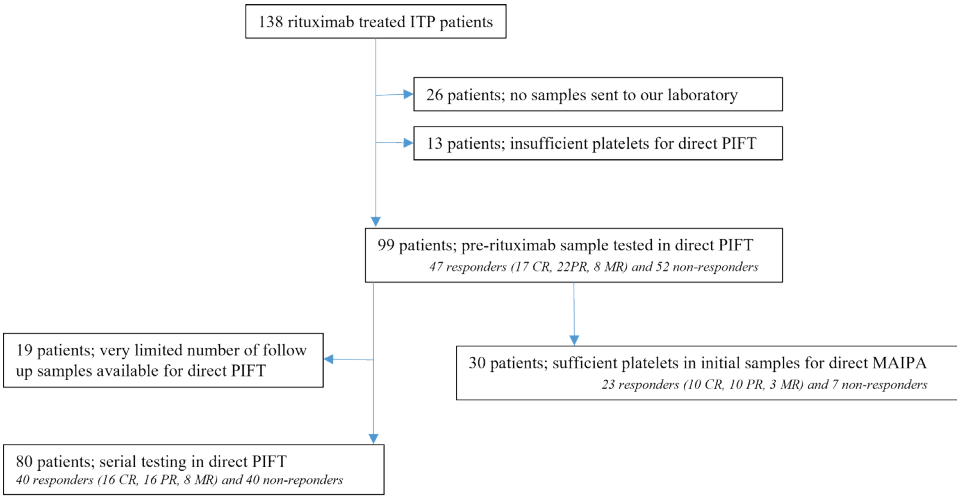
The members of the Dutch HOVON 64 Study Group are: Jaap J. Zwaginga (Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre and the Centre for Clinical Transfusion Research, Sanquin-Leiden University Medical Centre, Leiden, The Netherlands), Bronno van der Holt (HOVON Data Center, Erasmus MC Cancer Institute–Clinical Trial Centre, Rotterdam, The Netherlands), Peter A. te Boekhorst (Department of Hematology, Erasmus MC, Rotterdam, The Netherlands), Bart J. Biemond (Department of Hematology, Academic Medical Centre Amsterdam, Amsterdam, The Netherlands), Mark-David Levin (Department of Internal Medicine, Albert Schweitzer Hospital, Dordrecht, The Netherlands), Rene van der Griend (Department of ´ Internal Medicine, Diakonessenhuis, Utrecht, The Netherlands), Anneke Brand (Department of Immunohematology and Blood Transfusion, Leiden University Medical Centre and the Centre for Clinical Transfusion Research, Sanquin-Leiden University Medical Centre, Leiden, The Netherlands), Sonja Zweegman (Department of Hematology, VU University Medical Centre, Amsterdam, The Netherlands), Hans F. M. Pruijt (Department of Internal Medicine, Jeroen Bosch Hospital, Den Bosch, The Netherlands), Vera M. J. Novotny (Department of Hematology, Radboud University Medical Centre, Nijmegen, The Netherlands), Art Vreugdenhil (Department of Internal Medicine, Maxima Medical Centre, Veldhoven, The ´ Netherlands), Marco R. de Groot (Department of Internal Medicine, Medisch Spectrum Twente, Enschede, The Netherlands), Okke de Weerdt (Department of Internal Medicine, St. Antonius Hospital, Nieuwegein, The Netherlands), Elisabeth C. M. van Pampus (Department of Laboratory Medicine, Radboud University Medical Centre, Nijmegen, The Netherlands), Tanja M. van Maanen-Lamme (Department of Internal Medicine, Westfriesgasthuis, Hoorn, The Netherlands), Shulamiet Wittebol (Department of Internal Medicine, Meander Hospital, Amersfoort, The Netherlands), Martin R. Schipperus (Department of Internal Medicine, HagaZiekenhuis, Den Haag, The Netherlands), Matthijs H. Silbermann (Department of Internal Medicine, Tergooiziekenhuizen, Blaricum, The Netherlands), Peter C. Huijgens (Department of Hematology, VU University Medical Centre, Amsterdam, The Netherlands), Marleen Luten (HOVON Data Center, Erasmus MC Cancer Institute–Clinical Trial Centre, Rotterdam, The Netherlands), Rene Hollestein (HOVON Data Center, Erasmus MC Cancer Institute–Clinical Trial Centre, Rotterdam, The Netherlands), Jan A. C. Brakenhoff (Department of Internal Medicine, Waterland Hospital, Purmerend, The Netherlands), Jolanda G. Schrama (Department of Internal Medicine, Spaarne Hospital, Hoofddorp, The Netherlands), Fransje A. A. Valster (Department of Internal Medicine, Lievensberg Hospital, Bergen op Zoom, The Netherlands), Gerjo A. Velders (Department of Internal Medicine, Gelderse Vallei Hospital, Ede, The Netherlands), and Harry R. Koene (Department of Internal Medicine, St. Antonius Hospital, Nieuwegein, The Netherlands).

References

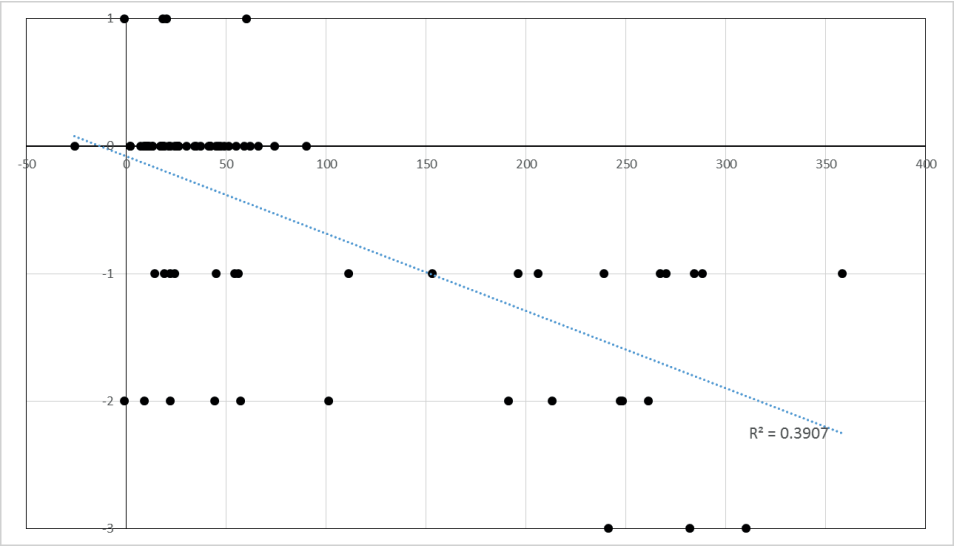
1. Auger S, Duny Y, Rossi JF, et al. Rituximab before splenectomy in adults with primary idiopathic thrombocytopenic purpura: a metaanalysis. Br J Haematol. 2012;158(3):386-398.
2. Patel VL, Mahévas M, Lee SY, et al. Outcomes 5 years after response to rituximab therapy in children and adults with immune thrombocytopenia. Blood. 2012;119(25):5989-5995.
3. Zwaginga JJ, van der Holt B, Te Boekhorst PA, Biemond BJ, Levin MD, van der Griend R, Brand A, Zweegman S, Pruijt HF, Novotny VM, Vreugdenhil A, de Groot MR, de Weerdt O, van Pampus EC, van Maanen-Lamme TM, Wittebol S, Schipperus MR, Silbermann MH, Huijgens PC, Luten M, Hollestein R, Brakenhoff JA, Schrama JG, Valster FA, Velders GA, Koene HR; Dutch HOVON 64 study group. Multi-center randomized open label phase II trial on three rituximab dosing schemes in immune thrombocytopenia patients. Haematologica. 2015 Mar;100(3):e90-2.
4. 2011 Clinical Practice Guideline on the Evaluation and Management of Immune Thrombocytopenia (ITP) The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia.
5. Porcelijn L, Folman CC, Bossers B, et al. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. Thromb Haemost. 1998;79(6):1101-5.
6. von dem Borne AEGK, Verheugt FWA, Oosterhof F, et al. A simple immunofluorescence test for the detection of platelet antibodies. Br J Haematol 1978; 39: 195–207.
7. Kiefel V, Santoso S, Weisheit M, Müeller-Eckhardt C. Monoclonal antibody--specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood. 1987 Dec;70(6):1722-6.
8. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold DM, Bussel JB, Cines DB, Chong BH, Cooper N, Godeau B, Lechner K, Mazzucconi MG, McMillan R, Sanz MA, Imbach P, Blanchette V, Kühne T, Ruggeri M, George JN. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood. 2009 Mar 12;113(11):2386-93.
9. Stasi R. Rituximab in autoimmune hematologic diseases: not just a matter of B cells. Semin Hematol. 2010 Apr;47(2):170-9.
10. Roche PA, Furuta K. The ins and outs of MHC class II-mediated antigen processing and presentation. Nat Rev Immunol. 2015 Apr;15(4):203-16.
11. Shlomchik MJ, Craft JE, Mamula MJ. From T to B and back again: positive feedback in systemic autoimmune disease. Nat Rev Immunol. 2001 Nov;1(2):147-53.
12. Martin F, Chan AC. B cell immunobiology in disease: evolving concepts from the clinic. Annu Rev Immunol. 2006;24:467-96.
13. Cooper N, Stasi R, Cunningham-Rundles S, Cesarman E, McFarland JG, Bussel JB. Platelet-associated antibodies, cellular immunity and FCGR3a genotype influence the response to rituximab in immune thrombocytopenia. Br J Haematol. 2012 Aug;158(4):539-47.
14. Stasi R, Cooper N, Del Poeta G, Stipa E, Laura Evangelista M, Abruzzese E,Amadori S. Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab. Blood. 2008 Aug 15;112(4):1147-50.
15. Mahévas M, Patin P, Huetz F, Descatoire M, Cagnard N, Bole-Feysot C, Le Gallou S, Khellaf M, Fain O, Boutboul D, Galicier L, Ebbo M, Lambotte O, Hamidou M, Bierling P, Godeau B, Michel M, Weill JC, Reynaud CA. B cell depletion in immune thrombocytopenia reveals splenic long-lived plasma cells. J Clin Invest. 2013 Jan;123(1):432-42.

16. Audia S, Samson M, Mahévas M, Ferrand C, Trad M, Ciudad M, Gautheron A, Seaphanh F, Leguy V, Berthier S, Salles B, Martin L, Lorcerie B, Ortega-Deballon P, Facy O, Caillot D, Soudry-Faure A, Michel M, Godeau B, Larmonier N, Saas P, Janikashvili N, Bonnotte B. Preferential splenic CD8(+) T-cell activation in rituximab-nonresponder patients with immune thrombocytopenia. Blood. 2013 Oct 3;122(14):2477-86.
17. Chow L, Aslam R, Speck ER, Kim M, Cridland N, Webster ML, Chen P, Sahib K, Ni H, Lazarus AH, Garvey MB, Freedman J, Semple JW. A murine model of severe immune thrombocytopenia is induced by antibody- and CD8+ T cell-mediated responses that are differentially sensitive to therapy. Blood. 2010 Feb 11;115(6):1247-53.
18. Arthur CM, Patel SR, Sullivan HC, Winkler AM, Tormey CA, Hendrickson JE, Stowell SR. CD8+ T cells mediate antibody-independent platelet clearance in mice. Blood. 2016 Apr 7;127(14):1823-7.
19. Choi PY, Roncolato F, Badoux X, Ramanathan S, Ho SJ, Chong BH. A novel triple therapy for ITP using high-dose dexamethasone, low-dose rituximab, and cyclosporine (TT4). Blood. 2015 Jul 23;126(4):500-3.

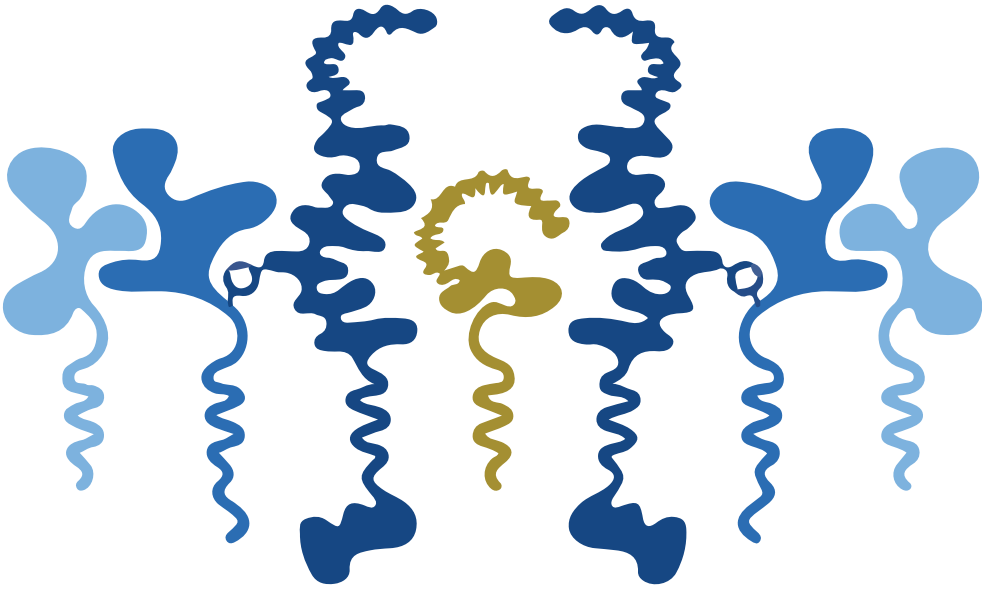
Supplemental data:



Supplemental Figure 1: *Of the 138 rituximab treated ITP patients included in the HOVON64 study, 99 pre-rituximab blood samples could be tested in the direct PIFT and 30 samples also in the direct MAIPA. For 80 patients sufficient follow up samples were available to perform serial testing in de direct PIFT. PIFT=Platelet Immunofluorescence Test, MAIPA=Monoclonal Antibody Immobilization of Platelet Antigens assay, CR=complete responder, PR=partial responder, MR=moderate responder, NR=non-responder*



Supplemental Figure 2: *Delta values, i.e. highest platelet count within ten weeks after rituximab treatment minus platelet count before rituximab treatment, compared with the corresponding direct PIFT result after rituximab treatment minus the result before treatment with rituximab. Spearman's correlation p<0.0001.*



07

CHAPTER 7

Anti-glycoprotein Ibα autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients

Porcelijn L, Schmidt DE, van der Schoot CE, Vidarsson G, de Haas M, Kapur R. Anti-glycoprotein Ibα autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients. Haematologica. 2020 Apr;105(4):e172-e174.

Anti-glycoprotein Ibα autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients

Leendert Porcelijn [1], David E Schmidt [2], C Ellen van der Schoot [2], Gestur Vidarsson [2], Masja de Haas [1 3 4], Rick Kapur [5]

[1]Department of Immunohematology Diagnostics, Sanquin Diagnostic Services, Amsterdam.
[2]Sanquin Research, Department of Experimental Immunohematology and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam.
[3]Sanquin Research, Center for Clinical Transfusion Research and Jon J van Rood Center for Clinical Transfusion Science, Leiden University Medical Center, Leiden.
[4]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leide the Netherlands.
[5]Sanquin Research, Department of Experimental Immunohematology and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam r.kapur@sanquin.nl.

To the editor:

A recently discovered mechanism showed that in mice, anti-glycoprotein (GP) Ib/IX platelet antibodies interfere with hepatocyte thrombopoietin (TPO) production. This mechanism may potentially also contribute to the relatively low TPO levels observed in patients suffering from immune thrombocytopenia (ITP), an autoimmune bleeding disorder in which anti-platelet autoantibodies are able to target platelets. To investigate this we reviewed a large cohort of thrombocytopenic patients which we assessed for anti-platelet autoantibodies and TPO levels (n=3490). We show for the first time that anti-GPIb/IX antibodies, occurring alone or together with other anti-platelet autoantibodies such as anti-GPV and/or with anti-GPIIb/IIIa antibodies, do not influence circulating TPO levels in ITP patients. This suggests that anti-GPIb/IX autoantibodies do not interfere directly with TPO production in humans. Platelet production is regulated mainly by TPO, a hematopoietic growth factor that interacts with the myeloproliferative leukemia protein receptor (Mpl; CD110) on megakaryocytes and circulating platelets.[1,2] The primary site of TPO synthesis is the liver, and to a lesser extent kidney, spleen and bone marrow cells.[3] Interestingly, it was suggested that TPO production is induced by the binding of desialylated aged platelets interacting with the hepatocyte asialoglycoprotein receptor (ASGPR), also known as the hepatic Ashwell-Morrell receptor (AMR).[4] Furthermore, circulating TPO levels are influenced by binding of TPO to platelet- and megakaryocyte-Mpl.[5,6]

ITP is an autoimmune bleeding disorder with a complex pathophysiology.[7] Many ITP patients show autoantibodies to platelet GPIIb/IIIa, GPIb/IX and GPV. In ITP patients, there appears to be an ongoing platelet destruction, but with normal

or mildly elevated TPO levels.[8,9] Recently, a novel mechanism of TPO production was described, in which platelet GPIb signals in an AMR-independent manner to induce hepatocytic TPO production, and was independent of platelet desialylation.[10] In this mouse study, monoclonal antibodies to GPIb were shown to inhibit hepatic TPO production.[10] This mechanism might play an additional role in the relatively low TPO levels observed in ITP patients. However, it has not been investigated if anti-GPIb antibodies are indeed able to interfere with circulating TPO levels in ITP patients. To address this unresolved question, we evaluated TPO levels in ITP patients with anti-platelet autoantibodies including a subgroup with only anti-GPIb IgG antibodies using a large cohort of thrombocytopenic patients evaluated in our national reference laboratory
(Sanquin Diagnostic Services, Amsterdam, The Netherlands) for antigen-specific platelet autoantibodies (years 2011-2019; 3490 patients and 201 healthy controls). Data were handled under national responsible use policies (Code of Conduct for Use of Data in Health Research; https://www.federa.org/codes-conduct). All of these thrombocytopenic samples were tested for platelet autoantibodies against GPIbIX, GPV and GPIIb/IIIa using a modified monoclonal antibody-immobilization of platelet antigens (MAIPA) assay.[11] In addition, circulating TPO levels were measured in fresh EDTA plasma by an in-house ELISA, as previously described.[12,13] Control samples were obtained from non-thrombocytopenic healthy blood donors. Unfortunately, platelet counts at the time of analysis were not available in our laboratory information system. A two-sided alpha value of 0.05 was used as cut-off for statistical significance. Children below 1 year of age were excluded. The total cohort which was analyzed comprised of 3490 individual thrombocytopenic patients with
2979 and 2239 samples for direct and indirect tests, respectively, and 201 healthy controls.

Platelet-associated IgG autoantibodies (direct test) and/or circulating anti-platelet IgG (indirect test) were assessed using MAIPA. Although not all ITP patients have detectable autoantibodies by MAIPA, we have previously reported that a direct antibody test has 98% specificity for clinically diagnosed ITP.[11]
In the current study we found that, in agreement with previous studies,[8,9] TPO levels in ITP patients were significantly increased compared to healthy controls (Figure 1A; P<3.5x10-3 versus healthy controls). However, all patients with detectable antibodies to GPIIb/IIIa, GPV or GPIb/IX, as determined with a direct test, showed similar TPO levels (Figure 1A). Among the majority of ITP patients with multiple anti-platelet glycoprotein antibodies, presence of anti-GPIb antibodies did not affect TPO levels (Figure 1B). Identically to the direct test, also using the indirect test, patients with circulating antibodies against GPIb alone displayed no differences in TPO levels compared to patients with anti-GPIIb/IIIa or anti-GPV antibodies (Figure 1C). In addition, we did not observe

any differences in TPO levels when anti-GPIb/IX antibodies co-occurred with antibodies against GPV and/or GPIIb/IIIa (Figure 1D). It is conceivable that a certain antibody level is required to achieve sufficient opsonization to block GPIb-hepatocyte interactions. However, in agreement with the results above, we did not observe any significant differences between patients with low or high anti-GPIb IgG antibody levels and TPO (Figure 1E).

Our findings in human ITP samples are not in agreement with the recently proposed mechanism stating that anti-GPIb autoantibodies impair TPO production in mice.[10] Alternatively, it may be possible that platelet activation, complement activation or a mechanical feature induced by anti-GPIb antibodies[14] determines the ability to induce Fc-independent platelet clearance, which just like anti-GPIIb/IIIa Fc-mediated platelet clearance, may not induce increased hepatic TPO generation. The limitations of our study are that no additional clinical information, such as co-morbidities and platelet counts, were available. Key strengths of our study are the large number of clinical patient samples available for analysis, the ability to distinguish antibodies against multiple platelet antigens, and the standardized analysis of anti-platelet antibodies in our laboratory. To our knowledge, this is the first time such as large scale analysis is performed investigating the association between anti-platelet GPIb, GPV, and GPII b/IIIa antibodies versus circulating TPO levels in thrombocytopenic patients. Our results further support the notion that the majority of ITP patients clearly demonstrate the simultaneous presence of antibodies to multiple platelet-glycoproteins, including anti-GPV antibodies which were found to be very prevalent, as also previously reported in ITP.[11,15]

In conclusion, our data show that in ITP patients anti-GPIb/IX antibodies, alone or co-occurring with anti-GPV and/or with anti-GPIIb/III a antibodies, do not influence circulating TPO levels. It therefore appears that in humans blocking of GPIb by anti-platelet GPIb antibodies does not directly account for the reduced TPO levels observed in ITP. More research is required to understand the mechanisms which account for the slightly elevated TPO levels in ITP patients.

Acknowledgements
We are thankful for the contributions of the staff of the Platelet and Leukocyte Serology Laboratory, Sanquin Research, in particular Gonda Oldert and Elly Huiskes. This work was supported by a research grant from the Landsteiner Foundation for Blood Transfusion Research (LSBR) and a doctoral stipend to D.E.S. by the Studienstiftung des Deutschen Volkes.

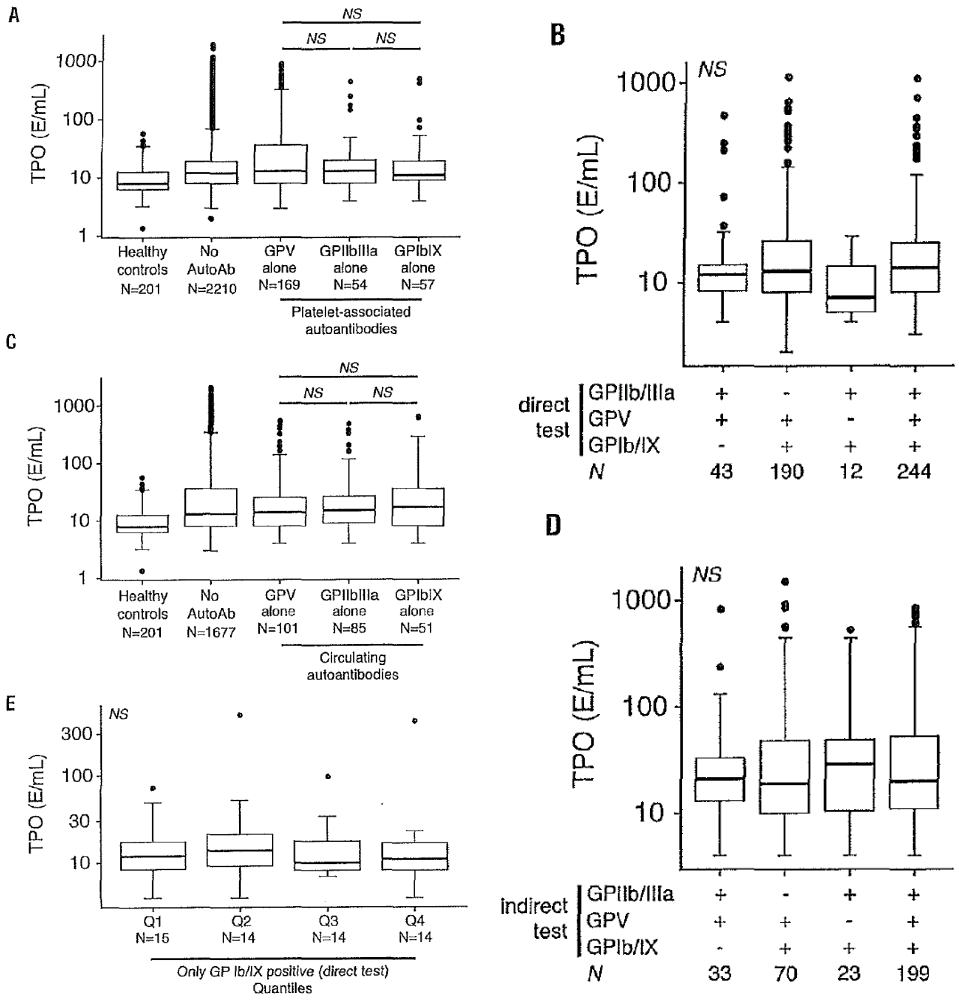
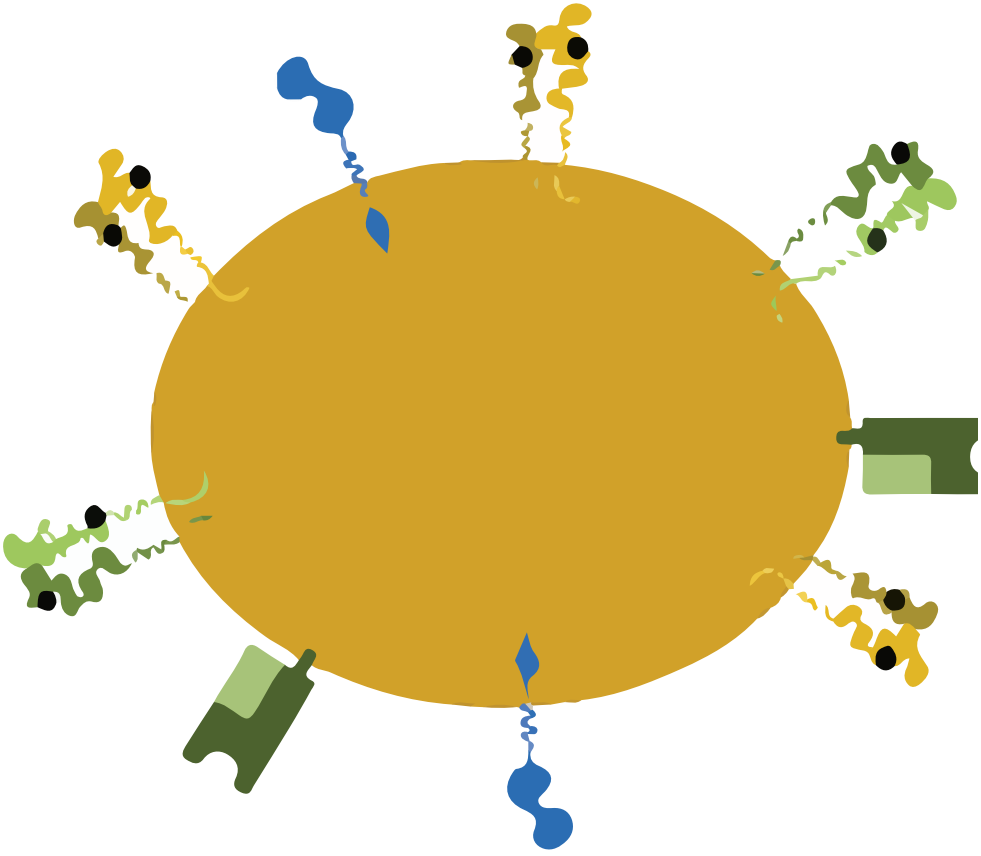


Figure 1. Anti-GPIbIX autoantibodies do not correlate with circulating TPO levels in patients with immune thrombocytopenia. (A) TPO levels, dependent on platelet-associated antibodies (direct test) for the indicated platelet antigens. Only single-positive antibody results are displayed. Exact P-values are (Post-hoc Nemenyi test): Anti-GPV vs anti-GPIb/IX, P=0.84; anti-GPIIb/IIIa vs anti-GPIb/IX, P=0.99; anti-GPV vs anti-GPIIb/IIIa, P=0.76. (B) TPO levels for multiple specificities of anti-platelet autoantibodies in a direct test. Kruskal-Wallis test, P=0.19. (C) TPO levels for single-positive circulating autoantibodies (indirect test). Exact P-values are (Post-hoc Nemenyi test): Anti-GPV vs anti-GPI b/IX, P=0.95; anti-GPIIb/IIIa vs anti-GPIb/IX, P=0.99; anti-GPV vs anti-GPIIb/IIIa, P=0.95. (D) TPO levels by multiple specificities of anti-platelet autoantibodies in an indirect test. Kruskal-Wallis test, P=0.93. (E) No dose-dependent effect of anti-GPIbI X autoantibodies (direct test) on TPO levels in immune thrombocytopenia. Patients were categorized in 25-percent quantiles based on observed antibody levels. Kruskal-Wallis test, P=0.80.

References

1. Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest. 2005;115(12):3339–3347.
2. Kaushansky K. Thrombopoietin. N Engl J Med. 1998;339(11):746–754.
3. Sungaran R, Markovic B, Chong BH. Localization and regulation of thrombopoietin mRNa expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. Blood. 1997;89(1):101–107.
4. Grozovsky R, Begonja AJ, Liu K, et al. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. Nat Med. 2015;21(1):47–54.
5. Folman CC, de Jong SM, de Haas M, Borne von dem AE. Analysis of the kinetics of TPO uptake during platelet transfusion. Transfusion. 2001;41(4):517–521.
6. Fielder PJ, Gurney AL, Stefanich E, et al. Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets. Blood. 1996;87(6):2154–2161.
7. Zufferey A, Kapur R, Semple JW. Pathogenesis and Therapeutic Mechanisms in Immune Thrombocytopenia (ITP). J Clin Med. 2017;6(2):16.
8. Porcelijn L, Folman CC, Bossers B, et al. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. Thromb. Haemost. 1998;79(6):1101–1105.
9. Kosugi S, Kurata Y, Tomiyama Y, et al. Circulating thrombopoietin level in chronic immune thrombocytopenic purpura. Br J Haematol. 1996;93(3):704–706.
10. Xu M, Li J, Neves MAD, et al. GPIbα is required for platelet-mediated hepatic thrombopoietin generation. Blood. 2018;132(6):622–634.
11. Porcelijn L, Huiskes E, Oldert G, et al. Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art. Br J Haematol. 2018;39(Suppl. 1):195.
12. Folman CC, Borne von dem AE, Rensink IH, et al. Sensitive measurement of thrombopoietin by a monoclonal antibody based sandwich enzyme-linked immunosorbent assay. Thromb Haemost. 1997;78(4):1262–1267.
13. Porcelijn L, Folman CC, de Haas M, et al. Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia. Pediatr Res. 2002;52(1):105–108.
14. Quach ME, Dragovich MA, Chen W, et al. Fc-independent immune thrombocytopenia via mechanomolecular signaling in platelets. Blood. 2018;131(7):787–796.
15. Vollenberg R, Jouni R, Norris PAA, et al. Glycoprotein V is a relevant immune target in patients with immune thrombocytopenia. Haematologica. 2019;104(6):1237-1243.



08

CHAPTER 8

Evolution and utility of antiplatelet autoantibody testing in patients with immune thrombocytopenia

Porcelijn L, Schmidt DE, Oldert G, Hofstede-van Egmond S, Kapur R, Zwaginga JJ, de Haas M. Evolution and Utility of Antiplatelet Autoantibody Testing in Patients with Immune Thrombocytopenia. Transfus Med Rev. 2020 Oct;34(4):258-269.

Evolution and utility of antiplatelet autoantibody testing in patients with immune thrombocytopenia

Leendert Porcelijn[a] , David E Schmidtb, Gonda Oldert[a], Suzanne Hofstede-van Egmond[a], Rick Kapur[b], Jaap Jan Zwaging[ac,d], Masja de Haasa,[d]

[a]Department of Immunohematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, the Netherlands.
[b]Sanquin Research, Department of Experimental Immunohematology, Amsterdam and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands.
[c]Department of Immuno-hematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands,
[d]Sanquin Research, Center for Clinical Transfusion Research, Leiden, The Netherlands, and Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands.

Abbreviations

ACE	antigen capture ELISA
AMR	Aswell-Morell receptor
ASCA	antigen specific capture assay
ASPA	antigen specific particle assay
BM	bone marrow
DNA	deoxyribonucleic acid
FNAIT	fetal/neonatal alloimmune thrombocytopenia
GP	glycoprotein
hc	healthy control
HPA	Human platelet antigen
ISTH	International Society on Thrombosis and Haemostasis
ITP	immune thrombocytopenia
IVIg	intravenous immunoglobuline
LBD	lectine binding domain
MACE	modified antigen capture ELISA
MAIPA	monoclonal antibody immobilization of platelet antigens
MK	megakoryocyte
moab	monoclonal antibody
OD	optical density
PIFT	platelet immunofluorescence test
SD	standard deviation
Tpo	thrombopoietin

Abstract

To this day, Immune Thrombocytopenia (ITP) remains a clinical diagnosis made by exclusion of other causes for thrombocytopenia. Reliable detection of platelet autoantibodies would support the clinical diagnosis, but the lack of specificity and sensitivity of the available methods for platelet autoantibody testing limits their value in the diagnostic work-up of thrombocytopenia. The introduction of methods for glycoprotein specific autoantibody detection has improved the specificity of testing and is acceptable for ruling in ITP, but not ruling it out as a diagnosis. The sensitivity of these assays varies widely, even between studies using comparable assays. A review of the relevant literature combined with our own laboratory's experience of testing large number of serum and platelet samples makes it clear that this variation can be explained by variations in the characteristics of the tests, including in the glycoprotein-specific monoclonal antibodies, the glycoproteins that are tested, the platelet numbers used in the assay and the cut-off levels for positive and negative results, as well as differences in the tested patient populations. In our opinion, further standardization and optimization of the direct autoantibody detection methods to increase sensitivity without compromising specificity seems possible, but will still likely be insufficient to distinguish the often very weak specific autoantibody signals from background signals. Further developments of autoantibody detection methods will therefore be necessary to increase sensitivity to a level acceptable to provide laboratory confirmation of a diagnosis of ITP.

Contents

General introduction
1. Introduction of GP-Specific Assays for Detection of Platelet Autoantibodies With Increasing Sensitivity of GPIIb/IIIa and GPIb/IX Autoantibody Detection
2. Other GPs as Targets for Autoantibodies and the Impact of GPV-Specific Platelet Autoantibodies
3. GP-Specific Autoantibody Binding Causing Lossof Platelet Function
4. GPIb-Specific Autoantibodies, Thrombopoietin Production, and Fc-Independent Platelet Destruction
5. The Impact of the Change in ITP Definition to <100 Instead of <150 × 109/L
6. The Impact of the GP-Specific Monoclonal Antibodies Used in the Assay on the Test Results
7. The Impact of Cut off Values in the Assay
8. The Impact of Autoantibodies on Platelet Production
9. Discussion
Declaration of Competing Interest
References

General introduction

Immune thrombocytopenia (ITP) is a benign hematological disorder, which may lead to severe hemorrhagic diathesis, sometimes requiring extensive therapy for many years.[1, 2] Although platelet autoantibodies are the major underlying cause for ITP, whether or not to use platelet autoantibody detection for diagnosing ITP has been the subject of discussion for over 40 years. More recent guidelines do indicate that performing glycoprotein specific autoantibody detection may be useful, still the general tendency remains to diagnose ITP by excluding other causes.[3-5] In this review, we provide a short history and discuss in detail the glycoprotein specific autoantibody methods for autoantibody detection in detail. For many decades, clinicians have managed without this test and ITP was confirmed on the basis of a lacking alternative diagnosis for the patient's thrombocytopenia. The leading questions in this review are if platelet autoantibody detection adds value to the diagnostic work-up of ITP? In other words, is the specificity and/or sensitivity of the presently available methods sufficient enough to serve as gold-standard to diagnose or discard ITP as diagnosis.

The value of a diagnostic gold-standard is proven in several studies. The McMaster ITP registry set up in Canada showed that 36 of 295 (12.2%) adult patients initially diagnosed as ITP were found not to have ITP at follow-up and that 10 of 319 (3.1%) patients initially diagnosed with other causes of thrombocytopenia eventually turned out to have ITP.[6] In patients referred to our laboratory for platelet autoantibody detection, we could recently confirm these findings. After analysis of clinical data received several months after the routine requests (i.e. independent of the autoantibody detection results), ITP could be excluded for 76 of 165 (46%) patients.[7] Also the ten-year retrospective chart review by Bryant et al. of a large cohort (n=492) children/adolescents (aged 0-18 years) initially diagnosed with ITP showed a different final diagnosis in 14%.[8] Most of these revised ITP diagnoses could well have been diagnosed earlier through a thorough evaluation of the clinical symptoms. However some needed extensive laboratory investigation before a final diagnosis could be made. In addition, due to the growing availability of large-scale DNA sequence methods, we now see that some patients have been diagnosed with and treated for ITP for many years, but for whom it has now been demonstrated that they suffer from an inherited disorder.[9] Overall, these data show that the sensitivity and specificity of a clinical diagnosis of ITP with 'exclusion of other causes' is still not optimal and that more reliable tests for the diagnosis of ITP would be helpful. The importance of good serological testing is evident, of course, while autoantibodies against platelets play a central role in the ITP pathology.[2, 10] Although cellular autoimmune responses with T cell cytotoxicity have been described[11-13] and may also be a cause of ITP, any reliable method for detecting platelet autoantibodies would support clinical diagnosis in the large majority of ITP patients.[4]

Historically, some of the first platelet autoantibody detection methods measured the serum-induced platelet-dependent endpoints such as aggregation, lysis or granule release; and two-step assays measured platelet-bound and circulating platelet-reactive antibodies, making use of fluorescence-labeled anti-human-immunoglobulines.[14-16] The two-step methods with fluorescently labeled anti-human-Ig antibodies were a major breakthrough and more reliable than platelet activation assays, but their sensitivity and specificity were still insufficient. Incubation of patient platelets with fluorescent-labeled anti-IgG in the direct (i.e., measurement of patient platelet-bound autoantibodies) platelet immunofluorescence test (PIFT) detects platelet autoantibodies in approximately 70-80% of ITP patients, with a specificity of approximately 50-60%.[16-18] Nonspecific antibody binding, e.g. by immune complexes binding via the platelet-IgG-Fcγ receptor type IIa, causes false-positive test results in many non-ITP patients.[19, 20] It was first shown by van Leeuwen et al. (1981) that a high percentage of autoantibodies in sera from ITP patients reacted positive in the PIFT with healthy donor platelets, but did not react with GPIIb/IIIa deficient platelets from Glanzmann thrombasthenia patients.[21] At that time, to reduce the problem of non-specific results, solubilization of the platelet membrane and extraction of the membrane proteins, retaining their antigenicity, with non-ionic detergents was described at the time.[22-25] Together with the availability of GP-specific monoclonal antibodies (moab), this led to the development of glycoprotein-specific platelet antibody detection methods. After a first experimental approach by Woods et al (1984) with immobilization of glycoproteins IIb/IIIa and Ib/IX on microtiterplates, two more sensitive methods were introduced, i.e. the immunobead assay by McMillan et al (1987) and the monoclonal antibody immobilization of platelet antigens (MAIPA) assay by Kiefel et al (1987).[26-29] In our laboratory the direct MAIPA is used for routine diagnostic detection of autoantibodies in ITP patients and shows good specificity (> 95%) and reasonable sensitivity (80%)[7], but varying results were found in different studies. In this review we will discuss our choices in platelet autoantibody assay design and the assay performance.

1. Introduction of GP specific assays for detection of platelet autoantibodies with increasing sensitivity of GPIIb/IIIa and GPIb/IX autoantibody detection.

Following the findings by van Leeuwen et al., Woods et al. attached isolated GPIIb/IIIa on microtiterplate wells coated with a GPIIb/IIIa specific moab, enabling them to confirm the presence of GPIIb/IIIa specific autoantibodies in plasma from five of 56 chronic ITP patients for the first time.[27] In the same year, they showed that GPIIb/IIIa was not the only target for autoantibodies, as three of 106 plasma's from chronic ITP patients were reactive with immobilized GPIb.[26] One of these GPIb reactive samples also reacted with GPIIb/IIIa, suggesting the presence of various specificities of antibodies in patients. Furthermore, in the

GPIIb/IIIa and GPIb studies all 34 and 59 samples, respectively, from patients with a variety of other platelet disorders showed negative results, indicating a high test specificity. Unfortunately, the number of positive samples detected with these early GP-specific assays turned out to be very small. Further optimization of the assay and the idea that free-circulating autoantibodies may be less detectable than platelet-bound autoantibodies became the subject for many follow-up studies. McMillan (1987) introduced moab-coated beads to specifically target GP for the detection of platelet-associated and free-circulating autoantibodies, which proved to be more sensitive.[28] By testing platelet-eluates in this technique platelet-associated autoantibodies were detected in 21 of 28 (75%) ITP patients, while free-circulating autoantibodies were detected in 34 of 59 (57.6%) patients. Again, none of the 31 non-ITP thrombocytopenic patients showed reactive autoantibodies. However, for unclear reasons, only two of 34 samples responded with both GP, which later turned out to be too low a percentage. The development of the antigen-capture ELISA (ACE) and MAIPA allowed for more standardized and reliable platelet antibody detection.[29, 30] Autoantibodies were detected in 58 of 81 (72%) sera from suspected ITP patients in MAIPA by Kiefel et al (1991), of which 17 (29%) GPIIb/IIIa specific, 19 (33%) GPIb/IX specific and 22 (38%) reactive with both GP.[31]

An overview of studies using GP-specific methods for the detection of autoantibodies is shown in Table1. In most studies, approximately 60-80% of autoantibodies react with GPIIb/IIIa and 50% with GPIb/IX. The majority of samples contain antibodies with both types of GP specificities, but still a significant percentage (10-40%) reacts with only one GP (Table 1). These findings indicated that it is necessary to test both GPIIb/IIIa and GPIb/IX for the detection of autoantibodies. However, despite initial reasonable results from McMillan and Kiefel with > 70% sensitivity, this percentage was no longer met in subsequent studies, triggering a search for other antibody binding sites and further optimization of the autoantibody detection assays.

Table 1: Autoantibody testing studies in patients with immune thrombocytopenia

Author	assay	material tested	number of pl used in test* x 10^6	patients	Healthy controls	non-ITP	Glycoprotein specific antibodies						pat pl counts	assay cut-off	GP specific MoAB
				pos/total (%)	pos/ total (%)	pos/ total (%)	GPIIb/IIIa	GPIb/IX	GPIa/IIa	GPV	GPIV	combi GP			
Woods 1984a[27]	ELISA	plasma		5/56 (9%)	0/16	0/34	5	nt	nt	nt	nt	nt		mean controls + 3SD	2G12 (IIb/IIIa)
Woods 1984b[26]	ELISA	plasma		3/73 (4%)	0/22	0/59	nt	3	nt	nt	nt	nt		mean controls + 3SD	AP1 (Ib)
McMillan 1987[28]	Immunobead	eluates		21/28 (75%)		0/15	13/21 (62%)	8/21 (38%)	nt	nt	nt	0/21 (0%)		mean controls + 2SD	2A9 (IIb), 3F5 (IIb/IIIa, 2G12 (IIb/IIIa), P3 (Ib)
		plasma		34/59 (58%)		0/20	23/34 (68%) 21 (62%) only#	13/34 (38%) 11 (32%) only	nt	nt	nt	2/34 (6%)		mean controls + 2SD	2A9 (IIb), 3F5 (IIb/IIIa, 2G12 (IIb/IIIa), P3 (Ib)
Kiefel 1991[31]	MAIPA	sera	100	58/81 (72%)			39/58 (67%) 17 (29%) only	41/58 (33%) 19 (33%) only	nt	nt	nt	22/58 (38%)		0.2	Gi5 (IIb/IIIa), FMC25 (IX)
He 1994[32]	Immunobead	sera		32/47 (68%)	1/43 (2%)	0/15	22/32 (69%) 5 (23%) only	24/32 (75%) 5 (23%) only	3/32 (9%) 0 (0%) only	nt	12/32 (38%) 2 (6%) only	20/32 (63%)	7-120	mean controls + 2SD	SZ22 (IIb), SZ21 (IIIa), SZ1 (Ib/ IX), SZ2 (Ib), Gi6 (Ia/IIa), FA6-152 (IV)

Gaiger 1994[89]	MAIPA	platelets	50	14/40 (35%)			14/16 (88%%) 7 (44%) only	9/16 (56%) 2 (13%) only	nt	nt	nt	7/16 (44%)	4-700	Mean controls + 6SD	VIPL1 (IIb/IIIa) , VIPL3 (IIb/IIIa), FMC25 (IX), SZ1 (Ib/IX)
		sera		5/45 (11%)					nt	nt	nt		4-700	Mean controls + 6SD	
Hou 1995[85]	MACE	platelets	100	30/60 (50%)	0/60		22/30 (73%) 14 (47%) only	16/30 (53%) 8 (27%) only	nt	nt	nt	8/30 (27%)	32 patients < 150 28 patients >150	Mean controls + 3SD	AP1 (Ib), AP2 (IIb/IIIa)
Stockelberg 1996[88]	MACE	sera	40	23/65	0/40		12/23 (52%) 8 (35%) only	15/23 (65%) 11 (48%) only	nt	nt	nt	4/23 (17%)	1-463	Mean controls + 3SD	AP1 (Ib), AP2 (IIb/IIIa)
Brighton 1996[60]	MAIPA	platelets	100	40/81 (49%)		11/51 (22%)	35/40 (88%) 19 (48%) only	21/40 (53%) 5 (13%) only	nt	nt	nt	16/40 (40%)	<140	mean controls + 3SD	SZ21 (IIIa), SZ22 (IIb), AP2 (IIb/IIIa, AK2 (Ibα), FMC25 (IX)
		sera	100	23/93 (25%)		2/53 (4%)	16/23 (70%) 7 (30%) only	16/23 (70%) 7 (30%) only	nt	nt	nt	9/23 (39%)	<140	mean controls + 3SD	SZ21 (IIIa), SZ22 (IIb), AP2 (IIb/IIIa, AK2 (Ibα), FMC25 (IX)
Crossley 1997[86]	MAIPA	platelets	10-100	11/23 (48%)			11/11 (100%)	nt	nt	nt	nt	nt	1-334	0.352	Clone 189/21-10 (IIb/IIIa)
Joutsi 1997[36]	simult. MAIPA	platelets	60	46/159 (29%)		simultaneous MAIPA for GPIIb/IIIa, GPIb/IX and GPIa/IIa								pat result/ mean contr. >1.5	AP1 (Ibα), AP2 (IIb/IIIa), Gi9 (Ia/IIa)
	MAIPA	platelets	60	13/125 (10%)			sufficient platelets to also test for GPV					nt		pat result/ mean contr. >1.5	SW16 (V)

Porcelijn 1998[17]	MAIPA	platelets	15 (IIb/IIIa) 40 (other GP)	19/47 (40%)		5/26 (19%)	11/19 (58%)	8/19 (42%)	0/19	12/19 (63%)	0/19	6/19 (32%)	<100	0.3	C17 (IIb/IIIa), MB45 (Ibα), 10G11 (Ia/IIa), SW16 (V), P58 (IV)
Warner 1999[61]	MAIPA	platelets		19/49 (39%)		3/32 (9%)	19/19 (100%)	nt	nt	nt	nt	nt		0.2	Raj-1 (IIb/IIIa)
	Antigen capture assay	platelets		19/49 (39%)		3/32 (9%)	19/19 (100%)	nt	nt	nt	nt	nt		0.2	Raj-1 (IIb/IIIa)
	Antigen capture assay	platelets		37/56 (66%)		2/26 (8%)	31/37 (84%) 8 (22%) only	28/37 (76%) 5 (14%) only	nt	nt	nt	23/37 (62%)		0.2	Raj-1 (IIb/IIIa), TW-1 (Ib)
Kosugi 2001[98]	MACE	eluates	50	18/47 (38%)			18/18 (100%)	nt	nt	nt	nt	nt		mean controls + 3SD	AP3 (IIIa)
Fabris 2002[99]	commercial MACE	platelets		39/65 (60%)		1/39 (3%)	29/39 (74%)	31/39 (79%)	0-21/39 not specified	nt	nt	21/39 (54%)	<100	Mean controls + 3SD	AP2 (IIb/IIIa), 142.1 (Ib/IX), 143.1 (Ia/IIa)
Chan 2003[100]	MACE	platelets		43/59 (73%)	0/30	5/31 (16%)	43/43 (100%)	nt	nt	nt	nt	nt		OD 0.200	Raj-1 (IIb/IIIa)
Fabris 2004[101]	commercial MACE	platelets		25/50 (50%)			18/25 (72%)	15/25 (60%)	2/25 (8%)			8/25 (32%)	< 100		
Davoren 2005[87]	commercial ELISA	eluates		114/216 (53%)		13/46 (28%)	95/114 (83%) 14 (12%) only	84/114 (74%) 3 (3%) only	94/114 (82%) 7 (6%) only			90/114 (79%)	1-834		
Tomer 2005[102]	Immunobead Flow	platelets	100	53/62 (86%)	0/60	0/14	53/62 (86%)	nt	nt	nt	nt	nt	3-166	ratio pat/3 hc>1.3	P2 (IIb/IIIa)
Meyer 2006a[94]	MAIPA	platelets	20	7/8 (88%)			7/7 (100%)	5/7 (71%)	3/7 (43%)	nt	nt	5/7 (71%)		0.2	P2 (IIb/IIIa), FMC25 (IX), Gi9 (Ia/IIa)
		sera	20	30/33 (91%)			24/30 (80%)	25/30 (83%)	12/30 (40%)	nt	nt	19/30 (63%)		0.2	P2 (IIb/IIIa), FMC25 (IX), Gi9 (Ia/IIa)
	ASPA	platelets	20	7/8 (88%)			7/7 (100%)	5/7 (71%)	1/7 (14%)	nt	nt	5/7 (71%)			P2 (IIb/IIIa), SZ1 (Ib/IX), AK7 (Ia/IIa)

		sera	20	32/33 (97%)	0/100		25/32 (78%)	29/32 (91%)	13/32 (41%)	nt	nt	23/32 ((72%)			
Meyer 2006[103]	MAIPA	platelets	20	14/28 (50%)			12/14 (86%)	11/14 (79%)	5/14 (36%)	nt	nt	9/14 (64%)		0.2	P2 (IIb/IIIa), FMC25 (IX), Gi9 (Ia/IIa)
	MAIPA	sera	20	7/39 (18%)			4/7 (57%)	3/7 (43%)	0/7	nt	nt	0/7		0.2	P2 (IIb/IIIa), FMC25 (IX), Gi9 (Ia/IIa)
	Antigen-specific Capture assay (ASCA)	sera	20	30/39 (77%)	0/70		25/30 (83%)	19/30 (63%)	9/30 (30%)	nt	nt	17/30 (57%)			P2 (IIb/IIIa), FMC25 (Ib/IX), AK7 (Ia/IIa)
Panzer 2007[90]	MAIPA	platelets		14/40 (35%)			12/14 (86%) 5 (36%) only	9/14 (64%) 2 (14%) only	nt	nt	nt	7/14 (50%)	3-223		
Najaoui 2012[91]	MAIPA	platelets	20	129/240 (54%)			104/129 (81%) 41 (39%) only	88/129 (68%) 25 (24%) only	nt	nt	nt	63/129 (49%)		0.15	Gi5 (IIb/IIIa), FMC25 (IX)
He 2013[104]	MAIPA	platelets	40	22/50 (44%)	20/86 (23%)		22/22 (100%)		nt	nt	nt	nt	<100	0.470-0.680 depending on moab	SZ21 (IIIa), SZ22 (IIb), SZ1 (Ib/IX), SZ2 (Ib)
Porcelijn 2018[7]	MAIPA	platelets	15 (IIb/IIIa) 40 (Ib/IX, V)	51/60 (85%)	1/462 (<1%)	0/43	40/51 (78%) 6 (12%) only	36/51 (71%) 4 (8%) only	nt	31/51 (61%) 3 (6%) only	nt	38/51 (75%)	<100	0.13	C17 (IIb/IIIa), MB45 (Ibα), SW16 (V)
	MAIPA	platelets	40	13/32			sufficient platelets to also test for GPIa/IIa					13/13 (100%)	<100	0.13	10G11 (Ia/IIa)
	MAIPA	platelets	40	Jul-26			sufficient platelets to also test for GPIV					7/7 (100%)	<100	0.13	P58 (IV)
Vollenberg 2019[38]	MAIPA	platelets	100	343/1140 (30%)			242/343 (71%) 71 (21%) only IIb/IIIa	232/343 (68%) 30 (9%) only Ib/IX	nt	222/343 (65%) 10 (3%) only V	nt	232/343 (68%)		0.2	Gi5 (IIb/IIIa, FMC25 (IX), SW16 (V)

Al-Samkari 2020[33]	commercial ELISA	eluates		205/228 (90%)		24/108 (22%)	269/280* (96%) 48 (17%) only IIb/IIIa	232/280 (83%) 11 (4%) only Ib/IX	145/280 (52%) 0 (0%) only IaIIa	nt	nt	221/280 (79%)	1-657		

**Number of platelets used in test: the number of platelets used per GP-specificity, to test the binding of GP-specific autoantibodies*
 # only: meaning only reactive with this GP, not with the other tested GP
Empty cells: no data are available in publication
nt: not tested

2. Other GP as targets for autoantibodies and the impact of GPV-specific platelet autoantibodies

In search of a more accessible autoantibody detection assay on the one hand and better sensitivity on the other hand, research was conducted using different methods into antibody binding to GPIa/IIa, GPIV and GPV.

He et al (1994) used an immunobead assay to detect autoantibodies in sera of ITP patients, not only against GPIIb/IIIa and GPIb/IX, but also against GPIa/IIa and GPIV.[32] Autoantibodies reactive with GPIa/IIa or GPIV were detected in three (9%) of 47 sera and 12 (38%) sera. None of the sera was only positive for anti-GPIa/IIa and 2 (6%) of the sera reacted only with GPIV. More recent studies by Porcelijn et al.[7], using the direct MAIPA and by Al-Samkari et al.[33], using a commercial GP-specific ELISA (PAKAuto) confirmed the almost non-occurrence of autoantibody binding exclusively to GPIa/IIa or GPIV.

Glycoprotein V as a target for autoantibodies was first reported by Beardsley (1988) in a case of childhood ITP.[34] In 1993, Meenaghan showed that the majority of GP reactive antibodies in multi-transfused patients with bone marrow failure (also) reacted with GPV.[35]

The first study investigating whether platelet-associated autoantibodies in adult ITP patients were also reactive with GPV was conducted by Joutsi et al (1997).[36] For those patients for whom sufficient platelets could be isolated, GPV reactivity was tested after performing a simultaneous direct MAIPA for GPIIb/IIIa, GPIb/IX and GPIa/IIa.Thirteen of 125 patients (10%) showed anti-GPV antibodies. In a follow-up study in 69 thrombocytopenia patients with strong reactive autoantibodies in the direct PIFT, they detected anti-GPV in 15 (22%) patients.[37] We (Porcelijn et al, 1998) detected GPV-associated autoantibodies in samples from 12 (63%, six specific and six in combination with GPIIb/IIIa and GPIb/IX) of 19 ITP patients with positive direct MAIPA results.[17] More recently, after optimization of the direct MAIPA, we detected platelet-associated autoantibodies in 51 of 60 (85%) well-categorized untreated ITP patients, of which 31 (61%) reacted positive with GPV.[7] The major role for GPV-associated autoantibodies in the pathogenesis of ITP was also confirmed by Vollenberg et al (2019).[38] In their study, platelet-associated autoantibodies were detected in 343 of 1140 (30%) patients suspected for ITP, 242 (71%) positive for anti-GPIIb/IIIa, 232 (68%) positive for anti-GPIb/IX and 222 (65%) positive for anti-GPV. For 10 (2.9%) samples only anti-GPV antibodies were detected.

In a cohort of 754 patients, referred to our laboratory for platelet autoantibody investigation, with positive direct MAIPA results (unpublished data), 625 (83%) were positive for anti-GPV, 481 (64%) for anti-GPIb/IX and 340 (45%) for anti-GPIIb/IIIa. For 178/754 (24%) patients only GPV-associated autoantibodies were

detected. The high percentage of anti-GPV might partly be due to the MAIPA settings as we see a higher average OD values for GPV compared with GPIb/IX and GPIIb/IIIa (Figure 1), which is in contrast to what was seen by Vollenberg et al.[38]

Considering these results and the limited number of available patient platelets, we have decided to include GPIIb/IIIa, GPIb/IX and GPV, but not GPIa/IIa and GPIV in our routine diagnostic autoantibody detection protocol.

3. GP-specific autoantibody binding causing loss of platelet function

The possibility to detect glycoprotein specific autoantibodies was not only a step forward in increasing the specificity of the detection of autoantibodies as a cause for platelet destruction, but could also be used to clarify some rarely encountered primary clotting disorders, which were thought to be caused by platelet function loss. These cases were shown to be based on blocking of functional binding sites at the different GP. First case reports of, so called, acquired Glanzmann disorder and acquired Bernard Soulier syndrome were already published in 1987 by Niessner et al., respectively Devine et al.[39, 40] Depending on the specific binding sites on GPIIb/IIIa or GPIb, the autoantibodies may inhibit GPIIb/IIIa-fibrinogen binding, leading to a condition resembling Glanzmann thrombasthenia, a genetic disorder causing GPIIb/IIIa deficiency or inhibit GPIb-von Willebrand Factor binding resembling Bernard Soulier syndrome, which is a genetic disorder causing GPIb/IX/V deficiency. Also a case of severe impaired response of platelets to collagen, due to GPIa/IIa specific autoantibodies blocking the collagen receptor, has been described by Deckmyn et al. (1990).[41] Interesting in these cases were the often normal platelet counts, despite the presence of autoantibodies. This could be explained by either the IgG-antibodies being of the IgG2 or IgG4 subclass and subsequently less Fc-Fcγ-receptor binding on macrophages or splenectomy preventing destruction of opsonized platelets.[42, 43] We questioned whether the blocking effect of autoantibodies on platelet function also plays a role in ITP patients, leading to a bleeding tendency, not only due to thrombocytopenia, but also due to a loss of function. We therefore developed a flow cytometry test for measuring platelet aggregation, in which 10- to 25-fold lower platelet counts were necessary than in the routine aggregation assays in an aggregometer.[44, 45] Indeed, a decreased platelet aggregation potential, both in adult and in pediatric ITP patients with GPIIb/IIIa specific autoantibodies could be demonstrated. To what extent the influence of blocking autoantibodies plays a role in the bleeding tendency in ITP patients is still unknown. More research is needed to objectify the clinical impact of this mechanism. Depending on the results of this research, it is conceivable that this aspect could be included in the treatment of patients with ITP.

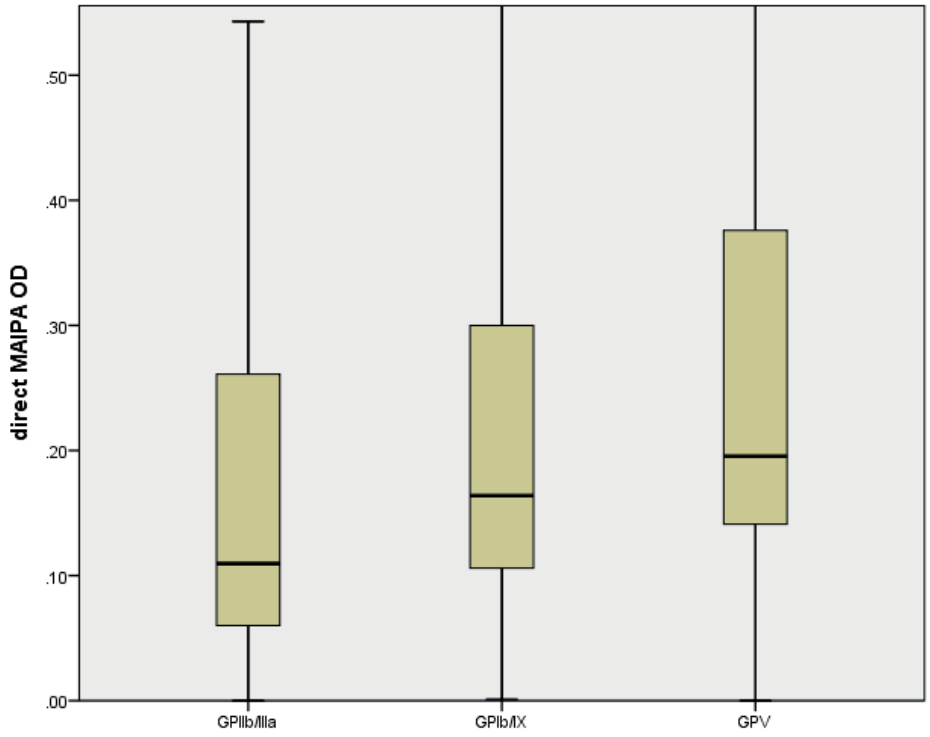


Figure 1: Routine autoantibody detection with positive results (i.e. at least one of the tested GP shows positive results) in direct MAIPA (n=754)
For 754 positive direct MAIPA results (i.e. at least one of the glycoproteins IIb/IIIa, Ib/IX or V shows OD values > 0.130) the results for the different GP are depicted in the boxplots. Remarkable is the difference in mean OD levels between the different GP. This is probably the result of test-specifics as, for reasons explained in the text, we use 15 x 106 platelets for GPIIb/IIIa and 40 x 106 platelets for GPIb/IX and GPV. The effect of raising the platelet number for GPIIb/IIIa is shown in Figure 4.

4. GPIb-specific autoantibodies, thrombopoietin production and Fc-independent platelet destruction

Regulation of platelet production depends on the levels of hematopoietic growth factor thrombopoietin (Tpo). Tpo, mainly produced in the liver, binds to the c-mpl-receptors on CD34+ stem cells, and stimulates platelet production.[46, 47] In previous studies, we showed that plasma Tpo levels are useful to discriminate thrombocytopenia caused by megakaryocyte and platelet production failure (highly elevated Tpo levels) from thrombocytopenia caused by elevated platelet

destruction as in immune thrombocytopenia (ITP) and FNAIT (normal or only slightly elevated Tpo levels).[17, 48, 49]

After initial reports in which the presence of GPIb/IX-specific autoantibodies was associated with a diminished response to intravenous immunoglobulin IgG (IVIg) therapy in ITP, several possible mechanisms explaining this observation have been studied.[50, 51] It was shown that de-sialylation of GPIb on senescent platelets triggers removal from circulation by the Ashwell-Morrell receptor (AMR) expressed on hepatocytes in the liver.[52, 53] Subsequently, it was demonstrated in mouse models that de-sialylation of GPIb also occurred after binding of moab specific for the ligand binding domain of GPIbα, causing platelet destruction via the AMR.[54] This, so called, Fc-independent platelet destruction route was thought to be a possible explanation for the lesser response on IVIg. Thereafter, Quach et al. found that, under shear conditions, binding of moab to the ligand binding domain of GPIbα can exert a pulling force causing activation of GPIb/IX, which can induce Fc-independent platelet clearance.[55] However, in both Al-Samkari et al. and Rogier et al. studies, the correlation between the clinical effectiveness of IVIg treatment and the presence of platelet-associated GPIb/IX autoantibodies could not be confirmed.[33, 56]

More recently, Xu et al. described a novel mechanism, in other words, GPIb directly inducing TPO production in hepatocytes.[57] In their mouse model, GPIb-specific moab caused inhibition of TPO production. We measured free plasma TPO levels in a large cohort of patients with positive autoantibody detection in the direct MAIPA and did not find a correlation between antibody GP-specificity and free plasma TPO levels.[33, 58] These conflicting results require further investigation into the influence of, in particular GPIb/IX-specific, platelet autoantibody binding on TPO production and on the Fc-independent platelet destruction pathway in humans.

5.The impact of the change in ITP definition < 100 instead of < 150 x 109/L

For several reasons, the platelet count of the patient is important in the detection of autoantibodies. First, for the categorization of patients in the group of patients with suspected ITP. For instance, in our laboratory we frequently receive autoantibody requests for pregnant women with platelet counts between 100 and 150 x 109/L. As in pregnancy, a physiological drop in platelet count is often seen. Unsurprisingly the test results for these cases are consistently negative.[59] Second, the sensitivity of the autoantibody detection assays seems inversely correlated with the patients platelet count. In 1996 already, Brighton showed a non-statistically significant trend toward higher positivity in direct MAIPA for ITP patients with lower platelet counts.[60] This was also seen in the prospective study by Warner et al. (1999), in which the glycoprotein specific antigen capture

assay was negative for ITP patients with platelet counts > 100 x 109/L.[61] Third, increased platelet counts as a result of therapy aimed at reducing the number of antibodies will of course reduce the number of positive test results. Indeed, in 170 known ITP patients, categorized in four platelet count groups, detection of autoantibodies became less sensitive for patients with platelet counts above 100 x 109/L (data not shown). In our routine diagnostic setting, we advise clinicians to request autoantibody detection if the platelet count is between 10 (if < 10 insufficient platelets can be isolated for direct testing) and 80 x 10 x 109/L.

6. The impact of the glycoprotein specific monoclonal antibodies used in the assay on the test results

Using GP specific mouse-anti-human moab that are known not to bind to restricted areas targeted for by patient autoantibodies is very important to prevent displacement of the latter from the platelet antigens.

Kiefel et al (1991) showed for three ITP patients with auto-antibodies against GPIb/IX that these antibodies were partially blocked by moab Gi10, directed against a fragment consisting of a part of GPIbα (after protease treatment) and GPIbβ.[31] He et al, 1995 showed for six of 16 anti-GPIb/IX antibodies specificity for the N-terminal glycocalicin part of GPIbα.[62] These authors were able to specify the main autoepitope for these six antibodies to the short fragment AA326-346. We found a significant correlation between the indirect and direct MAIPA OD values for the antibodies directed against GPIIb/IIIa, GPV and GPIa/IIa, but noticed a deviating pattern in patients with strong autoantibodies against GPIb/IX (unpublished data; Figure 2). We therefore periodically tested the presence of free-circulating autoantibodies using moab MB45 (glycocalicin part of GPIbα) and moab FMC25 (GPIX). All positive results, either with MB45 or FMC25, are shown in Figure 3. For the majority of samples, indirect MAIPA OD values were higher with FMC25. This is probably caused by autoepitope loss and/or moab binding epitope loss as also shown by Kiefel and He.[31, 62] Intriguing is that we do not see this deviating pattern for GPIb/IX if MB45 is used in the direct MAIPA. In the direct MAIPA anti-GPIb/IX does not seem to prevent MB45 from binding and vice versa MB45 does not seem to displace the autoantibodies. Because we use frozen (-196 °C) platelets for the indirect MAIPA, we also investigated whether the freeze-storage-thaw procedure affects the MAIPA results, e.g. by degradation of the glycocalicin part of GPIbα. This did not solve the problem and further investigation into the exact mechanism causing this discrepancy is necessary. These results again support the importance of carefully selecting the moab for antigen binding in GP-specific assays.

Although varying results were found for autoepitope localization on GPIIb/IIIa, the epitopes for a high percentage of autoantibodies seem to be restricted to some specific areas depending on an intact heterodimeric complex structure.[63-65]

Several studies have indicated that a significant percentage of GPIIb/IIIa reactive autoantibodies actually bind to GPIIb. Already in 1983, Varon and Karpatkin noticed a decreased binding of the GPIIb specific moab 3B2 on platelets from ITP patients.[66] After a first experiment by McMillan et al. (2001), observing that autoantibodies from ITP patients reacted with αIIbβ3 but not with αvβ3 expressed on Chinese ovary (CHO) cells, a more specific antibody-binding localization between the amino acids L1 and Q449 of the N-terminal half of the β-propeller domain in αIIb was shown (McMillan, 2002).[67, 68] This restricted region was confirmed by Kiyomizu et al (2012) and mapped to specific loops and critical amino acids in this region.[69] Restricted locations for autoantibody binding were also noticed using anti-GPIIb/IIIa F(ab')2 fragments from two ITP patients and Fab fragments from two human monoclonal anti-GPIIb/IIIa, both inhibiting the binding of anti-GPIIb/IIIa from other ITP patients (Hou 1995, Escher 1998, McMillan 2007).[70-72] The restricted binding of platelet antibodies is further supported by IgG light chain restriction and limited numbers of B cell clones producing autoantibodies in ITP patients.[73, 74]

In 2012 the Scientific Subcommittee of the ISTH recommended to use moab to each of the GPIIb (e.g. SZ22) and GPIIIa (e.g. SZ21) subunits or to the intact GPIIb/IIIa (e.g. Gi5, AP2, Raj-1); GPIb/IX (e.g. the GPIbα specific AP1 or the GPIX specific FMC25).[75] In our hands C17 (GPIIb/IIIa) SW16 (GPV), and FMC25 (GPIX) replacing MB45 (GPIbα) show best results in the MAIPA.

7. The impact of cut-off values in the assay

To differentiate specific signals from the noise, the assay cut-off value to be used is, of course, very dependent on the test specifics. E.g. longer incubation steps in the '2-day' MAIPA[29] for autoantibody detection, in comparison with the '1-day' MAIPA[76] which we use for HPA alloantibody detection, give better signals to noise ratios for the often weakly reactive autoantibodies. Reported assay cut-off values to determine positive vs negative results vary among papers, even when comparable MAIPA assays are used.

The essence of choosing specific ODs of course is to have the best (trade of between) sensitivity and specificity which should respectively be validated by true ITP patients and true non-ITP patients with varying platelet numbers. By testing a large group of healthy subjects and non-ITP thrombocytopenia patients, we were able to set the cut-off value to OD = 0.13 (mean 462 healthy controls + 3SD), without compromising specificity.[7] In a series of 754 routine request samples with at least one of the glycoproteins IIb/IIIa, Ib/IX or V reacting positive in direct MAIPA, the highest OD was only between 0.130 and 0.200 for 273 (36%) samples (unpublished data). The importance of having low background signals can also be seen in Figure 2. Remarkably, correlation between direct and indirect

MAIPA results can still be observed for OD levels between 0.050 and 0.130, indicating specific autoantibody signals even with very low OD values that would be classified as negative. These results could mean that, especially for the indirect MAIPA, the cut-off value of the mean of healthy controls + 3SD is still too high to sensitively detect platelet autoantibodies. Illustrating in this context is the high sensitivity of 90% at the expense of specificity (78%) found by Al-Samkari when testing platelet eluates of suspected ITP patients in the commercial PAKAuto assay.[33]
Therefore, we can conclude that with the available GP-specific assays, without compromising specificity, an acceptable sensitivity for ruling out ITP will not be possible and other methods will be necessary to distinguish background from noise.

8. The impact of autoantibodies on platelet production
It must be emphasized that ITP is not only a disorder causing increased platelet destruction, but also decreased platelet production. Glycoproteins are already expressed on megakaryocytes (MK) during maturation[77], and GPIIb/IIIa, GPIb/IX and GPIa/IIa autoantibodies are known to cause inhibition of MK maturation, as well as pro-platelet and platelet formation.[78, 79] Although, most ITP patients show normal MK numbers in the bone marrow (BM), Houwerzijl et al. (2006) found MK in ITP patients having characteristics of apoptosis-like programmed cell death.[80] Lev et al. and Grodzielski et al. (2018) studied the interference of autoantibodies with the MK binding to their ligands.[81, 82] Anti-GPIa/IIa antibodies caused a decrease in adhesion of GPIa/IIa to collagen I and a decrease in phosphor-MLC2 levels, leading in the early phase of MK maturation, in the osteoblast niche, to premature platelet release. Anti-GPIIb/IIIa and –GPIb/IX interfered with the MK-fibrinogen, respectively -von Willebrand Factor interaction, leading to functional abnormalities and inhibited pro-platelet production.

Shestra et al. (2020) hypothesized that a percentage of autoantibodies might be sequestered in the BM, targeting platelet progenitor cells and newly produced platelets, which could be one of the reasons for the absence of detectable autoantibodies in peripheral blood.[83] They investigated the presence of autoantibodies in BM, testing cell-free BM fluid and a mixture of mononuclear cells, platelets and MK for the presence of GPIIb/IIIa and GPIb/IX autoantibodies, in the indirect, respectively direct antigen capture assay. Seven of 18 (39%) patients had detectable antibodies in the direct ACE and 3 (17%) in the indirect ACE. Five out of ten patients with detectable antibodies in the BM could not be detected in the peripheral blood. All controls, i.e. healthy controls (n=6) and non-ITP thrombocytopenic patients (n=3) had no detectable autoantibodies in the BM. BM testing increased the sensitivity for autoantibody detection with ACE from 60 to 72%.

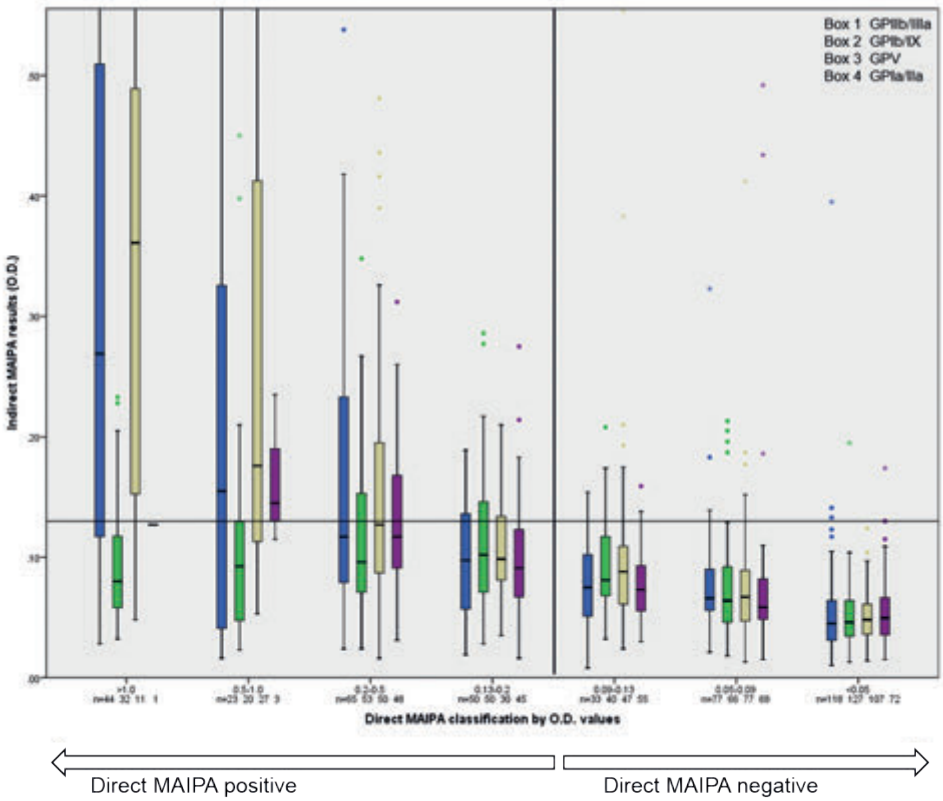


Figure 2: Indirect versus direct autoantibody detection with MAIPA (n=400)
Comparing direct (platelet-associated, groups categorized in OD ranges) and indirect (free-circulating) MAIPA results, shows a highly significant correlation. Interesting is the correlation continuing below the cut-off level of 0.130 (indicated by lines). Remarkable is the deviating correlation between the strongly reactive direct and less reactive indirect GPIX MAIPA results. For this reason (see text) we decided to change from moab MB45 (GPIbα) to moab FMC25 (GPIX) (see Figure 3).

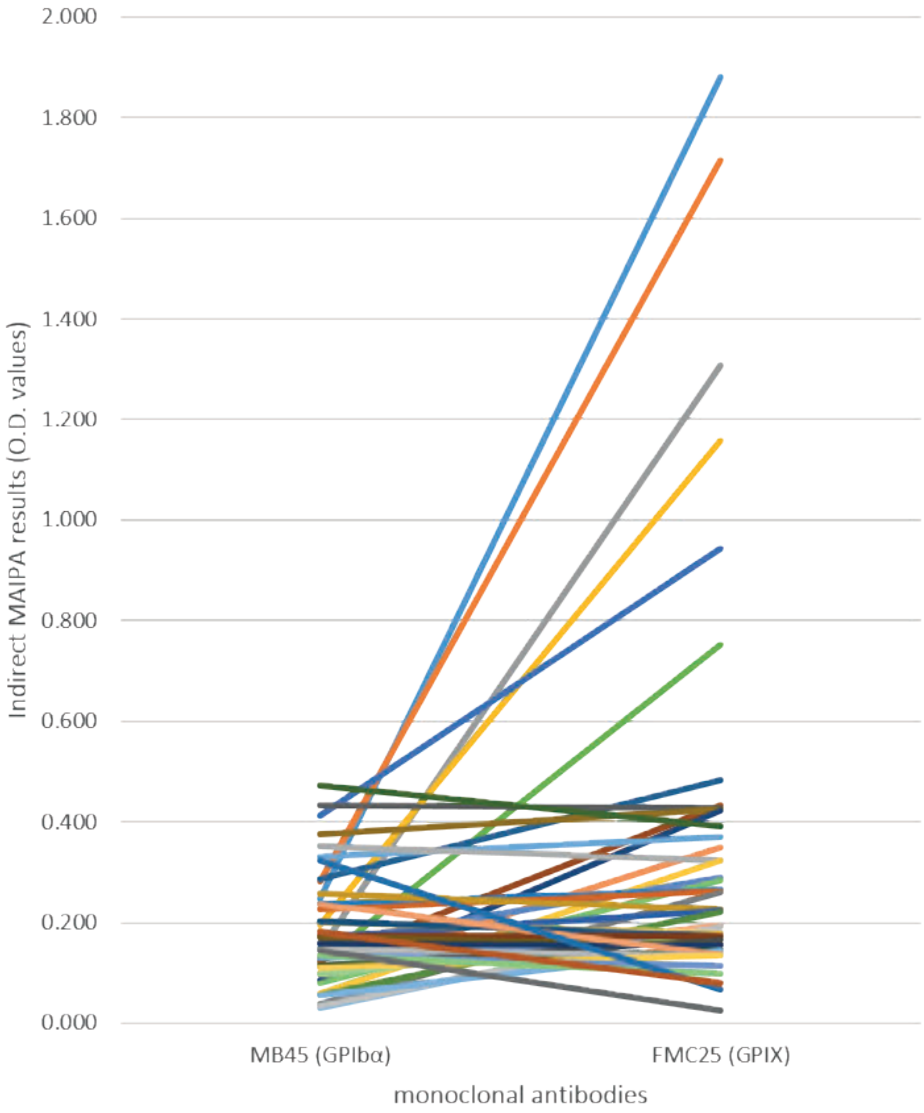


Figure 3: Comparison anti-GPIb/IX positive indirect MAIPA results if using the moab MB45 or FMC25 (n=45)
The indirect MAIPA results, using MB45 and FMC25 for 45 positive (i.e. positive with MB45 and/or positive with FMC25) are shown.

9. Discussion

We would like to emphasize that auto antibody detection can make a valuable contribution to diagnosing ITP. Recently, Vrbensky et al (2019) published a systematic review and meta-analysis of platelet autoantibody tests in the diagnosis of ITP in which eighteen eligible studies were included.[4] They concluded that autoantibody testing in ITP patients has a high specificity, but a low sensitivity and that a positive autoantibody test can be useful for ruling in ITP, but a negative test does not rule out ITP. Notably, we have recently performed a systematic review of platelet autoantibody assays in childhood ITP and reached a more or less similar conclusion for autoantibody detection.[84]

Now, we have revisited the eighteen studies included in the paper of Vrbensky and completed the series with the four studies introducing GP-specific assays[26-28, 31] and nine studies also giving information on the GP specificity of platelet autoantibodies, including the recent papers by Vollenberg and Al-Samkaria.[17, 32, 36, 85-88] To understand the significant differences in sensitivity found in the studies, we compared some important aspects. First, five of nine eligible studies used by Vrbensky for the calculation of the sensitivity only tested for GPIIb/IIIa and GPIb/IX.[60, 61, 89-91] Three also for GPIa/IIa[92-94] and only one also tested for GPV[7]. As was recently confirmed, GPV is an important target for autoantibodies.[38, 95] The exact increase in sensitivity by including GPV is probably very much depending on the test characteristics. For instance, Vollenberg et al. did not see any differences in antibody load for the different GP[38], which triggered us to investigate the GP specific OD values for the direct MAIPA positive results. In our series GPV shows higher OD levels than GPIb/IX and GPIIb/IIIa (Figure 1). Importantly, if GPV would not have been included in our routine setting, we would have missed 178 of 754 (24%) positive results. In addition, we considered the relatively low GPIIb/IIIa OD values might be the result of a difference in platelet numbers used in the direct MAIPA, because the expression of GPIIb/IIIa is higher than the other GP. For optimal use of the limited patient platelets available for testing, we use 15 x 106 platelets in the MAIPA for GPIIb/IIIa, versus 40 x 106 for GPIb/IX and for GPV. The effect of increasing the input from 15 to 40 x 106 platelets per test is shown in Figure 4. These results made us decide to increase the platelet numbers used in the indirect MAIPA for the detection of GPIIb/IIIa reactive autoantibodies to 40 x 106 per test. As shown in Table 1, the platelet numbers used for testing vary significantly between studies, which also complicates comparing the results. Second, patients with platelet counts > 100 x 109/L were included in three[60, 89, 90] of the nine studies and platelet counts were not mentioned in three other studies[61, 91, 94]. In our opinion, it is important to limit testing to the group of patients with platelet counts below 100 or better still below 80 x 109/L (see below). Third, the low sensitivity of autoantibody detection is mainly due to insufficient signal to noise ratios. In our routine series, 36% (273/754 positive results) showed OD

values between 0.130 and 0.200. Most studies used mean of healthy controls (hc) + 3SD or 0.200 as cut-off value.[60, 61, 89, 94] The mean of healthy controls is, of course, dependent on the background signals and varies significantly between studies. Using hc + 3SD can compromise sensitivity in case of high background signals.

Finally, detection of free circulating autoantibodies in all available assays is less sensitive than detection of platelet-associated antibodies. This is somewhat surprising conceptually, because after transfusing a platelet concentrate to ITP patients, the one hour increment is often zero, indicating platelets are almost instantly opsonized and removed from circulation. One explanation could be

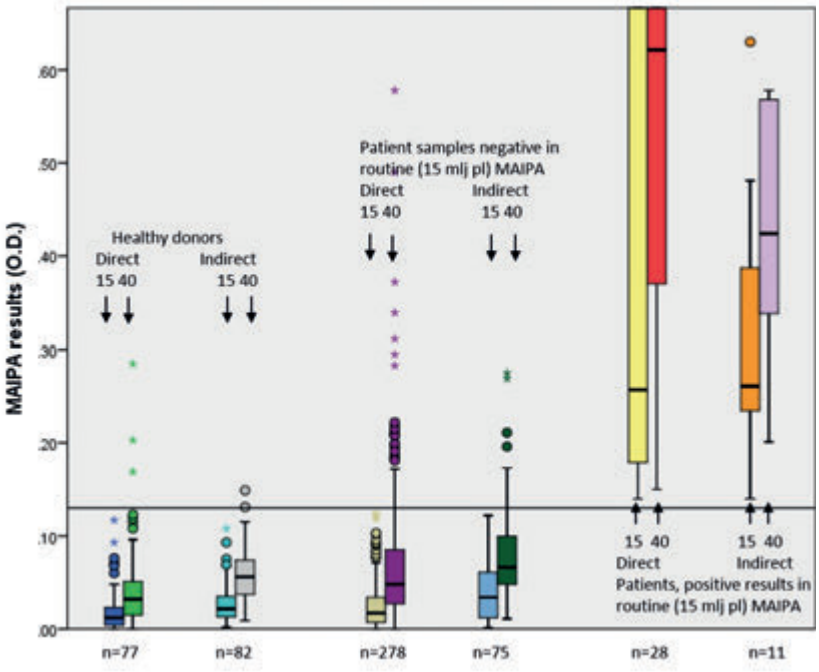


Figure 4: 15 or 40 x 106 platelets used in MAIPA for detection of GPIIb/IIIa reactive autoantibodies
The effect of increasing the number of platelet from 15 to 40 x 106 used in the direct and indirect MAIPA is shown.

that antibody production, platelet opsonization and removal occurs locally in the spleen and free-circulating autoantibodies in the peripheral blood are less detectable. Considering that, direct and indirect autoantibody test results (Figure 2) correlate even below our cut-off level of 0.130, one could assume that for a percentage of patients (especially free-circulating) autoantibodies are present, but are simply too weak to be detected. More sensitive methods will be necessary to detect these antibodies.

In contrast to what was found by Al-Samkari et al (2020)[33] using testing of platelet eluates in the PAKAuto (Immucor), the correlation between circulating and platelet associated autoantibodies is highly significant in our MAIPA assay (Figure 2). These different results might well be caused by the different assays used. In the PAKAuto GP are already isolated and bound to the microtiterplate wells, whereas in MAIPA intact platelets are used. The number of GP per well may vary significantly in PAKAuto, but are reasonably comparable between indirect and direct MAIPA, using the same number of platelets.

In addition to the detection of glycoprotein specific autoantibodies being of value for the diagnosis of ITP, it is interesting to zoom in on the usefulness of monitoring autoantibodies during treatment and whether the glycoprotein specificity of the autoantibodies can be valuable for choice of treatment. We performed serial antibody detection for patients treated with rituximab and found a strong correlation between platelet counts and direct MAIPA OD values.[96, 97] Al-Samkari et al. also showed a strong correlation between the absence of detectable platelet-associated autoantibodies and clinical remission (sensitivity 87%, specificity 90%) and argued that test results can help clinicians in their choice of treatment.[33] Indeed, knowing that there is a strong correlation between test results and the effect of treatment (i.e. platelet counts), serial testing can be supportive for treatment policy, especially for patients with more possible causes for thrombocytopenia. The benefits of knowing the glycoprotein specificity of the antibodies is something that needs further investigation. The presence of strong GPIIb/IIIa reactive autoantibodies, possibly causing inhibition of fibrinogen binding, can be a reason to opt for treatment that reduces antibody production, rather than for treatment to reduce platelet destruction. Before such choices can be made, we need to better understand the effect of treatment (e.g. splenectomy and thrombopoietin) on antibody production. Studying well categorized ITP patients, using reliable glycoprotein-specific autoantibody detection methods for serial testing during treatment, will hopefully provide more insight in the near future. The interaction between the Fc-independent platelet destruction pathway via the AMR, thrombopoietin production, GPIbα de-sialylation and platelet autoantibody specificity is intriguing, but varying and sometimes even contradictory results in human studies need to be further

investigated before this can be taken into account in clinical practice .

In summary, we conclude that with the caveats indicated above, detection of platelet autoantibodies is truly a powerful diagnostic tool in the work-up of patients suspected for ITP. In this respect, we agree with Vrbensky et al. that the available GP-specific assays can at least be used as a 'rule in' test for ITP. We also conclude that we can and must further improve platelet autoantibody testing assays. For comparison of test accuracy in terms of sensitivity and specificity between laboratories, further standardization is necessary. In this regard, next to the GP tested, key parameters, like patient platelet counts and test cut-off levels, platelet numbers used for solubilization, GP specific moab and patient characteristics (routine laboratory requests or clinical cohorts patients; adults or children) should be standardized and reported. In addition to diagnosing ITP, a reliable glycoprotein-specific platelet autoantibody detection method can be used to further investigate the effects of the antibodies which will contribute to a more individualized treatment.

References

1. D.B. Cines, J.B. Bussel, H.A. Liebman, E.T. Luning Prak. The ITP syndrome: pathogenic and clinical diversity, Blood 2009; 113:6511-6521.
2. A. Zufferey, R. Kapur, J.W. Semple. Pathogenesis and Therapeutic Mechanisms in Immune Thrombocytopenia (ITP), J Clin Med 2017; 6.
3. J.G. Kelton, J.R. Vrbensky, D.M. Arnold. How do we diagnose immune thrombocytopenia in 2018?, Hematology Am Soc Hematol Educ Program 2018; 2018:561-567.
4. J.R. Vrbensky, J.E. Moore, D.M. Arnold, J.W. Smith, J.G. Kelton, I. Nazy. The sensitivity and specificity of platelet autoantibody testing in immune thrombocytopenia: a systematic review and meta-analysis of a diagnostic test, J Thromb Haemost 2019; 17:787-794.
5. D. Provan, D.M. Arnold, J.B. Bussel, B.H. Chong, N. Cooper, T. Gernsheimer, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia, Blood Adv 2019; 3:3780-3817.
6. D.M. Arnold, I. Nazy, R. Clare, A.M. Jaffer, B. Aubie, N. Li, et al. Misdiagnosis of primary immune thrombocytopenia and frequency of bleeding: lessons from the McMaster ITP Registry, Blood Adv 2017; 1:2414-2420.
7. L. Porcelijn, E. Huiskes, G. Oldert, M. Schipperus, J.J. Zwaginga, M. de Haas. Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art, Br J Haematol 2018; 182:423-426.
8. N. Bryant, R. Watts. Thrombocytopenic syndromes masquerading as childhood immune thrombocytopenic purpura, Clin Pediatr (Phila) 2011; 50:225-230.
9. K. Downes, K. Megy, D. Duarte, M. Vries, J. Gebhart, S. Hofer, et al. Diagnostic high-throughput sequencing of 2396 patients with bleeding, thrombotic, and platelet disorders, Blood 2019; 134:2082-2091.
10. D.B. Cines, A. Cuker, J.W. Semple. Pathogenesis of immune thrombocytopenia, Presse Med 2014; 43:e49-59.
11. C. Zhao, X. Li, F. Zhang, L. Wang, J. Peng, M. Hou. Increased cytotoxic T-lymphocyte-mediated cytotoxicity predominant in patients with idiopathic thrombocytopenic purpura without platelet autoantibodies, Haematologica 2008; 93:1428-1430.
12. B. Olsson, P.O. Andersson, M. Jernas, S. Jacobsson, B. Carlsson, L.M. Carlsson, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura, Nat Med 2003; 9:1123-1124.
13. L. Chow, R. Aslam, E.R. Speck, M. Kim, N. Cridland, M.L. Webster, et al. A murine model of severe immune thrombocytopenia is induced by antibody- and CD8+ T cell-mediated responses that are differentially sensitive to therapy, Blood 2010; 115:1247-1253.
14. J.G. Kelton, P.J. Powers, C.J. Carter. A prospective study of the usefulness of the measurement of platelet-associated IgG for the diagnosis of idiopathic thrombocytopenic purpura, Blood 1982; 60:1050-1053.
15. C. Mueller-Eckhardt, W. Kayser, K. Mersch-Baumert, G. Mueller-Eckhardt, M. Breidenbach, H.G. Kugel, et al. The clinical significance of platelet-associated IgG: a study on 298 patients with various disorders, Br J Haematol 1980; 46:123-131.
16. J.G. Kelton, W.G. Murphy, A. Lucarelli, J. Garvey-Williams, A. Santos, R. Meyer, et al. A prospective comparison of four techniques for measuring platelet-associated IgG, Br J Haematol 1989; 71:97-105.

17. L. Porcelijn, C.C. Folman, B. Bossers, E. Huiskes, M.A. Overbeeke, C.E. v d Schoot, et al. The diagnostic value of thrombopoietin level measurements in thrombocytopenia, Thromb Haemost 1998; 79:1101-1105.
18. A.E. von dem Borne, F.W. Verheugt, F. Oosterhof, E. von Riesz, A.B. de la Riviere, C.P. Engelfriet. A simple immunofluorescence test for the detection of platelet antibodies, Br J Haematol 1978; 39:195-207.
19. H. Hagenstrom, P. Schlenke, H. Hennig, H. Kirchner, H. Kluter. Quantification of platelet-associated IgG for differential diagnosis of patients with thrombocytopenia, Thromb Haemost 2000; 84:779-783.
20. F.M. Helmerhorst, R.J. Smeenk, C.E. Hack, C.P. Engelfriet, A.E. von dem Borne. Interference of IgG, IgG aggregates and immune complexes in tests for platelet autoantibodies, Br J Haematol 1983; 55:533-545.
21. E.F. van Leeuwen, J.T. van der Ven, C.P. Engelfriet, A.E. von dem Borne. Specificity of autoantibodies in autoimmune thrombocytopenia, Blood 1982; 59:23-26.
22. C. Tanford, J.A. Reynolds. Characterization of membrane proteins in detergent solutions, Biochim Biophys Acta 1976; 457:133-170.
23. O.J. Bjerrum. Immunochemical investigation of membrane proteins. A methodological survey with emphasis placed on immunoprecipitation in gels, Biochim Biophys Acta 1977; 472:135-195.
24. I. Hagen, O.J. Bjerrum, N.O. Solum. Characterization of human platelet proteins solubilized with Triton X-100 and examined by crossed immunoelectrophoresis. Reference patterns of extracts from whole platelets and isolated membranes, Eur J Biochem 1979; 99:9-22.
25. L. Porcelijn, E. Huiskes, M. de Haas. Progress and development of platelet antibody detection, Transfus Apher Sci 2020; 59:102705.
26. V.L. Woods, Jr., Y. Kurata, R.R. Montgomery, P. Tani, D. Mason, E.H. Oh, et al. Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura, Blood 1984; 64:156-160.
27. V.L. Woods, Jr., E.H. Oh, D. Mason, R. McMillan. Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP, Blood 1984; 63:368-375.
28. R. McMillan, P. Tani, F. Millard, P. Berchtold, L. Renshaw, V.L. Woods, Jr. Platelet-associated and plasma anti-glycoprotein autoantibodies in chronic ITP, Blood 1987; 70:1040-1045.
29. V. Kiefel, S. Santoso, M. Weisheit, C. Mueller-Eckhardt. Monoclonal antibody--specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies, Blood 1987; 70:1722-1726.
30. K. Furihata, D.J. Nugent, A. Bissonette, R.H. Aster, T.J. Kunicki. On the association of the platelet-specific alloantigen, Pena, with glycoprotein IIIa. Evidence for heterogeneity of glycoprotein IIIa, J Clin Invest 1987; 80:1624-1630.
31. V. Kiefel, S. Santoso, E. Kaufmann, C. Mueller-Eckhardt. Autoantibodies against platelet glycoprotein Ib/IX: a frequent finding in autoimmune thrombocytopenic purpura, Br J Haematol 1991; 79:256-262.
32. R. He, D.M. Reid, C.E. Jones, N.R. Shulman. Spectrum of Ig classes, specificities, and titers of serum antiglycoproteins in chronic idiopathic thrombocytopenic purpura, Blood 1994; 83:1024-1032.
33. H. Al-Samkari, R.P. Rosovsky, R.S. Karp Leaf, D.B. Smith, K. Goodarzi, A.E. Fogerty, et al. A modern reassessment of glycoprotein-specific direct platelet autoantibody testing in immune thrombocytopenia, Blood Adv 2020; 4:9-18.

34. D. Beardsley. Characterisation of platelet autoantigens (abstract). XX Congress ISBT. London, S-T-6-2 1988.

35. M. Meenaghan, P.A. Judson, K. Yousaf, L. Lewis, D.H. Pamphilon. Antibodies to platelet glycoprotein V in polytransfused patients with haematological disease, Vox Sang 1993; 64:167-170.

36. L. Joutsi, R. Kekomaki. Comparison of the direct platelet immunofluorescence test (direct PIFT) with a modified direct monoclonal antibody-specific immobilization of platelet antigens (direct MAIPA) in detection of platelet-associated IgG, Br J Haematol 1997; 96:204-209.

37. L. Joutsi-Korhonen, K. Javela, P. Hormila, R. Kekomaki. Glycoprotein V-specific platelet-associated antibodies in thrombocytopenic patients, Clin Lab Haematol 2001; 23:307-312.

38. R. Vollenberg, R. Jouni, P.A.A. Norris, M. Burg-Roderfeld, N. Cooper, M.J. Rummel, et al. Glycoprotein V is a relevant immune target in patients with immune thrombocytopenia, Haematologica 2019; 104:1237-1243.

39. H. Niessner, K.J. Clemetson, S. Panzer, C. Mueller-Eckhardt, S. Santoso, P. Bettelheim. Acquired thrombasthenia due to GPIIb/IIIa-specific platelet autoantibodies, Blood 1986; 68:571-576.

40. D.V. Devine, M.S. Currie, W.F. Rosse, C.S. Greenberg. Pseudo-Bernard-Soulier syndrome: thrombocytopenia caused by autoantibody to platelet glycoprotein Ib, Blood 1987; 70:428-431.

41. H. Deckmyn, S.L. Chew, J. Vermylen. Lack of platelet response to collagen associated with an autoantibody against glycoprotein Ia: a novel cause of acquired qualitative platelet dysfunction, Thromb Haemost 1990; 64:74-79.

42. R. McMillan, R.D. Bowditch, P. Tani, H. Anderson, S. Goodnight. A non-thrombocytopenic bleeding disorder due to an IgG4-kappa anti-GPIIb/IIIa autoantibody, Br J Haematol 1996; 95:747-749.

43. L. Porcelijn, E. Huiskes, R. Maatman, A. de Kreuk, M. de Haas. Acquired Glanzmann's thrombasthenia caused by glycoprotein IIb/IIIa autoantibodies of the immunoglobulin G1 (IgG1), IgG2 or IgG4 subclass: a study in six cases, Vox Sang 2008; 95:324-330.

44. I.M. De Cuyper, M. Meinders, E. van de Vijver, D. de Korte, L. Porcelijn, M. de Haas, et al. A novel flow cytometry-based platelet aggregation assay, Blood 2013; 121:e70-80.

45. E.R. van Bladel, A.G. Laarhoven, L.B. van der Heijden, K.M. Heitink-Polle, L. Porcelijn, C.E. van der Schoot, et al. Functional platelet defects in children with severe chronic ITP as tested with 2 novel assays applicable for low platelet counts, Blood 2014; 123:1556-1563.

46. K. Kaushansky. Thrombopoietin, N Engl J Med 1998; 339:746-754.

47. I.S. Hitchcock, K. Kaushansky. Thrombopoietin from beginning to end, Br J Haematol 2014; 165:259-268.

48. L. Porcelijn, C.C. Folman, M. de Haas, H.H. Kanhai, M.F. Murphy, A.E. von dem Borne, et al. Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia, Pediatr Res 2002; 52:105-108.

49. L. Porcelijn, E. Huiskes, L. Onderwater-Van Den Hoogen, C.C. Folman, J.J. Zwaginga, M. De Haas. Plasma thrombopoietin levels as additional tool in clinical management of thrombocytopenic neonates, Platelets 2020; 31:62-67.

50. J. Peng, S.H. Ma, J. Liu, Y. Hou, X.M. Liu, T. Niu, et al. Association of autoantibody specificity and response to intravenous immunoglobulin G therapy in immune thrombocytopenia: a multicenter cohort study, J Thromb Haemost 2014; 12:497-504.

51. M.L. Webster, E. Sayeh, M. Crow, P. Chen, B. Nieswandt, J. Freedman, et al. Relative efficacy of intravenous immunoglobulin G in ameliorating thrombocytopenia induced by antiplatelet GPIIbIIIa versus GPIbalpha antibodies, Blood 2006; 108:943-946.

52. V. Rumjantseva, K.M. Hoffmeister. Novel and unexpected clearance mechanisms for cold platelets, Transfus Apher Sci 2010; 42:63-70.

53. K.M. Hoffmeister. The role of lectins and glycans in platelet clearance, J Thromb Haemost 2011; 9 Suppl 1:35-43.

54. J. Li, D.E. van der Wal, G. Zhu, M. Xu, I. Yougbare, L. Ma, et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia, Nat Commun 2015; 6:7737.

55. M.E. Quach, M.A. Dragovich, W. Chen, A.K. Syed, W. Cao, X. Liang, et al. Fc-independent immune thrombocytopenia via mechanomolecular signaling in platelets, Blood 2018; 131:787-796.

56. T. Rogier, M. Samson, G. Mourey, N. Falvo, N. Magy-Bertrand, S. Ouandji, et al. Antiplatelet Antibodies Do Not Predict the Response to Intravenous Immunoglobulins during Immune Thrombocytopenia, J Clin Med 2020; 9. https://doi.org/10.3390/jcm9061998.

57. M. Xu, J. Li, M.A.D. Neves, G. Zhu, N. Carrim, R. Yu, et al. GPIbalpha is required for platelet-mediated hepatic thrombopoietin generation, Blood 2018; 132:622-634.

58. L. Porcelijn, D.E. Schmidt, C.E. van der Schoot, G. Vidarsson, M. de Haas, R. Kapur. Anti-glycoprotein Ibalpha autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients, Haematologica 2020; 105:e172-e174.

59. T.B. Gernsheimer. Thrombocytopenia in pregnancy: is this immune thrombocytopenia or...?, Hematology Am Soc Hematol Educ Program 2012; 2012:198-202.

60. T.A. Brighton, S. Evans, P.A. Castaldi, C.N. Chesterman, B.H. Chong. Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias, Blood 1996; 88:194-201.

61. M.N. Warner, J.C. Moore, T.E. Warkentin, A.V. Santos, J.G. Kelton. A prospective study of protein-specific assays used to investigate idiopathic thrombocytopenic purpura, Br J Haematol 1999; 104:442-447.

62. R. He, D.M. Reid, C.E. Jones, N.R. Shulman. Extracellular epitopes of platelet glycoprotein Ib alpha reactive with serum antibodies from patients with chronic idiopathic thrombocytopenic purpura, Blood 1995; 86:3789-3796.

63. K. Fujisawa, P. Tani, R. McMillan. Platelet-associated antibody to glycoprotein IIb/IIIa from chronic immune thrombocytopenic purpura patients often binds to divalent cation-dependent antigens, Blood 1993; 81:1284-1289.

64. S. Kosugi, Y. Tomiyama, M. Shiraga, H. Kashiwagi, H. Mizutani, Y. Kanakura, et al. Platelet-associated anti-glycoprotein (GP) IIb-IIIa autoantibodies in chronic immune thrombocytopenic purpura mainly recognize cation-dependent conformations: comparison with the epitopes of serum autoantibodies, Thromb Haemost 1996; 75:339-345.

65. R. Kekomaki, B. Dawson, J. McFarland, T.J. Kunicki. Localization of human platelet autoantigens to the cysteine-rich region of glycoprotein IIIa, J Clin Invest 1991; 88:847-854.

66. D. Varon, S. Karpatkin. A monoclonal anti-platelet antibody with decreased reactivity for autoimmune thrombocytopenic platelets, Proc Natl Acad Sci U S A 1983; 80:6992-6995.

67. R. McMillan, J. Lopez-Dee, J.C. Loftus. Autoantibodies to alpha(IIb)beta(3) in patients with chronic immune thrombocytopenic purpura bind primarily to epitopes on alpha(IIb), Blood 2001; 97:2171-2172.

68. R. McMillan, L. Wang, J. Lopez-Dee, S. Jiu, J.C. Loftus. Many alphaIIbbeta3 autoepitopes in chronic immune thrombocytopenic purpura are localized to alphaIIb between amino acids L1 and Q459, Br J Haematol 2002; 118:1132-1136.

69. K. Kiyomizu, H. Kashiwagi, T. Nakazawa, S. Tadokoro, S. Honda, Y. Kanakura, et al. Recognition of highly restricted regions in the beta-propeller domain of alphaIIb by platelet-associated anti-alphaIIbbeta3 autoantibodies in primary immune thrombocytopenia, Blood 2012; 120:1499-1509.

70. M. Hou, D. Stockelberg, J. Kutti, H. Wadenvik. Glycoprotein IIb/IIIa autoantigenic repertoire in chronic idiopathic thrombocytopenic purpura, Br J Haematol 1995; 91:971-975.

71. R. Escher, D. Muller, M. Vogel, S. Miescher, B.M. Stadler, P. Berchtold. Recombinant human natural autoantibodies against GPIIb/IIIa inhibit binding of autoantibodies from patients with AITP, Br J Haematol 1998; 102:820-828.

72. R. McMillan. The pathogenesis of chronic immune thrombocytopenic purpura, Semin Hematol 2007; 44:S3-S11.

73. J.H. Roark, J.B. Bussel, D.B. Cines, D.L. Siegel. Genetic analysis of autoantibodies in idiopathic thrombocytopenic purpura reveals evidence of clonal expansion and somatic mutation, Blood 2002; 100:1388-1398.

74. D. Stockelberg, M. Hou, S. Jacobsson, J. Kutti, H. Wadenvik. Light chain-restricted autoantibodies in chronic idiopathic thrombocytopenic purpura, but no evidence for circulating clone B-lymphocytes, Ann Hematol 1996; 72:29-34.

75. D.M. Arnold, S. Santoso, A. Greinacher, I. Platelet Immunology Scientific Subcommittee of the. Recommendations for the implementation of platelet autoantibody testing in clinical trials of immune thrombocytopenia, J Thromb Haemost 2012; 10:695-697.

76. K. Campbell, K. Rishi, G. Howkins, D. Gilby, R. Mushens, C. Ghevaert, et al. A modified rapid monoclonal antibody-specific immobilization of platelet antigen assay for the detection of human platelet antigen (HPA) antibodies: a multicentre evaluation, Vox Sang 2007; 93:289-297.

77. W. Vainchenker, J.F. Deschamps, J.M. Bastin, J. Guichard, M. Titeux, J. Breton-Gorius, et al. Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in in vivo cells from normal and leukemic patients, Blood 1982; 59:514-521.

78. R. McMillan, L. Wang, A. Tomer, J. Nichol, J. Pistillo. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP, Blood 2004; 103:1364-1369.

79. M. Chang, P.A. Nakagawa, S.A. Williams, M.R. Schwartz, K.L. Imfeld, J.S. Buzby, et al. Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro, Blood 2003; 102:887-895.

80. E.J. Houwerzijl, N.R. Blom, J.J. van der Want, E. Vellenga, J.T. de Wolf. Megakaryocytic dysfunction in myelodysplastic syndromes and idiopathic thrombocytopenic purpura is in part due to different forms of cell death, Leukemia 2006; 20:1937-1942.

81. M. Grodzielski, C.A. Di Buduo, N.P. Goette, P.R. Lev, P.M. Soprano, P.G. Heller, et al. Autoantibodies in immune thrombocytopenia affect the physiological interaction between megakaryocytes and bone marrow extracellular matrix proteins, Br J Haematol 2018; 183:319-323.

82. P.R. Lev, M. Grodzielski, N.P. Goette, A.C. Glembotsky, Y.R. Espasandin, M.S. Pierdominici, et al. Impaired proplatelet formation in immune thrombocytopenia: a novel mechanism contributing to decreased platelet count, Br J Haematol 2014; 165:854-864.

83. S. Shrestha, I. Nazy, J.W. Smith, J.G. Kelton, D.M. Arnold. Platelet autoantibodies in the bone marrow of patients with immune thrombocytopenia, Blood Adv 2020; 4:2962-2966.

84. D.E. Schmidt, A.J. Lakerveld, K.M.J. Heitink-Polle, M.C.A. Bruin, G. Vidarsson, L. Porcelijn, et al. Anti-platelet antibody immunoassays in childhood immune thrombocytopenia: a systematic review, Vox Sang 2020; 115:323-333.

85. M. Hou, D. Stockelberg, J. Kutti, H. Wadenvik. Antibodies against platelet GPIb/IX, GPIIb/IIIa, and other platelet antigens in chronic idiopathic thrombocytopenic purpura, Eur J Haematol 1995; 55:307-314.

86. A. Crossley, J.E. Calvert, P.R. Taylor, A.M. Dickinson. A comparison of monoclonal antibody immobilization of platelet antigen (MAIPA) and immunobead methods for detection of GPIIb/IIIa antiplatelet antibodies in immune thrombocytopenic purpura, Transfus Med 1997; 7:127-134.

87. A. Davoren, J. Bussel, B.R. Curtis, M. Moghaddam, R.H. Aster, J.G. McFarland. Prospective evaluation of a new platelet glycoprotein (GP)-specific assay (PakAuto) in the diagnosis of autoimmune thrombocytopenia (AITP), Am J Hematol 2005; 78:193-197.

88. D. Stockelberg, M. Hou, S. Jacobsson, J. Kutti, H. Wadenvik. Detection of platelet antibodies in chronic idiopathic thrombocytopenic purpura (ITP). A comparative study using flow cytometry, a whole platelet ELISA, and an antigen capture ELISA, Eur J Haematol 1996; 56:72-77.

89. A. Gaiger, A. Neumeister, H. Heinzl, I. Pabinger, S. Panzer. HLA class-I and -II antigens in chronic idiopathic autoimmune thrombocytopenia, Ann Hematol 1994; 68:299-302.

90. S. Panzer, L. Hocker, M. Rieger, R. Vormittag, D. Koren, D. Dunkler, et al. Agonist-inducible platelet activation in chronic idiopathic autoimmune thrombocytopenia, Eur J Haematol 2007; 79:198-204.

91. A. Najaoui, T. Bakchoul, J. Stoy, G. Bein, M.J. Rummel, S. Santoso, et al. Autoantibody-mediated complement activation on platelets is a common finding in patients with immune thrombocytopenic purpura (ITP), Eur J Haematol 2012; 88:167-174.

92. F. Fabris, A. Casonato, M.L. Randi, G. Luzzatto, A. Girolami. Clinical significance of surface and internal pools of platelet-associated immunoglobulins in immune thrombocytopenia, Scand J Haematol 1986; 37:215-220.

93. F. Fabris, A. Casonato, R. Zanchetta, S. Cortellazzo, F. Busolo, A. Girolami. A microplate enzyme-linked immunospecific assay (ELISA) detecting unbound anti-platelet antibodies, Folia Haematol Int Mag Klin Morphol Blutforsch 1984; 111:66-71.

94. O. Meyer, A. Agaylan, H.H. Borchert, T. Aslan, S. Bombard, H. Kiesewetter, et al. A simple and practical assay for the antigen-specific detection of platelet antibodies, Transfusion 2006; 46:1226-1231.

95. P. Nurden, A.T. Nurden. Is the mysterious platelet receptor GPV an unsuspected major target for platelet autoantibodies?, Haematologica 2019; 104:1103-1105.

96. L. Porcelijn, E. Huiskes, G. Oldert, R. Fijnheer, M.R. Schipperus, J.J. Zwaginga, et al. Detection of platelet autoantibodies revisited to identify immune thrombocytopenia, Blood 2015; 126:1142.

97. L. Porcelijn, E. Huiskes, M. Schipperus, B. van der Holt, M. de Haas, J.J. Zwaginga, et al. Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients, Blood 2017; 129:3389-3391.

98. S. Kosugi, Y. Tomiyama, S. Honda, H. Kashiwagi, M. Shiraga, S. Tadokoro, et al. Anti-alphavbeta3 antibodies in chronic immune thrombocytopenic purpura, Thromb Haemost 2001; 85:36-41.

99. F. Fabris, R. Scandellari, M.L. Randi, G. Carraro, G. Luzzatto, A. Girolami. Attempt to improve the diagnosis of immune thrombocytopenia by combined use of two different platelet autoantibodies assays (PAIgG and MACE), Haematologica 2002; 87:1046-1052.

100. H. Chan, J.C. Moore, C.N. Finch, T.E. Warkentin, J.G. Kelton. The IgG subclasses of platelet-associated autoantibodies directed against platelet glycoproteins IIb/IIIa in patients with idiopathic thrombocytopenic purpura, Br J Haematol 2003; 122:818-824.

101. F. Fabris, R. Scandellari, E. Ruzzon, M.L. Randi, G. Luzzatto, A. Girolami. Platelet-associated autoantibodies as detected by a solid-phase modified antigen capture ELISA test (MACE) are a useful prognostic factor in idiopathic thrombocytopenic purpura, Blood 2004; 103:4562-4564.

102. A. Tomer, J. Koziol, R. McMillan. Autoimmune thrombocytopenia: flow cytometric determination of platelet-associated autoantibodies against platelet-specific receptors, J Thromb Haemost 2005; 3:74-78.

103. O. Meyer, A. Agaylan, S. Bombard, H. Kiesewetter, A. Salama. A novel antigen-specific capture assay for the detection of platelet antibodies and HPA-1a phenotyping, Vox Sang 2006; 91:324-330.

104. Y. He, Y.X. Zhao, M.Q. Zhu, Q. Wu, C.G. Ruan. Detection of autoantibodies against platelet glycoproteins in patients with immune thrombocytopenic purpura by flow cytometric immunobead array, Clin Chim Acta 2013; 415:176-180.

09

CHAPTER 9

Antibodies against platelet glycoproteins in clinically-suspected VITT patients

Meier RT/Porcelijn L (co-first), Hofstede-van Egmond S, Caram-Deelder C, Coutinho JM, Henskens YMC, Kruip MJHA, Stroobants AK, Zwaginga JJ, van der Schoot CE, de Haas M, Kapur R. Antibodies against Platelet Glycoproteins in Clinically Suspected VITT Patients. Antibodies (Basel). 2024 May 1;13(2):35. doi: 10.3390/antib13020035. PMID: 38804303; PMCID: PMC11130846.

Antibodies against platelet glycoproteins in clinically-suspected VITT patients

Romy T. Meier [1]/Leendert Porcelijn [2] (co-first), Suzanne Hofstede-van Egmond [2], Camila Caram-Deelder [3], Jonathan M. Coutinho [4], Yvonne M.C. Henskens [5], Marieke J.H.A. Kruip [6], An K. Stroobants [7], Jaap J. Zwaginga [8], C. Ellen van der Schoot [1], Masja de Haas [2,8], Rick Kapur[1,*]

[1] Dept. of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, Amsterdam, University of Amsterdam, The Netherlands
[2] Sanquin Diagnostic Services, Department of Immunohematology Diagnostics, Sanquin The Netherlands
[3] Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands
[4] Department of Neurology, Amsterdam UMC, Amsterdam, The Netherlands
5 Central Diagnostic Laboratory Maastricht University Medical Centre+, Maastricht, The Netherlands
[6] Department of Haematology, Erasmus MC, Erasmus University Medical Center, Rotterdam, The Netherlands
[7] Department of Clinical Chemistry, Radboud University Medical Center, Nijmegen, The Netherlands
[8] Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands
**Correspondence: r.kapur@sanquin.nl*

Abstract:
Vaccine-induced thrombotic thrombocytopenia (VITT) is a rare but severe complication following COVID-19 vaccination, marked by thrombocytopenia and thrombosis. Analogous to heparin-induced thrombocytopenia (HIT), VITT shares similarities in anti-platelet factor 4 (PF4) IgG-mediated platelet activation via the FcγRIIa. To investigate the involvement ofplate-let-antibodies in VITT, we analyzed the presence of platelet-antibodies directed against glyco-proteins (GP) IIb/IIIa, GPV and GPIb/IX in the serum of 232 clinically-suspected VITT patients determined based on (suspicion of) occurrence of thrombocytopenia and/or thrombosis in rela-tion to COVID-19 vaccination. We found that 19% of clinically-suspected VITT patients tested positive for anti-platelet GPs: 39%, 32% and 86% patients tested positive for GPIIb/IIIa, GPV and GPIb/IX, respectively. No HIT-like VITT patients (with thrombocytopenia and thrombosis) tested positive for platelet-antibodies. Therefore, it seems unlikely that platelet-antibodies play a role in HIT-like anti-PF4-mediated VITT. Platelet-antibodies were predominantly associated with the occurrence of thrombocytopenia. We found no association between the type of vaccination (ad-enoviral vector vaccine versus mRNA vaccine) or different vaccines (ChAdOx1 nCoV-19, Ad26.COV2.S, mRNA-1273, BTN162b2) and the development of platelet-antibodies. It is essential to conduct more research on the pathophysiology of VITT, to improve diagnostic approaches and identify preventive and therapeutic strategies.

Keywords:
keyword 1; Platelet-autoantibodies 2; Thrombocytopenia 3; Thrombosis 4; COVID-19 5; Vaccination.

1. Introduction

Vaccine-induced thrombotic thrombocytopenia (VITT) is a disorder that has been rec-ognized since the global vaccination strategy against SARS-CoV-2 started [1, 2]. VITT was initially characterized by thrombocytopenia and thrombosis, and shows similarities with heparin-induced thrombocytopenia (HIT) in terms of clinical characteristics and underlying mechanism [3, 4]. In HIT, antibodies are directed against platelet factor 4 (PF4)/heparin complexes resulting in FcγRIIa-dependent platelet activation, while in VITT PF4-antibodies have been identified [1]. Interestingly, besides the more recognized role for PF4-antibodies, a possible role for antibodies against platelet membrane glyco-proteins (GPs) has recently been suggested [5]. Platelet-autoantibodies have been im-plicated in diseases including sepsis and the autoimmune disorder immune thrombo-cytopenia (ITP), in which platelet clearance is mediated by platelet-autoantibodies [6]. In addition, platelet-associated IgG was shown to be elevated in thrombocytopenic pa-tients with sepsis [7]. Whereas healthy individuals generally do not test positive for platelet antibodies in the MAIPA, 18% of ITP patients test positive for GPV, 15% for GPIIb/IIIa and 15% for GPIb/IX in the indirect MAIPA [8, 9]. Given the role of plate-let-autoantibodies in thrombocytopenia, it is possible these platelet-autoantibodies play a role in the pathophysiology of VITT.

A study found that healthy recipients of both adenoviral vector and mRNA vaccines, developed platelet-autoantibodies without a clear preference for one of the tested platelet glycoproteins (GP) IIb/IIIa, Ib/IX and Ia/IIa [10]. In another study with 30% of the 27 proven VITT patients vaccinated with ChAdOx1 nCov-19 tested positive for free circulating platelet-antibodies targeting platelet GPIIb/IIIa, GPIb/IX or GPIa/IIa [5]. To gain more insight into the significance of antibodies against platelet glycoproteins, we conducted an analysis in all known clinically-suspected VITT individuals determined by physicians based on the (suspicion of) occurrence of thrombocytopenia/thrombosis upon COVID-19 vaccination in the Netherlands.

2. Materials and Methods

We tested clinically-suspected VITT patients for the presence of plate-let-antibodies. Due to lack of availability of patient platelets, we used an indirect mon-oclonal antibody immobilization of platelet antigens (MAIPA) assay [11]. This assay is considered the gold standard reference technique in platelet immunology and is used in the Netherlands to support the diagnosis of immune thrombocytopenia (ITP) [11, 12]. The MAIPA was performed as described by Kiefel et al. [11], in brief: microtiter plates were coated with goat-anti-mouse (GαM) for 12 hours at 4°C. Following this, platelets were washed and patient serum was added to the plate. Subsequently, monoclonal an-tibodies directed against circulating antibodies (GPIIb/IIIa (αIIbβ3, CD41/CD61, CLB/Thromb1 (C17), Sanquin Reagents), GPV (CD42d, SW16, Sanquin Reagents) and GPIb/

IX (CD42c/CD42a, FMC25, ThermoFisher)) were introduced [8]. After washing and centrifugation, a GαM-HRP conjugate was added to the plate. After further washing, extinction was measured using an ELISA reader (Epoch ELISA reader). An extinction of ≥ 0.130 was interpreted as positive, while an extinction of ≤ 0.130 was regarded as neg-ative.

Furthermore, we measured free circulating plasma thrombopoietin (TPO) levels to gain insights into platelet production or platelet breakdown. TPO levels were measured in EDTA-anticoagulated plasma samples using an in-house developed TPO sandwich ELISA, as described by Folman et al. [13]: microtiter plates were coated with two non-cross-reactive monoclonal antibodies. After washing and blocking the plates, a third biotinylated monoclonal antibody and patient plasma were added. Following further washing,, a streptavidin-horseradish-peroxidase was added and H2SO4 was added to stop the reaction. The extinction was determined using an ELISA-reader (Epoch ELISA reader). Results were reported as “normal” (0-60 U/ml plasma) and “el-evated” (>60 U/ml plasma).
Since D-dimer data were missing at the time that the samples were collected, we were unable to adhere to the later and currently established VITT classification [14, 15]. We therefore categorized clinically-suspected VITT patients based on the occurrence of thrombocytopenia and/or thrombosis. For VITT diagnostic testing we used an in-house developed anti-PF4 in which patient serum was added to a PF4-coated (Chromatec, Greifswald, Germany) microtiter plate. PF4-antibodies were detected measuring exci-tation after adding GaH-HRP IgG to the plate. Patients with an OD ≥1.0 were considered positive. In the PIPAA, performed as described by Greinacher et al. [1] with slight modifications, we incubated washed donor platelets with PF4 and with and without FcγRIIa (CD32)-blocking monoclonal antibody clone IV.3 (Sanquin Research, Amster-dam, The Netherlands). Patients with both thrombocytopenia and thrombosis, and testing positive in both diagnostic tests, were classified as HIT-like VITT patients. This classification aligns with the confirmation criteria for HIT patients, who are identified by a positive anti-heparin/PF4-ELISA and a positive FcγRIIa-dependent hepa-rin-induced platelet activation assay (HIPAA) [16, 17].
To estimate the incidence of platelet-antibodies in COVID-19 vaccinated individuals we used data on the total number of vaccines within our study period which was obtained from the National Institute for Public Health and the Environment (RIVM) and encompasses all COVID-19 vaccination data within The Netherlands. This study was conducted in line with the ethical guidelines of the institutional research committee and in compliance with the 1964 Helsinki declaration and its sub-sequent revisions or similar ethical standards. Clinical data was only collected at ad-mission. Samples (residual material) were obtained from Sanquin Diagnostics, which functions as the national reference center for VITT, HIT and platelet-antibody testing.

3. Results

3.1. Patient characteristics

We examined 232 patients clinically-suspected of VITT of whom we received samples for diagnostic testing between March 22th and November 26th 2021 (Table 1). Our cohort consisted of 111 females and 121 males with a median age of 62 (IQR: 53-68). Of the 232 VITT suspected patients 112 (48%) were vaccinated with ChAdOx1 nCoV-19, 7 (3%) with Ad26.COV2.S, 34 (15%) with mRNA-1273, and 79 (34%) with BTN162b2. Patients were admitted, on average, 21 days after vaccination.

Our cohort contained seven confirmed HIT-like VITT patients (for patients' description: Table S1). All other patients tested negative in both the anti-PF4 IgG ELISA and FcγRIIa-dependent PIPAA or did not have both thrombocytopenia and thrombosis (for patients' description: Table S2).

	Clinically suspected patients (n=232)	Positive for platelet antibodies (n=44)	Negative for platelet antibodies (n=188)
Demographics			
Median age (IQR)	62 (53-68)	62 (54-69)	60 (53-69)
Female sex (no.(%))	111 (48%)	24 (55%)	87 (46%)
Male sex (no.(%))	121 (53%)	20 (45%)	101 (54%)
Vaccination			
Vaccine type (no.(%))			
Adenoviral vector vaccines	119 (51%)	20 (45%)	99 (53%)
ChAdOx1 nCoV-19	112 (48%)	19 (43%)	93 (50%)
Ad26.COV2.S	7 (3%)	1 (2%)	6 (3%)
mRNA vaccines	113 (49%)	24 (55%)	89 (47%)
mRNA-1273	34 (15%)	7 (16%)	27 (14%)
BTN162b2	79 (34%)	17 (39%)	62 (33%)
Days between admission and vaccination			
Mean (IQR)	21 (8-28)	24 (9-29)	21 (8-28)
Number of vaccination (no.(%))			
First dose	37 (16%)	10 (23%)	31 (17%)
Second dose	68 (29%)	15 (34%)	55 (29%)
Third dose	2 (1%)	-	2 (1%)
No information on dose	125 (54%)	19 (43%)	100 (53%)

Clinical characteristics (no.(%))			
Thrombocytopenia (<100x10^9/L)	151 (65%)	34 (77%)	117 (62%)
Median platelet count (IQR)	51 (18-99)	35 (8-63)	55 (21-108)
No thrombocytopenia	55 (24%)	4 (9%)	51 (27%)
No data on platelet count	26 (11%)	6 (14%)	20 (11%)
Thrombosis	71 (31%)	7 (16%)	64 (34%)
No thrombosis	129 (56%)	31 (71%)	98 (52%)
No data on thrombosis available	32 (14%)	6 (14%)	26 (14%)
Thrombocytopenia and thrombosis	32 (14%)	3 (7%)	29 (15%)
Thrombocytopenia only	119 (51%)	31 (71%)	88 (47%)
Thrombosis only	39 (17%)	4 (9%)	35 (19%)
Neither thrombocytopenia nor thrombosis	19 (8%)	1 (2%)	18 (10%)
No data on both thrombocytopenia and thrombosis	23 (10%)	5 (11%)	18 (10%)
Laboratory tests			
Anti-PF4 ELISA negative (OD < 1.0)	212 (91%)	42 (96%)	170 (90%)
PIPAA negative	206 (97%)	38 (90%)	168 (99%)
PIPAA positive	6 (3%)	4 (10%)	2 (1%)
Anti-PF4 ELISA weak-positive (1.0 ≤ OD < 2.0)	7 (3%)	1 (2%)	6 (3%)
PIPAA negative	6 (86%)	1 (100%)	5 (83%)
PIPAA positive	1 (14%)	-	1 (17%)
Anti-PF4 ELISA positive (OD ≥ 2.0)	13 (6%)	1 (2%)	12 (6%)
PIPAA negative	3 (23%)	1 (100%)	2 (17%)
PIPAA positive	10 (77%)	-	10 (83%)

Table 1: Baseline and clinical characteristics of 232 VITT-suspected patients who were tested in the indirect MAIPA.

We did not observe platelet-antibodies in HIT-like VITT patients (N=7). However, we found that 44 clinically-suspected VITT patients in our cohort tested positive for plate-let-antibodies; 26% (N=31) of patients with isolated thrombocytopenia (platelet count <100x10^9/L), 10% (N=4) of patients with thrombosis only, 9% (n=3) of patients with both thrombocytopenia and thrombosis, and 5% (n=1) of patients with neither throm-bocytopenia nor thrombosis (Figure 1).

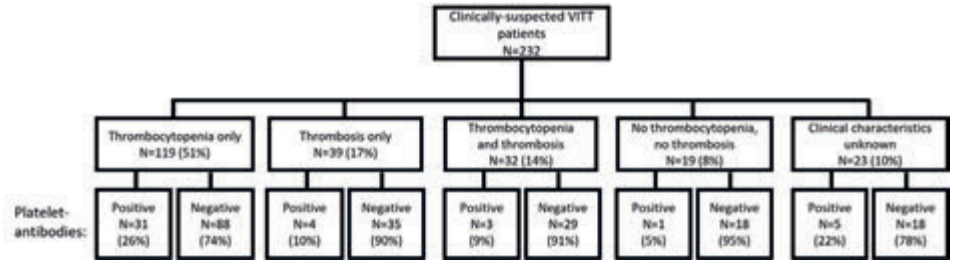


Figure 1: *Anti-platelet GP in clinically-suspected VITT patients after vaccination with ChAdOx1 nCoV-19, BNT162b2, mRNA-1273 or Ad26.COV2.S. Serum samples of 232 unique and clinical-ly-suspected VITT patients were analyzed for the presence of platelet-autoantibodies.*

3.3. Clinical characteristics in clinically-suspected VITT patients with platelet-antibodies

Within the 44 platelet-antibody positive patients, we observed a higher incidence of thrombocytopenia (77%), compared to the group testing negative for plate-let-antibodies (62%) (Table 1). Remarkably, a smaller proportion of the platelet-antibody positive group (16%) presented with thrombosis, compared to the platelet-antibody negative group (34%). The combination of thrombocytopenia and thrombosis was less common in patients positive for platelet-antibodies. It should be noted that data on thrombocytopenia and/or thrombosis was not available for all patients, and these pa-tients were not included in this analyses.

3.4. Presence of platelet-antibodies in relation to vaccines

In our cohort, 17% (n=19) of ChAdOx1 nCov-19 vaccinees, 22% (n=17) of BNT162b2 vaccinees, 21% (n=7) of mRNA-1273 vaccinees and 14% (n=1) Ad26.COV2.S vaccinees tested positive for platelet-antibodies (Table 1). Within this cohort, twenty patients vaccinated with adenoviral vector vaccines tested positive for platelet-antibodies out of a total 3,304,944 doses given nationwide during the study period (0.61 cases per 100,000 adenoviral vector-based

COVID-19 vaccine doses). Additionally, 24 patients vaccinated with mRNA-based vaccines tested positive for platelet-antibodies out of a total of 20,670,060 given doses (0.12 cases per 100,000 mRNA-based COVID-19 vaccine doses).
To determine whether there was a relationship between the presence of platelet-antibodies and the type of vaccine (adenoviral vector vaccine vs. mRNA vaccine) we performed a multivariate logistic regression to determine the effects of age and sex on the likelihood that clinically-suspected VITT patients vaccinated with adenoviral vector vaccines will develop platelet-antibodies versus suspected VITT patients vaccinated with mRNA vaccines (Figure 2, panel A). We found no difference in the risk of devel-oping platelet-antibodies between being vaccinated with the adenoviral vector- and the mRNA vaccine (OR=1.43, 95%CI [0.73; 2.79]) as the logistic regression model was not significant (p-value=0.465) and explained 1.1% (pseudo R2) of the variance of the presence of platelet-antibodies.

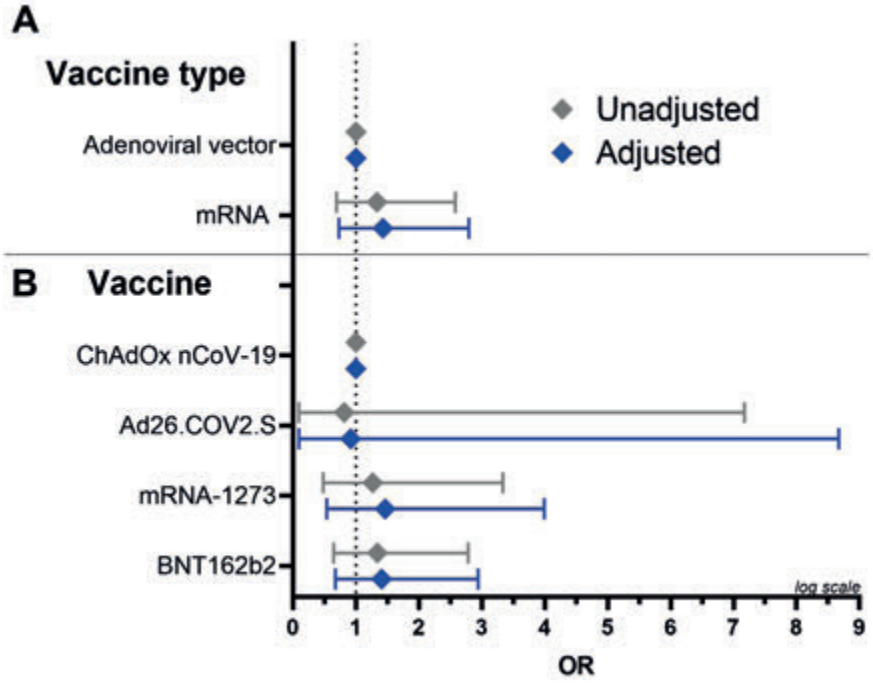


Figure 2: *Forest plot for odds ratios with 95% CI for the effect on presence of platelet-antibodies. We corrected for age (continuous) and sex (female vs male). A) mRNA vaccines were compared with adenoviral vector vaccines (baseline). B) BNT162b2, mRNA-1273 and Ad26.COV2.S were compared to ChAdOx1 nCoV-19 (baseline).*

We performed a similar analysis to investigate the relationship between the presence of platelet-antibodies and the four different vaccines (Figure 2, panel B). With ChAdOx1 nCov-19 as our reference, we found no difference in risk of developing platelet-antibodies between patients vaccinated with the four different vaccines; the BNT162b2 vaccine (OR=0.92, 95%CI [0.10; 8.7]), the mRNA-1273 vaccine (OR=1.46, 95%CI [0.54; 4.0]) and the Ad26.COV2.S vaccine (OR=1.41, 95%CI [0.68; 2.94]). The logistic re-gression model was not significant (p-value=0.766) and explained 1.1% (pseudo R2) of the variance of the presence of platelet-antibodies. However, it is important to note that in this analysis the small group size and poor model performance (small pseudo R2) diminishes the power of detecting a possible relevant and significant change is limited.

3.5. Platelet-antibody profiles

To further investigate whether the platelet-antibody positive patients in our cohort were ITP patients, we compared antibody profiles of suspected VITT patients with an-tibody profiles of suspected ITP patients. Out of the 44 suspected-VITT patients positive for platelet-antibodies; 14% tested positive for GPIIb/IIIa, 5% for GPV, 41% for GPIb/IX-antibodies and 11% tested positive for all three platelet-antibodies (Figure 3). In comparison, of patients tested in the MAIPA in our institute in the years 2022 and 2023 due to suspected ITP, 518 out of 1507 (34 %) patients tested positive for plate-let-antibodies; 16% for GPIIb/IIIa, 12% for GPV, 25% for GPIb/IX, and 22% tested positive for all three platelet-antibodies. Although we found that anti-GPIb/IX antibodies were increased in clinically suspected VITT patients (41%) vs in suspected ITP patients (25%), overall antibody profiles between clinically-suspected VITT patients and suspected ITP patients were not statistically significant (X-squared = 10.592, df = 6, p-value = 0.1018).

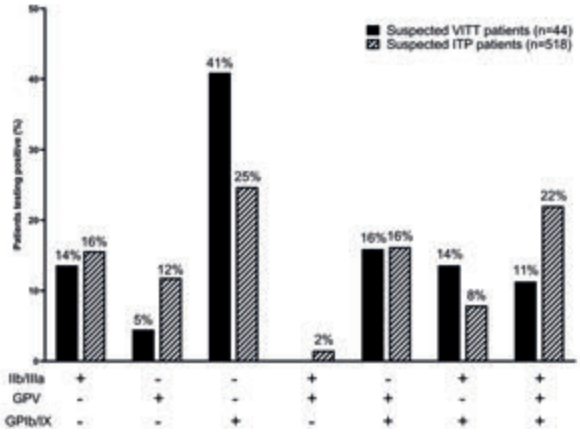


Figure 3: *Percentage of suspected VITT or ITP patients (Y-axis) positive for glycoprotein specific platelet-antibodies (X-axis). Solid bars are suspected VITT patients, dashed bars are suspected ITP patients. Glycoprotein specific anti-platelet (GPIIb/IIIa, GPV, GPIb/IX) detection stratified to type of vaccine in clinically-suspected VITT (n=44) and suspected ITP (n=518) were not significantly different (X-squared = 10.592, df = 6, p-value = 0.1018).*

3.6. TPO levels of clinically-suspected VITT patients

We examined the levels of thrombopoietin (TPO) in the plasma of 42 patients to de-termine the probability of identifying patients positive for platelet antibodies as ITP patients, in which TPO levels are normal/non-elevated [18, 19]. We determined the TPO levels of 42 of 44 platelet-antibody positive patients and 178 platelet-antibody negative patients, of which 7 HIT-like VITT patients. Out of the seven HIT-like VITT patients, two (29%) patients had high TPO levels and five (71%) patients had normal TPO levels. Out the 42 patients testing positive for platelet-antibodies, the majority of 25 (59%) patients with normal TPO levels, and four (10%) patients with elevated TPO levels presented with thrombocytopenia (Figure S1). Since ITP patients generally do not have elevated TPO levels, we cannot rule out that patients in our cohort with normal TPO levels are ITP patients.

4. Discussion

In our investigation into the potential role for platelet-autoantibodies in VITT patho-physiology, we analyzed the presence of platelet-antibodies in a cohort of 232 clinical-ly-suspected VITT patients, including seven HIT-like VITT patients. We did not detect circulating platelet-autoantibodies in HIT-like VITT patients, implying that plate-let-autoantibodies may not be involved in the pathophysiology of HIT-like VITT. In-terestingly, three out of seven HIT-like VITT patients (43%) were diagnosed with in-tracranial thrombosis which is found to be a hallmark for VITT (Table S1) [20]. We found that forty-four patients (19%) in our cohort of clinically-suspected VITT patients tested positive for platelet-antibodies. These platelet-antibodies were predominantly detected in patients with thrombocytopenia, raising the possibility of a mechanism of anti-body-mediated platelet clearance. It therefore seems likely that other plate-let-antibody-independent mechanisms may underlie the development of thrombosis (with or without thrombocytopenia) in VITT patients. Analysis of platelet-antibody levels in non-thrombocytopenic and COVID-19 vaccinated control group would be re-quired to further study this, however, this group was unfortunately not available to us.

Considering platelet-autoantibodies have been found in both adenoviral vector and mRNA COVID-19 vaccine recipients [21–23], but not healthy individuals [8, 24], we examined the association between the (type of) vaccine(s) and the presence of plate-let-autoantibodies. We found that the risk of developing antibodies was independent of the (type of) vaccine and we therefore concluded there is no association between the (type of) vaccine or the presence of platelet-antibodies in clinically-suspected VITT pa-tients. Thus, it remains unclear what may have caused the presence of these plate-let-antibodies in clinically-suspected and non-HIT like VITT patients.

Since testing for platelet-autoantibodies is generally done to support an ITP diagnosis, it is plausible that some of the patients testing positive for the plate-let-antibodies could be (de novo/preexisting) ITP patients. Since data on underlying conditions in patients is not available to us, we explored whether these patients could be ITP patients; we analyzed the platelet-autoantibody profile in our cohort of clinical-ly-suspected VITT patients and compared it to those of ITP patients (Figure 2). Although we did not find overall differences in antibody profiles between suspected VITT patients and suspected ITP patients, we did find that 41% of the 44 suspected VITT patients positive for platelet-antibodies, tested positive for antibodies directed against GPIb/IX. This discrepancy suggest that vaccination could result in the production of plate-let-autoantibodies with a preference for epitopes located on platelet-GPIb/IX.

Furthermore, we analyzed TPO levels in patient plasma to further determine the likelihood of platelet-antibody positive patients being classified as ITP patients, which in ITP patients generally demonstrate normal/non-significantly elevated TPO levels [18, 19]. TPO, a protein produced mainly in the liver and secreted into the circulation, is the main regulator of thrombopoiesis and can bind to TPO receptors on circulating platelets and megakaryocytes and megakaryocyte precursors [25]. Circulating TPO is primarily cleared by platelets through binding to the TPO receptor followed by internalization and consumption of TPO. Although TPO levels in the blood and bone marrow are inversely related to platelet count, high TPO levels are more likely to indicate an issue in the production of platelets [18, 19]. Considering that ITP patients commonly show normal or slightly elevated TPO levels, the 25 (59%) patients with thrombocytopenia who tested positive for platelet-antibodies and had normal TPO levels, might be ITP cases. How-ever, taking into account that ITP is diagnosed through the exclusion of other condi-tions, and follow-up data is missing, further clinical information is necessary for con-firmation [26].

Given the surge in de novo ITP cases and pre-existing ITP exacerbations after COVID-19 vaccination and the rise in positive platelet-antibody tests since January-June 2021 (Table S3), it remains plausible that the clinically-suspected non-HIT like VITT patients testing positive for platelet-antibodies in our cohort were ultimately diagnosed with ITP [27–30]. ITP cases have not only been described after vaccination with COVID-19 vaccines (1.13 per 100,000 ChAdOx1 nCoV-19 doses; 0.80 cases of thrombo-cytopenia per million doses of both BNT162b2 and mRNA-1273), but also after other vaccinations including for hepatitis A, varicella, and measles-mumps-rubella vaccines (1-4 cases per 100,000 MMR doses) [27, 31–34]. Although virus vaccine components and virus-induced molecular mimicry have been mentioned as potential causes for vaccine-induced ITP, it is unclear what triggers the formation of platelet GP-specific

anti-bodies upon vaccination with COVID-19- and other vaccines.
Reports of ITP occurring after infection with COVID-19 [35, 36] lead us to investigate fluctuations in ITP reference testing in our laboratory, in order to clarify whether COVID-19 vaccine administration may have contributed to the increase in positive ITP reference tests. Starting June 2020 the Dutch ITP guideline required testing for plate-let-autoantibodies in the MAIPA to support an ITP diagnosis [37], which likely resulted in an increase in platelet-autoantibody tests in the second half of 2020. Requests for platelet-autoantibody tests continued to increase in the following years, which is most likely related to the start of the COVID-19 vaccination strategy in January 2021 and the concomitant clinical awareness for serious adverse effects [27, 28, 38]. Although the increase in confirmed COVID-19 infections in January/February 2022 [39] appears to coincide with the continuous increase of positive platelet-autoantibody tests, more data on whether the patients in our cohort experienced COVID-19 infections needs to be investigated in subsequent studies.

4. Conclusions

We tested 232 clinically-suspected VITT patients, of which seven patients were confirmed HIT-like VITT patients, for the presence of platelet-antibodies. We found 44 pa-tients tested positive for platelet-antibodies, of which none were confirmed HIT-like VITT patients. Therefore, the role of anti-platelet GPs in HIT-like and anti-PF4 mediated VITT appears unlikely. Although further investigation is needed, the presence of platelet-antibodies seemed primarily associated with the occurrence of thrombocyto-penia, indicating a potential mechanism of antibody-mediated platelet clearance not directly linked to the development of VITT. Investigating a possible connection between the administered (type of) vaccine(s) and the presence of platelet-antibodies, we found no significant correlation. Similarly, our analysis comparing platelet-antibody profiles of suspected ITP patients to those of suspected VITT patients showed no overall dis-tinctions. In addition, analysis of TPO levels showed the majority of patients with platelet-antibodies and thrombocytopenia had normal TPO levels which could be in-dicative of ITP, and analysis of ITP reference test requests revealed an increase since the start of the COVID-19 vaccination strategy. Taken together, it is possible that thrombocytopenic patients testing positive for platelet-antibodies who were suspected of having VITT, are de novo or preexisting ITP patients. However, as ITP is a diagnosis of exclusion and we lack data on preexisting conditions we cannot conclusively say the patients testing positive for platelet-antibodies are ITP patients. New studies with better clinically defined patients and longitudinal analysis of the presence of plate-let-antibodies could reveal more about the presence of platelet-antibodies after COVID-19 vaccination. Overall, more research into the pathophysiological mechanisms of VITT is highly warranted for strengthening diagnostic approaches and identifying therapeutic targets.

References

1. Greinacher A, Thiele T, Warkentin TE, Weisser K, Kyrle PA, Eichinger S. Thrombotic Thrombocytopenia after ChAdOx1 nCov-19 Vaccination. N Engl J Med. 2021;384:2092–101.
2. Schultz NH, Sørvoll IH, Michelsen AE, Munthe LA, Lund-Johansen F, Ahlen MT, et al. Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. N Engl J Med. 2021;384:2124–30.
3. Arepally GM. Clinical platelet disorders heparin-induced thrombocytopenia. Blood. 2017;129:2864–72.
4. Arepally GM, Cines DB. Pathogenesis of heparin-induced thrombocytopenia. Transl Res. 2020;225:131–40.
5. Nicolai L, Leunig A, Pekayvaz K, Esefeld M, Anjum A, Rath J, et al. Thrombocytopenia and splenic platelet-directed immune responses after IV ChAdOx1 nCov-19 administration. Blood. 2022;140:478–90.
6. Kiefel V. Platelet antibodies in immune thrombocytopenia and related conditions. J Lab Med. 2020;44:273–84.
7. Stéphan F, Cheffi MA, Kaplan C, Maillet JM, Novara A, Fagon JY, et al. Autoantibodies against platelet glycoproteins in critically ill patients with thrombocytopenia. Am J Med. 2000;108:554–60.
8. Porcelijn L, Schmidt DE, Oldert G, Hofstede-van Egmond S, Kapur R, Zwaginga JJ, et al. Evolution and Utility of Antiplatelet Autoantibody Testing in Patients with Immune Thrombocytopenia. Transfus Med Rev. 2020;34:258–69.
9. Porcelijn L, Schmidt DE, Ellen van der Schoot C, Vidarsson G, de Haas M, Kapur R. Anti-glycoprotein Ibα autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients. Haematologica. 2020;105:e172.
10. Petito E, Colonna E, Falcinelli E, Mezzasoma AM, Cesari E, Giglio E, et al. Anti-severe acute respiratory syndrome coronavirus-2 adenoviral-vector vaccines trigger subclinical antiplatelet autoimmunity and increase of soluble platelet activation markers. Br J Haematol. 2022;198:257–66.
11. Kiefel V, Santoso S, Weisheit M, Mueller-Eckhardt C. Monoclonal antibody--specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood. 1987;70:1722–6.
12. Foxcroft Z, Campbell K, Merieux Y, Urbaniak S, Brierley M, Rigal D, et al. Report on the 13th international society of blood transfusion platelet immunology workshop. Vox Sang. 2007;93:300–5.
13. Folman CC, Von Dem Borne AEGK, Rensink IHJAM, Gerritsen W, Van Der Schoot CE, De Haas M, et al. Sensitive measurement of thrombopoietin by a monoclonal antibody based sandwich enzyme-linked immunosorbent assay. Thromb Haemost. 1997;78:1262–7.
14. Chen, R. T. Brighton C., & Black S. Updated Proposed Brighton Collaboaration process for developing a standard case definition for study of new clinical syndrome X, as applied to Thrombosis with Thrombocytopenia Syndrome (TTS). 2021; March:1–19. Available from https://brightoncollaboration.us/wp-content/uploads/2021/05/TTS-Interim-Case-Definition-v10.16.3-May-23-2021.pdf.
15. Pavord S, Scully M, Hunt BJ, Lester W, Bagot C, Craven B, et al. Clinical Features of Vaccine-Induced Immune Thrombocytopenia and Thrombosis. N Engl J Med. 2021;385:1680–9.
16. Bissola AL, Daka M, Arnold DM, Smith JW, Moore JC, Clare R, et al. The clinical and laboratory diagnosis of vaccine-induced immune thrombotic thrombocytopenia. Blood Adv. 2022;6:4228–35.

17. Nazy I, Sachs UJ, Arnold DM, McKenzie SE, Choi P, Althaus K, et al. Recommendations for the clinical and laboratory diagnosis of VITT against COVID-19: Communication from the ISTH SSC Subcommittee on Platelet Immunology. J Thromb Haemost. 2021;19:1585–8.
18. Emmons RVB, Reid DM, Cohen RL, Meng G, Young NS, Dunbar CE, et al. Human Thrombopoietin Levels Are High When Thrombocytopenia Is Due to Megakaryocytic Deficiency and Low When Due to Increased Platelet Destruction. Blood. 1996;87:4068–71.
19. Kuter DJ, Phil D, Gernsheimer TB. Thrombopoietin and Platelet Production in Chronic Immune Thrombocytopenia. 2009. https://doi.org/10.1016/j.hoc.2009.09.001.
20. Rogers P, Walker I, Yeung J, Khan A, Gangi A, Mobashwera B, et al. Thrombus Distribution in Vaccine-induced Immune Thrombotic Thrombocytopenia after ChAdOx1 nCov-19 Vaccination. Radiology. 2022;305:590–6.
21. Saudagar V, Patil S, Goh S, Pothiawala S. Vigilance regarding immune thrombocytopenic purpura after COVID-19 vaccine. Ir J Med Sci. 2022;191:919.
22. Shah SRA, Dolkar S, Mathew J, Vishnu P. COVID-19 vaccination associated severe immune thrombocytopenia. Exp Hematol Oncol. 2021;10:1–5.
23. Liao PW, Teng CLJ, Chou CW. Immune Thrombocytopenia Induced by the Chimpanzee Adenovirus-Vectored Vaccine against SARS-CoV-2 Infection. Vaccines. 2021;9.
24. Shrestha S, Nazy I, Smith JW, Kelton JG, Arnold DM. Platelet autoantibodies in the bone marrow of patients with immune thrombocytopenia. Blood Adv. 2020;4:2962.
25. Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest. 2005;115:3339.
26. Provan D, Arnold DM, Bussel JB, Chong BH, Cooper N, Gernsheimer T, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. Blood Adv. 2019;3:3780–817.
27. Kuter DJ. Exacerbation of immune thrombocytopenia following COVID-19 vaccination. Br J Haematol. 2021;195:365–70.
28. Lee EJ, Beltrami-Moreira M, Al-Samkari H, Cuker A, DiRaimo J, Gernsheimer T, et al. SARS-CoV-2 vaccination and ITP in patients with de novo or preexisting ITP. Blood. 2022;139:1564–74.
29. Kapur R, Kustiawan I, Vestrheim A, Koeleman CAM, Visser R, Einarsdottir HK, et al. A prominent lack of IgG1-Fc fucosylation of platelet alloantibodies in pregnancy. Blood. 2014;123:471–80.
30. Moulis G, Crickx E, Thomas L, Massy N, Mahévas M, Valnet-Rabier MB, et al. De novo and relapsed immune thrombocytopenia after COVID-19 vaccines: results of French safety monitoring. Blood. 2022;139:2561–5.
31. Mingot-Castellano ME, Butta N, Canaro M, Del Castillo Solano MDCG, Sánchez-González B, Jiménez-Bárcenas R, et al. COVID-19 Vaccines and Autoimmune Hematologic Disorders. Vaccines. 2022;10.
32. Lee EJ, Cines DB, Gernsheimer T, Kessler C, Michel M, Tarantino MD, et al. Thrombocytopenia following Pfizer and Moderna SARS-CoV-2 vaccination. Am J Hematol. 2021;96:534.
33. The ITP Support Association - Vaccinations and ITP. Cited 2023 Apr 26. Available from https://www.itpsupport.org.uk/index.php/en/vaccinations-and-itp.
34. Welsh KJ, Baumblatt J, Chege W, Goud R, Nair N. Thrombocytopenia including immune thrombocytopenia after receipt of mRNA COVID-19 vaccines reported to the Vaccine Adverse Event Reporting System (VAERS). Vaccine. 2021;39:3329.
35. Bhattacharjee S, Banerjee M. Immune Thrombocytopenia Secondary to COVID-19: a Systematic Review. Sn Compr Clin Med. 2020;2:2048.

36. Alharbi MG, Alanazi N, Yousef A, Alanazi N, Alotaibi B, Aljurf M, et al. COVID-19 associated with immune thrombocytopenia: a systematic review and meta-analysis. Expert Rev Hematol. 2022;15:1.
37. Schipperus MR, Amini SN, Schipperus LK, Nelson VS. ITP Richtlijn. 2020.
38. Koilpillai S, Dominguez B, Khan A, Carlan S. Severe case of refractory immune thrombocytopenic purpura requiring splenectomy after the COVID-19 vaccine. BMJ Case Rep. 2022;15:e250153.
39. Confirmed cases | Coronavirus Dashboard | Government.nl. Cited 2023 Feb 8. Available from https://coronadashboard.government.nl/landelijk/positief-geteste-mensen.

Supplemental data:

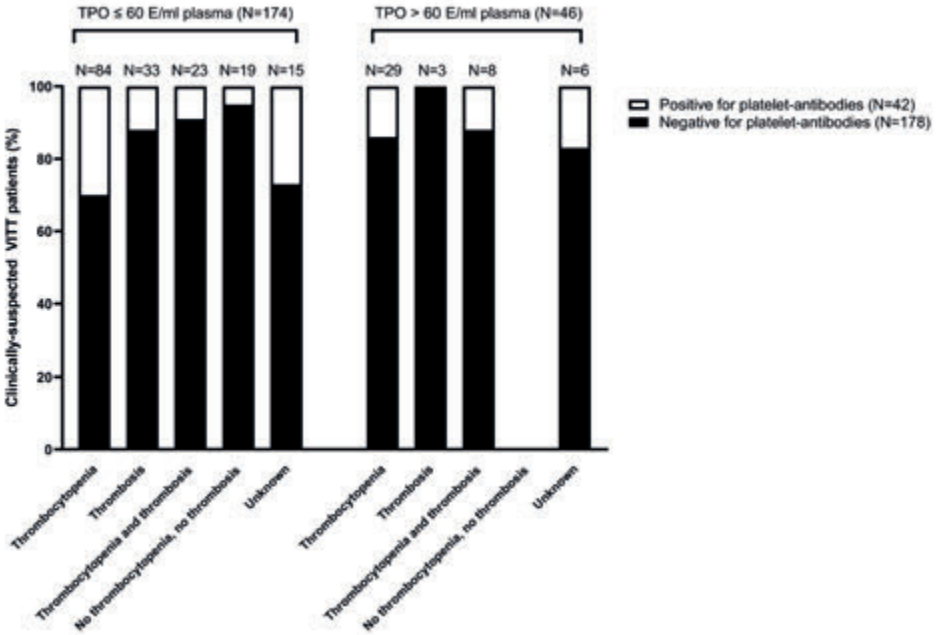


Figure S1: *Analysis of TPO levels of 42 platelet-antibody positive patients and of 178 platelet-antibody negative patients. Out of the 42 platelet-antibody positive patients 36 patients had normal TPO levels and six patients had elevated TPO levels. Of the patients that tested negative for platelet-antibodies: 138 patients had normal TPO levels and 40 patients had elevated TPO levels.*

Patient	Sex (M/F)	Age	Vaccine	Dose	Days since vaccination	anti-PF4 ELISA (OD)	FcγRIIa-dependent PIPAA(+/-)	Platelet count at admission	Site thrombosis	Indirect MAIPA (+/-)	TPO levels
1	M	67	ChAdOx1 nCov-19	-	12	>3.0	+	<100	CVST	-	28
2*	F	32	ChAdOx1 nCov-19	-	42	2.1	+	<100	Mesenteric thrombosis	-	24
3	F	75	ChAdOx1 nCov-19	-	-	>3.0	+	<100	DVT	-	67 (elevated)
4	M	64	ChAdOx1 nCov-19	1	9	>3.0	+	<100	Intracranial	-	115 (elevated)
5	M	61	ChAdOx1 nCov-19	-	-	>3.0	+	<100	Multiple	-	14
6	F	28	Ad26. COV2.S	1	11	2.24	+	<100	CVST	-	22
7	F	53	Ad26. COV2.S	1	-	>3.0	+	<100	Other	-	15

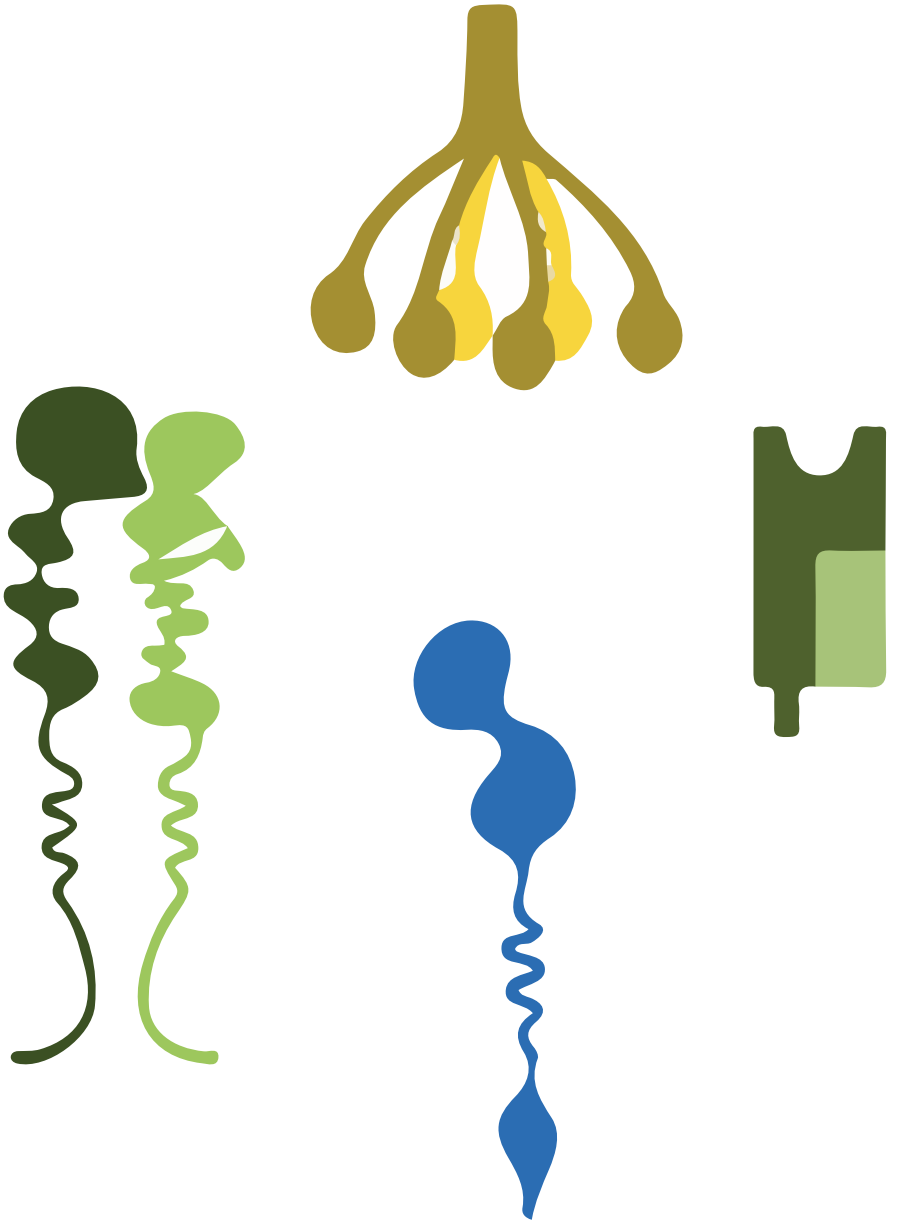
Supplemental table 1: *Details HIT-like VITT patients tested for platelet-antibodies. Patients who were considered HIT-like VITT patients (with thrombocytopenia and thrombosis) and were tested for the presence of platelet-antibodies in the indirect MAIPA. *Patient 2 was tested at 20 days after vaccination but was not considered probable VITT after a negative FcγRIIa-dependent PIPAA at 22 days after vaccination, the second FcγRIIa-dependent PIPAA at 42 days after vaccination was positive.*

Clinical characteristics	anti-PF4 IgG ELISA	FcγRIIa-dependent PIPAA (pos/neg)	Indirect MAIPA (pos/neg)
Thrombocytopenia only (n=119)	Neg (n=111)	Neg (n=108)	Neg (n=79)
			Pos (n=29)
		Pos (n=3)	Neg (n=1)
			Pos (n=2)
	Pos (n=8)	Neg (n=5)	Neg (n=5)
		Pos (n=3)†	Neg (n=3)
Thrombosis only (n=39)	Neg (n=38)	Neg (n=36)	Neg (n=33)
			Pos (n=3)
		Pos (n=2)	Neg (n=1)
			Pos (n=1)
	Pos (n=1)	Pos (n=1)†	Neg (n=1)
Thrombocytopenia and thrombosis (n=32)	Neg (n=22)	Neg (n=21)	Neg (n=20)
			Pos (n=1)
		Pos (n=1)	Pos (n=1)
	Pos (n=10)	Neg (n=3)	Neg (n=2)
			Pos (n=1)
		Pos (n=7)*	Neg (n=7)
Clinical characteristics unknown (n=23)	Neg (n=22)	Neg (n=22)	Neg (n=18)
			Pos (n=4)
			Pos (n=1)
No thrombocytopenia, no thrombosis (n=19)	Neg (n=19)	Neg (n=19)	Neg (n=18)
			Pos (n=1)

Supplemental table 2: *Results from anti-PF4 IgG ELISA, FcγRIIa-dependent PIPAA and indirect MAIPA. * considered HIT-like VITT patients. † tested positive in both the anti-PF4 IgG ELISA and PIPAA but did not have thrombocytopenia and thrombosis or clinical characteristics were unknown.*

Time period	Patients tested positive for platelet-autoantibodies of total number of tests performed N(%)
July-December 2019	132 (42.6)
January-June 2020	128 (46.9)
July – December 2020	166 (38.1)
January – June 2021	240 (44.6)
July-December 2021	228 (42.0)
January -June 2022	273 (48.3)

Supplemental table 3: *Requests for ITP diagnostic reference testing. Increase in platelet-autoantibody testing and positive tests after implementation of the ITP guidelines in June 2020 and a further increase in testing and positive tests since the start of the vaccination on January 8, 2021.*



10

CHAPTER 10

General discussion

In this thesis development of laboratory tests aiming to support the diagnosis of immune thrombocytopenia (ITP) is described. It has appeared to be more complex to design tests that have high accuracy for platelet bound or circulating autoantibodies against platelet antigens. This is largely caused by the fact that in addition to specific antibodies, non-specific antibodies also bind, causing false positive reactions while platelet autoantibodies on the other hand have a low affinity and can therefore be released during the required platelet washing steps in the various tests, which can lead to false negative results.1 However, since 2021 platelet autoantibody detection in samples from patients suspected for ITP is recognized as a 'rule-in' test for ITP.2 Also our results, regarding the optimization of a glycoprotein specific platelet autoantibody detection method in which we showed that sensitivity, but especially specificity could be largely increased, were used to come to this consensus. Several factors can influence the pathogenic response of platelet-antibodies, such as antibody titer, the relative affinity of the antibody isotype to FcγRs, the expression level and polymorphisms of FcγRs which can modulate the antibody binding affinity in their turn, infections directing antibody cross-reactivity by molecular mimicry, C-reactive protein levels for enhancing antibody-mediated platelet phagocytosis, and inhibitory immune receptors.3–5 A current research question is if autoantibody specificity in ITP dictates different mechanisms of platelet clearance and destruction. Not only platelet autoantibody levels and specificity, but also plasma thrombopoietin (TPO) levels can be used to guide the diagnosis of underlying causes for thrombocytopenia, e.g. ITP, in which TPO levels are relatively normal/non-elevated.6 Therefore, we set out a series of studies to evaluate the value of both the antibody and TPO tests in supporting the diagnosis of ITP, with possibly with it also take a step towards a more individualized treatment approach.

Plasma TPO level measurements: methods, results,
The search for a method for measuring platelet production by plasma TPO levels is described in our thesis. Instead and already for decades reticulocytes are used as marker to evaluate compensated loss of red blood cells. The possibility to distinguish young platelets, named reticulated platelets (RP) referring to reticulocytes for red blood cells, from mature platelets was already described by Ingram and Coopersmith in 1969.7 They showed that, up to approximately 36 hours after being released from the bone marrow, platelets are larger and contain more ribonucleic acid (RNA). In the past decades, the diagnostic value of the absolute RP count or percentage of the total platelet number (immature platelet fraction, IPF) has been extensively investigated. Nowadays RP can be measured by automated hematology analyzers, which initiated comparison of results generated for patients suffering from ITP and bone marrow failure.8,9 Unfortunately, outcomes are still difficult to interpret. Although results from quality assurance exercises support the interpretation and standardization of

RP measurements in automated analyzers, for improving the sensitivity and specificity of IPF for uncomplicated ITP and bone marrow failure patients, overlapping results and differences between analyzers are observed.

An alternative indirect approach to see whether the production of platelets in the bone marrow is sufficient or suppressed arose after the discovery of TPO in 1994 10,11 followed by the development of a sensitive ELISA for measuring free circulating TPO levels, by Folman et al (1997) .12 TPO, mainly produced in the liver, binds to c-mpl on platelets and platelet-precursor cells. If sufficient platelets/precursor cells are produced, the free plasma TPO level will not increase in contrast to thrombocytopenia due to reduced production, which increases the TPO level. After an initial study already in 1998 6, in which we confirmed this correlation, we started to routinely measure free circulating plasma TPO levels in thrombocytopenic patients. Now, two and a half decades later, measuring TPO has proven to be a valuable diagnostic assay, not only to indicate a decreased platelet production if TPO levels are increased, but also to support the clinical diagnosis ITP, where the TPO values are normal to only slightly elevated (via incompletely understood mechanisms). By investigating cohorts of healthy controls (n=95) and serologically and clinically well-defined thrombocytopenic patients (n=178), we were able to calculate the best plasma TPO cut-off levels. Normal TPO levels are 11 ± 8 (mean ± Std, range 4 -32 AU/ml, one arbitrary unit equals 9 pg recombinant TPO (Research Diagnostics Flanders, NJ, U.S.A.)).6

In neonates, the TPO plasma level is, for some not yet fully understood reasons, higher compared to levels found in adults, i.e. 27 ± 24 (range 2-93 AU/ml). For patients suffering from thrombocytopenia as a result of increased platelet destruction (e.g. immune mediated), free plasma TPO levels are normal or only slightly increased (< 60 AU/ml and for neonates < 128 AU/ml). Using these cut-off values, the measurement of free plasma TPO levels provides clinicians with an additional tool to decide on the differential diagnosis. 13,14

Table 1: Plasma TPO values in adult and neonatal thrombocytopenia patients

	plasma TPO level (A.U./ml)	range (A.U./ml)
Adults		
healthy controls	11	4-32
ITP	13	2-54
decreased hematopoiesis	> 60	
Neonates		
healthy controls	27	2-93
FNAIT/maternal ITP/ bacterial infection	< 128	a
amegakaryocytosis/severe asphyxia/congenital viral infections	> 128	b

a ruling out b with NPV 96% (95% CI 90-99)

However, a distinction between an increased platelet destruction and a decreased platelet production by measuring TPO levels can only be made if the platelet count, and subsequently the 'TPO-sink', is significantly decreased (we advise clinicians only to request TPO measurements if the patient platelet count is sufficiently decreased i.e. < 80 x 109/L). In ITP, the production of platelets is normal or only slightly decreased and TPO can still bind to c-mpl on platelets before the antibody binding causes platelet destruction by macrophage-mediated phagocytosis. This is because we associate normal or only slightly increased TPO levels in ITP patients with increased platelet destruction. Furthermore, the cut-off level of 60 AU/ml for ITP does not apply to young children. A somewhat elevated TPO level, up to 200 A.U., can be seen in young children (i.e. children under 10 years old) with acute ITP, decreasing with age until approximately five years of age, as we show in the pre-print paper by Schmidt et al. (2020) 'Age at diagnosis shapes the prognosis of Childhood immune thrombocytopenia'.15 In young children, in contrast to autoantibodies of the IgG-class in adults, mainly autoantibodies of the IgM class are detected.15–17 It is tempting to hypothesize that autoantibodies of the IgM-class with additional complement activation might cause intravascular platelet destruction and a release of internalized TPO. This could be an explanation for the increased TPO levels in acute ITP. Unfortunately, in contrast to assays designed in autoimmune hemolytic anemia to investigate the capacity of autoantibodies for complement activation and red blood cell destruction, such assays are lacking to study if platelet autoantibodies (of the

IgG and/or IgM class) can induce complement activation and subsequently lyse platelets in the pathophysiology in ITP. For most ITP patients however, the free circulating TPO levels can provide valuable information and it may be that, after further optimization of RP assays, measurement of both RP and TPO will provide even more accurate information on either increased platelet production or insufficient platelet production.

Optimization of autoantibody detection

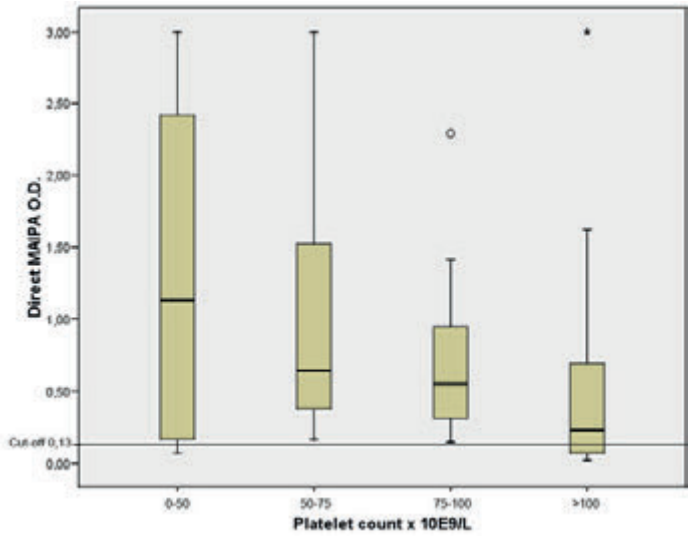
As described in this thesis, we made three observations that underlie major improvement of platelet autoantibody detection.18 First we redesigned the glycoprotein-specific monoclonal antibody immobilization of platelet antigens (MAIPA) assay for platelet autoantibody detection by validating the optimal threshold for identification of positive signals, showing that for platelet autoantibodies the detection can be set much lower than was used previously for platelet alloantibody detection. With a lowered cut-off O.D. level from 0.300 (which is used for platelet alloantibody detection) to 0.130, being the healthy controls mean + 3SD the sensitivity of the MAIPA to detect autoantibodies was improved, also in comparison with earlier published international studies in which often cut-off levels of 0.200 or even 0.500 were used. In a series of 754 routine request samples with at least one of the GPs IIb/IIIa, Ib/IX, or V reacting positively in direct MAIPA, the highest OD was only between 0.130 and 0.200 for 273 (36%) samples (unpublished data).

Second, although it was generally assumed that platelet autoantibodies are mainly directed against GPIIb/IIIa and GPIb/IX and therefore in most studies research was limited to those two GPs. We showed the importance of including the detection of GPV-associated antibodies.19–21 Although the presence of a significant number of patients with detectable GPV-associated platelet autoantibodies in ITP patients was already described in 1997 by Joutsi et al.21, GPV-autoantibody detection was not routinely implemented in platelet autoantibody detection. After optimization of the direct MAIPA, we detected platelet-associated autoantibodies in 51 of 60 (85%) well-categorized untreated ITP patients, of which 31 (61%) reacted positive with GPV.[7] The major role for GPV-associated autoantibodies in the pathogenesis of ITP was later confirmed by Vollenberg et al (2019).20

Third, the implementation of the new definition of ITP in 200922, with lowering of the platelet count threshold to below 100 x 109/L instead of 150 x 109/L. This of course changed the composition of cohort of patients tested for platelet autoantibodies by omitting patients with only moderately lowered platelet counts caused by other reasons than ITP. Indeed like in pregnancy, there is a physiological drop in platelet counts which relatively often results in platelet counts between 100 and 150 x 109/L. Furthermore, as can be expected, several studies have shown that the detection of autoantibodies is inversely related to

the number of platelets.23,24 We also showed this inverse correlation in 170 known ITP patients (Figure 1, data not published).

Figure 1: correlation platelet count and mean autoantibody level



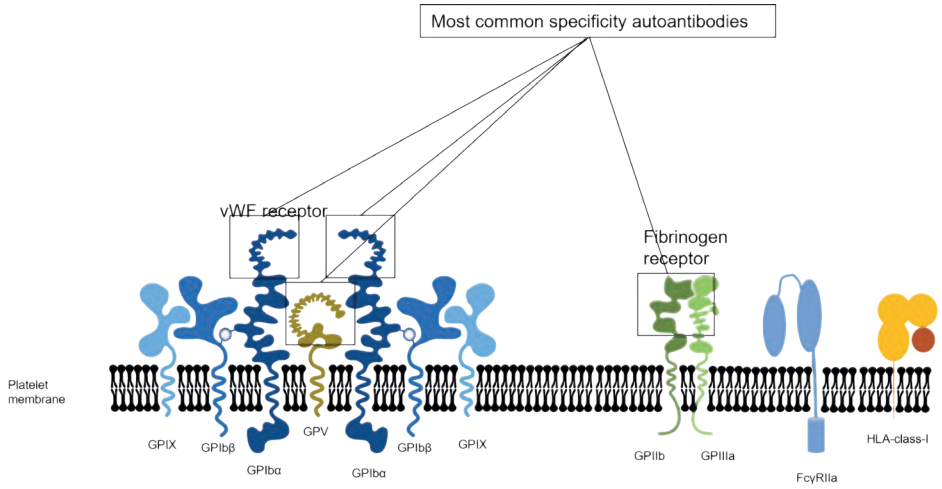
The mean direct (i.e. detecting autoantibodies bound on the patient platelets) monoclonal antibody immobilization of platelet antigens (MAIPA) results (optical densitiy) used for this figure are the highest O.D. values detected for either GPIIb/IIIa, GPIb/IX or GPV.

By using the modified MAIPA assay with a lower cut-off value for determining positive test results and testing the relevant platelet GPs for bound autoantibodies, the sensitivity of direct MAIPA based platelet autoantibody detection could be increased from 60% to a level of approximately 75-80%. Although this sensitivity is still not optimal, a major advantage is the high test specificity of >95%, making the assay suitable for 'ruling-in' of the diagnosis of ITP.18,19

The glycoprotein specific autoantibody detection method is now included in the Dutch ITP guideline2 and with it, Sanquin Diagnostic Services, receive approximately 1500 requests per year. For each request, we investigate, using the direct and indirect MAIPA, the presence of GPIIb/IIIa, GPIb/IX and GPV specific autoantibodies of the IgG-class on the platelets and in the serum of the patient. If sufficient patient platelets can be isolated, we also perform the direct and indirect platelet immunofluorescence test (PIFT) for the detection of autoantibodies of the IgG- and IgM-class. In addition, a plasma TPO level is measured for each application. After this, we reflect on the outcome of the test results, the eventual diagnosis made in the patients, and finally the usefulness of this diagnostic test panel.

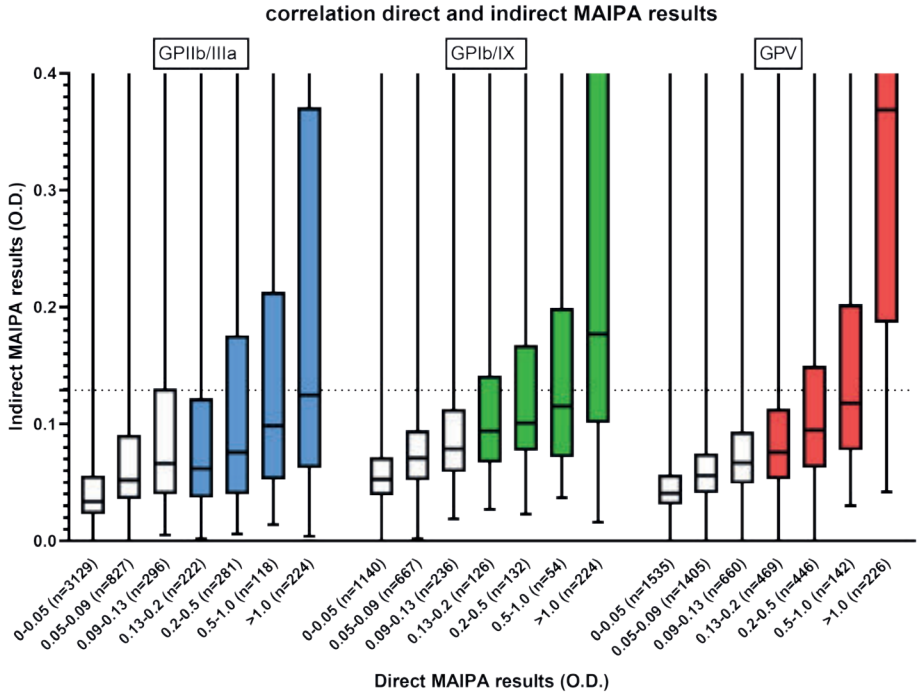
Unfortunate, but intriguing, is the fact that no antibodies can be detected in approximately 20% of patients with strongly suspected ITP. Partly, this can be explained by limitations of the tests to distinguish weak binding autoantibodies from background 'noise'.18 As shown in chapter 8, comparing the results of the detection of free circulating and platelet-associated autoantibodies with indirect MAIPA and direct MAIPA respectively show a strong correlation, even for results that encorporate the low cut-off level (O.D. = 0.130) that we use.18 This indicates that the cut-off value, calculated by the mean plus three times the standard deviation of a large group of healthy controls, is probably still too high. So far, our efforts to improve the signal to noise ratios have not yielded any useful results. Mapping the association between the direct and indirect MAIPA revealed another unexpected finding, i.e. that for GPIIb/IIIa and GPV the correlation from low to high O.D. values remained present, but not for GPIb/IX. A deviation in indirect MAIPA reactivity was seen for the high O.D. direct MAIPA values (Chapter 8). Further investigation showed the monoclonal antibody MB45, which is binding to the ligand binding domain (LBD) of GPIbα to be responsible for this problem. It is known that the LBD is one of the preferred locations for autoantibody binding (Figure 2) and most likely MB45 blocks or displaces a significant percentage of GPIb/IX specific autoantibodies. After replacing MB45 for the GPIX specific FMC25, this problem was solved and a strong correlation between the direct and indirect MAIPA results, like for the other GP, was seen (Figure 3).

Figure 2: Preferred locations for platelet autoantibody binding



Most common autoantibody binding sites are the terminal part (i.e. the ligand binding domain (LBD) of GPIbα, GPV and the terminal part of GPIIb/IIIa (mainly GPIIb).18

Table 1: Plasma TPO values in adult and neonatal thrombocytopenia patients



The indirect MAIPA results (y-axis) compared to the direct MAIPA results categorized by reaction strength (X-axis) from negative (O.D.< 0.130, white boxes) to strongly positive. A strong correlation (p<0.001) between the direct and indirect MAIPA results is seen, even for results below the cut-off O.D. level of 0.130 indicating that an even lower cut-off value might be possible if the signal to noise ratio can be increased.
Results shown are after replacing GPIb/IX specific moab MB45 with FMC25. The figure showing deviating results with MB45 can be found in chapter 8.

Another possibility for the 20% unexpected negative results is that in a group of ITP patients cytotoxic CD8+ T cells cause platelet-precursor damage and/or destruction of platelets.25 Evidence for such exclusive mechanism (i.e. not complementary with autoantibody mediated destruction) was limited until recent and summarized in a paper by Vrbensky et al. (2021).26 However, more recently, Malik et al. (2023) were able to identify disease-associated T cell clones and managed culturing isolated CD8+ T cells from ITP patients, indicating an important role for CD8+ T cell mediated platelet activation and apoptosis.27 Further research for investigating the role of T cell mediated platelet destruction and possibilities for diagnostic assays to detect such mechanisms, is necessary.

TPO and autoantibody measurements: clinical indication and implications for therapies.

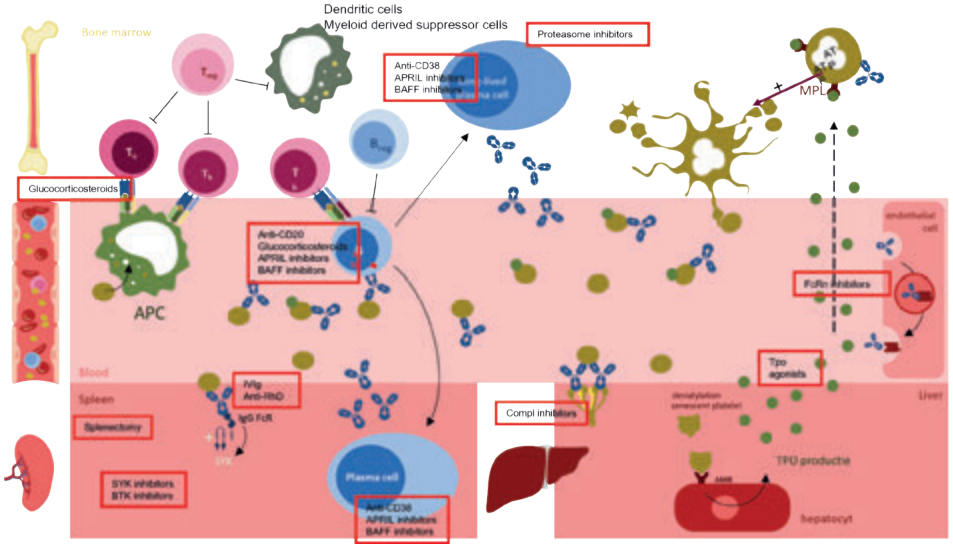
In recent decades, new therapeutic drugs, such as TPO-RA, anti-CD20 , SYK-inhibitors, FcRn-inhibitors, CTLA4-Ig fusion proteins and very recent BAFF and APRIL inhibitors (Table..) became available or are currently being investigated in clinical trials. To increase comparability between these studies, in 2009 an international working group of expert clinicians defined standard terminology and definitions for primary ITP and its different phases and criteria for the grading of severity, and clinically meaningful outcomes and response. With this redefinition, among other things the name 'Idiopathic Thrombocytopenic Purpura (ITP)' was changed to 'Immune ThrombocytoPenia (ITP)' to emphasize the underlying immune disorder, which can frequently occur without purpura.

Table 2: ITP treatment, presumed working mechanisms and effect on autoantibody levels

Drug	Presumed working mechanism	Effect on autoantibody level
Corticosteroids	Inhibits a broad range of immune responses	Strong decrease within days[28]
Splenectomy	Removal of platelet destruction and part of antibody production site	Varying effect[28,29]
IVIg	Blocking FcγR inhibiting platelet phagocytosis	Not likely*
Anti-RhD	Blocking FcγR inhibiting platelet phagocytosis	Not likely*
Anti-CD20	Destruction of B lymphocytes and possibly decreased splenic CD8+ T cell proliferation	Strong decrease within days in percentage of patients[30–32]
Anti-CD38	Destruction of plasma cells	Expected decrease*
TPO-RA	Stimulates platelet production and possible immunomodulation	First results show some decline
SYK-inhibitor	Inhibits platelet phagocytosis	Not likely*
FcRn-inhibitors	Inhibits re-entering of IgG from endothelial cells into the circulation	First results show some decline
Complement inhibitors	Inhibits complement activation pathway	Not likely*
BAFF inhibitors	Inhibits B cell maturation, proliferation and survival	Expected decrease*[31,33]
APRIL inhibitors	Inhibits B cell maturation, proliferation and survival and plasma cell survival	Expected decrease*[29–31,33,34]
Proteasome inhibitors	Destruction of plasma cells	Expected decrease*[35]
Bruton tyrosin kinase inhibitors	Inhibits platelet phagocytosis	Not likely*

** studies are still to be conducted*

Figure 4: Overview of ITP treatment options with points of intervention for the various drugs



To study the mechanisms of action of the new drugs and to monitor autoantibody levels during clinical trials, there was a renewed interest in autoantibody detection methods. The glycoprotein-specific MAIPA assay we have optimized is therefore increasingly used here. Notwithstanding the effect of treatment on autoantibody levels has only been studied for a few drugs (Table 2).

Glucocorticoids, that may have inhibitory effects on a broad range of immune responses including interfering with the binding of interleukins to B cells, keep B cells from proliferating and subsequently prevent antibody production. A strong and rapid decrease of platelet autoantibody levels is a result of prednisone and dexamethasone and was already shown by the group of Robert McMillan in the 1990s. 28

In a multi-center randomized open label phase II trial comparing three rituximab dosing schemes, with138 ITP patients included 36, we noticed three different autoantibody patterns in direct PIFT and direct MAIPA in serial patient samples (chapter 6). First, detectable autoantibodies (partly) disappeared within several days. This pattern was seen for all 16 tested ITP patients with complete remission. However, for 32 of 39 non-responsive patients either autoantibodies were detectable before and remained detectable despite rituximab treatment or no autoantibodies were detectable before and during rituximab treatment.37 Interesting in this respect is our finding that a significant higher percentage of ITP patients, not responding to rituximab, lack detectable platelet autoantibodies (i.e. 16 of 52 (31%) versus 4 of 47 (9%)). Our results show a strong inversed

correlation between autoantibody levels and platelet counts in rituximab-responding patients, next to a group non-responsive patients, which can be explained by insufficient eradication of antibodies producing plasma cells or/ and a different underlying, possibly 'non-autoantibody', possibly cytotoxic T cell mediated platelet destruction (chapter 6).37

Our observations were not in line with a study by Arnold et al. (2017).38 They investigated 55 rituximab treated ITP patients, 25 (45%) with detectable platelet associated autoantibodies at baseline, for whom no correlation between non-responsiveness and lack of detectable antibodies at baseline was seen. Remarkable is their finding that response to rituximab was correlated with a decline in GPIIb/IIIa specific autoantibodies, but not with a decline in GPIb/IX autoantibodies.38 A significant (p=0.007) higher response rate to rituximab was observed by Feng et al. (2017) for ITP patients (n=86) with detectable GPIIb/IIIa specific autoantibodies (n=36) at baseline.39 They also showed a somewhat better, however not significant (p=0.052), response for patients with GPIb/IX specific autoantibodies (n=31).

Unfortunately, comparison between these study results is difficult because different antibody detection methods were used (e.g. different cut-off values) and GPV was not included in all studies.

More recent it was shown, as we indeed suspected from our serial autoantibody level results, that the presence of autoreactive long-lived plasma cells (LLPCs) in the bone marrow and spleen may be due to non-responsiveness to rituximab and splenectomy induced B-cell depletion and possible reactivation of memory B cells generating new autoreactive plasma cells. Clinical trials with drugs like anti-CD38, BAFF- and APRIL-inhibitors will provide an opportunity to see how the autoantibody titers will correlate with deep B cell and plasma cell depletion.29–31,33,34

Thrombopoietin agonists (TPO-RA) increase platelet production by stimulating thrombopoiesis from stem cell to megakaryocyte and have been used for some time as a second-line treatment for ITP. At this moment, data of the STIP (Stop TPO-RA in ITP patients) study, in which the effect of discontinuing TPO-RA on remission is investigated, are being processed. Part of the study is serial testing of platelet autoantibodies with our direct MAIPA. First results show a decrease in platelet autoantibody levels as a result of TPO-RA treatment, indeed indicating a possible immune modulating effect of TPO-RA, in line with a previous study in which a clinically-relevant murine model of active ITP was used.40

Approximately 20% of ITP patients show a sustained treatment free response rate after discontinuation of TPO-RA treatment.41–47 This is not unique as other treatments, like corticosteroids, rituximab and IVIg, are also known to have a 20-30% treatment free response rate. Somehow, an (partial) immunological reset seems to occur in a percentage of patients after treatment. Whether this 'successful reset' occurs in the same patients after treatment with different

medications, is unknown. The underlying mechanism causing this sustained remission is also not clear. Studies show a possible role of the restauration of the regulatory (reg) T cell imbalance resulting in an increase in transforming-growth-factor-β1 (TGF-β1), an increase in reg B cells and/or an increase of Fcγ receptor (FcγR) IIb with a decrease in FcγRI and IIa levels resulting in a decrease in platelet phagocytosis.48,49

The spleen, being a major site for antibody production and platelet destruction via Fc-RcγR binding on macrophages, plays an important role in ITP.49 Splenectomy, with a 60% long term remission, is shown to decrease platelet glycoprotein-reactive T and B cells and accomplishes a (transient) decrease in anti-platelet autoantibodies.29,50

Evidence that for a percentage of patients the removal of the platelet breakdown site is decisive, is shown by two patients that we have followed in our laboratory. One woman with severe ITP, where the platelet count normalized after splenectomy, but where we (even years later) demonstrated a high level of autoantibodies of IgG1 class and another woman with strong GPIIb/IIIa specific autoantibodies who showed a normalization of the platelet number after splenectomy, but continued to have a bleeding tendency, because of high levels of GPIIb/IIIa function (fibrinogen binding) blocking antibodies (acquired Glanzmann phenotype).

The effects of the more recent developed drugs on platelet autoantibody levels are still to be investigated.

Autoantibodies influencing production, platelet function and different clearance and destruction pathways

It is now known that the physiologic clearance of aging platelets takes place in the liver by binding of desialylated glycoprotein Ibα to the Aswell-Morrell receptor (AMR), and that AMR binding also leads to an increase in thrombopoietin production.51–56 This, together with the varying observations (both affirmative and negative) that ITP patients with GPIbα specific antibodies respond less well to IVIg treatment, has led to several hypotheses and studies about underlying mechanisms of action.57–63 Among other things, it was described that GPIbα specific autoantibodies can cause desialylation of GPIbα, which makes possible the so-called Fc-independent AMR degradation pathway in addition to the antibody-mediated platelet degradation via macrophages.63 As a result, the production of TPO would be stimulated. However, as shown by Marini et al. desialylation of GPIbα can also be caused by anti-GPIIb/IIIa autoantibodies but TPO production was not measured in this study.62 It has also been postulated that (in mice) on the contrary, anti-GPIbα platelet autoantibodies block the GPIbα binding to the AMR and therefore prevent the increase in TPO production.59 In our routine ITP diagnostic workflow, we investigate the presence of anti-GPIb/IX antibodies, together with anti-GPIIb/IIIa and anti-GPV antibodies. To see if anti-GPIb/IX

antibodies alone or in combination with other GP specific antibodies influence TPO levels, we retrospectively looked at the correlation between plasma TPO levels and autoantibody specificity in 3940 suspected ITP patients. We did not find this correlation (see chapter 7).64 However, in this retrospective study, patient platelet counts were not available. Hence, although ITP is defined as platelet count < 100 x 109/L and therefore most referred suspected ITP patients were thrombocytopenic, we could not classify the group by platelet counts and therefore we decided to further investigate this in a prospective study65. In a cohort of 53 ITP patients, a positive association was observed between GPIb/IX-antibodies and slightly increased TPO levels, but only in patients with severe thrombocytopenia (β 0.092 [95%CI 0.012-0.172], p=0.03), and not in patients with moderate or mild thrombocytopenia. This may indicate that at least most GPIbα-binding autoantibodies (possibly depending on the exact binding site) do not block TPO production, but rather increase TPO production under severe thrombocytopenic conditions due to GP desialylation causing platelet destruction via the Fc -independent degradation pathway via AMR.

To further investigate the influence of the glycoprotein specificity of the autoantibodies and the possible related AMR degradation pathway, we investigated the specificity of platelet autoantibodies in 74 ITP patients, using Indium-111 labeled autologous platelet scans, to see if GPIIb/IIIa, GPIb/IX or GPV specificity is correlated with splenic or hepatic platelet clearance.66 Indeed the presence of GPIb/IX autoantibodies was more pronounced in patients with a hepatic sequestration pattern, however this association was not significant. Surprisingly, we detected a significant association between the presence of GPV specific autoantibodies and splenic platelet sequestration.66 Further investigations are necessary to find out what the underlying mechanism for this association is.

Being appointed as central laboratory for performing vaccine induced thrombotic thrombocytopenia (VITT) diagnostics67, we were able to investigate the presence and glycoprotein-specificity of platelet autoantibodies for a large cohort of individuals in whom thrombocytopenia occurred within 30 days after Covid-19 vaccination (chapter 9).68 The indirect (free circulating) platelet autoantibody detection showed positive results for 44 of 231 patients. The presence of platelet autoantibodies could not be associated with the diagnosis of VITT, but showed a deviation in glycoprotein-specificity compared to a large cohort of pre-covid-19 vaccination ITP suspected patients. In the VITT suspected (vaccine-related) cohort we detected less multi-glycoprotein specific autoantibodies and significantly more directed only against GPIb/IX, i.e. 41% compared to 25%. We can now only speculate about the reasons for this shift. Possibly this could be caused by molecular mimicry. However, ITP after vaccination is very rare and the only vaccine for which there is a demonstrable cause-effect relationship is the measles, mumps and rubella (MMR) vaccine69–74. Indeed there may

be human epitopes showing molecular mimicry with SARS-CoV-2 spikes and, although up to now these epitopes are not detected for GPIb/IX, it is suggested that immunogenic epitopes in SARS-COV-2 vaccines may trigger autoimmune diseases in predisposed individuals.75 Further investigation in the presence, course and glycoprotein specificity of platelet autoantibodies in post-vaccination thrombocytopenia, which with the more sensitive MAIPA assay has now become feasible, is necessary to gain more insight in the underlying mechanism.

General comments and future developments

This thesis reflects our search for optimization of diagnostics to provide treating physicians with better methods for recognizing the cause of thrombocytopenia, to better map the effect of new medications and to gain more insight into the immunological abnormalities leading to ITP.

Routinely we perform plasma TPO measurements to differentiate thrombocytopenia due to decreased platelet production or increased platelet destruction. By all hence generated data, we were able to calculate normal values for both children and adults the various causes of thrombocytopenia. By measuring plasma TPO levels, which only requires 50 µl blood, we can provide the treating physician with an indication of the underlying cause of thrombocytopenia.

Our optimized MAIPA detection of platelet autoantibodies is now a recognize diagnostic tool, included in the Dutch ITP directives as strong support for the diagnosis of ITP diagnosis while without platelet autoantibodies ITP still needs exclusion of other causes which is known to cause an initial misdiagnosis of approximately 10% .1,2 Also due to the more sensitive and extremely specific results, we are now using the direct and indirect MAIPA for autoantibody detection in clinical trials to test new ITP medication and for research objectives.

In our long history as a reference laboratory for platelet serology these developments can be seen as logical steps, building on a history starting already in the 50's of the last century. In the last 70 years, our laboratory, among other things, discovered the first HPA alloantibodies against the antigen defined as Zw(a), later named HPA-1a) by prof van Loghem in 1959.76 In the 1970's,prof Albert von dem Borne developed more / additional ? assays for the detection of platelet-specific allo- and autoantibodies using the PIFT.77 Moreover, detection of these antibodies eventually showed to give additional insights in the increased clearance and destruction of platelets, and the sometimes observed compromised production and function of the remaining platelets. Finally, the detection of antibodies and the intriguingly normal TPO levels in ITP are starting to influence therapy choices and targeted therapy development.

However, many questions are still to be answered.

Although measuring plasma TPO levels is useful, it is still only an indirect measure

to detect a decrease in platelet production. As written above, the value of measuring immature platelet fractions (IPF), analogue to the reticulocyte count for RBCs, is improving, but not yet optimal. Combining TPO and IPF may possibly provide further improvement .
Next, to increase the (now only 80%) sensitivity for the detection of platelet autoantibodies would be of great value. Although we have to realize that the maximum sensitivity might not be 100% because of patents with (only) T-cell dependent ITP. Despite various improvement attempts, it has not yet been possible to improve the signal to noise ratio in our antibody assays.
In this context, it is also interesting to see how antibody and TPO levels are influenced by deep depletion of B cells and plasma cells in ITP patients with BAFF- or APRIL-inhibitors or by specific B-cell targeting CAR-T cells. As said, it might well be that a significant percentage of ITP is not caused by autoantibodies and for those patients we need to expand diagnostics to detect the putative role of cytotoxic T cells and how it is influenced by more or less successful response to therapy. Similarly, we need better detection of the potential presence of complement activation mediated platelet destruction. With these eventually more reliable methods for the detection of autoantibody (IgG and IgM) and complement-mediated platelet destruction, it would be worthwhile to study in a large cohort a work-up for ITP analogue to the work-up for autoimmune hemolytic anemia, i.e. measuring reticulocytes and performing a direct antiglobulin test (DAT).

And in this cohort, we could additionally look how the balance between platelet autoantibodies, complement activation and glycoprotein desialylation is reflected in the platelet destruction percentages in spleen and/or liver.
Indeed these additional diagnostics are also needed to account for unexpected results of our improved and more used antibody assays. For example, in a group of ITP patients who were highly refractory to routine treatment, we see only a low percentage of autoantibodies. The question is whether this is due to previous therapy or whether the refractoriness may be because this is an ITP subgroup with a different underlying pathology. Another example is that surprisingly we detected autoantibodies in a significant percentage of patients with active systemic lupus erythematosis (SLE), but without thrombocytopenia.78 SLE is known as a condition in which secondary ITP can occur. This is one of the first studies in which autoantibody detection was performed in the serum of patients with another autoimmune disorder, but without thrombocytopenia. Further research will have to reveal what the presence of the autoantibodies means in these patients.

Although this thesis concerns a relatively small field, some of its findings can be extended to other autoimmune conditions, such as autoimmune hemolytic

anemia, granulocytopenia or even to conditions such as rheumatoid arthritis or SLE. Mapping different groups of patients with apparently the same condition will hopefully lead to insight into differences through such research and thus to an understanding of the optimal therapy for the individual. From the gained insights into immunology and the optimization of diagnostic options so far, we can conclude that the discussed aims for the future in this respect can only be attained by the increasing synergy between clinic and laboratory.

References

1. Arnold DM, Nazy I, Clare R, et al. Misdiagnosis of primary immune thrombocytopenia and frequency of bleeding: lessons from the McMaster ITP Registry. Blood Adv. 2017;1(25):2414-2420. doi:10.1182/bloodadvances.2017010942
2. Schipperus M, Nelson V, Amini S. Immuuntrombocytopenie (ITP): hoofdpunten uit de richtlijn van 2020 met aanbevelingen voor diagnostiek en behandeling. Nederlands tijdschrift voor Hematologie. 2021;18:20-28.
3. Kapur R, Kim M, Shanmugabhavananthan S, Liu J, Li Y, Semple JW. C-reactive protein enhances murine antibody–mediated transfusion-related acute lung injury. Blood. 2015;126(25):2747-2751. doi:10.1182/blood-2015-09-672592
4. Kapur R, Einarsdottir HK, Vidarsson G. IgG-effector functions: "The Good, The Bad and The Ugly." Immunol Lett. 2014;160(2):139-144. doi:10.1016/j.imlet.2014.01.015
5. Cines DB. Pathogenesis of refractory ITP : Overview. Br J Haematol. 2023;203(1):10-16. doi:10.1111/bjh.19083
6. Porcelijn L, Folman CC, Bossers B, et al. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. Thromb Haemost. 1998;79(6). doi:10.1055/s-0037-1615023
7. Ingram M, Coopersmith A. Reticulated Platelets following Acute Blood Loss. Br J Haematol. 1969;17(3):225-229. doi:10.1111/j.1365-2141.1969.tb01366.x
8. Schmoeller D, Picarelli MM, Paz Munhoz T, Poli de Figueiredo CE, Staub HL. Mean Platelet Volume and Immature Platelet Fraction in Autoimmune Disorders. Front Med (Lausanne). 2017;4. doi:10.3389/fmed.2017.00146
9. Buttarello M, Mezzapelle G, Freguglia F, Plebani M. Reticulated platelets and immature platelet fraction: Clinical applications and method limitations. Int J Lab Hematol. 2020;42(4):363-370. doi:10.1111/ijlh.13177
10. Lok S, Kaushansky K, Holly RD, et al. Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature. 1994;369(6481):565-568. doi:10.1038/369565a0
11. Wendling F, Maraskovsky E, Debili N, et al. cMpl ligand is a humoral regulator of megakaryocytopoiesis. Nature. 1994;369(6481):571-574. doi:10.1038/369571a0
12. Folman CC, von dem Borne AE, Rensink IH, et al. Sensitive measurement of thrombopoietin by a monoclonal antibody based sandwich enzyme-linked immunosorbent assay. Thromb Haemost. 1997;78(4):1262-1267. https://www.ncbi.nlm.nih.gov/pubmed/9364995
13. Porcelijn L, Folman CC, De Haas M, et al. Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia. Pediatr Res. 2002;52(1). doi:10.1203/00006450-200207000-00019
14. Porcelijn L, Huiskes E, Onderwater-Van Den Hoogen L, Folman CC, Zwaginga JJ, De Haas M. Plasma thrombopoietin levels as additional tool in clinical management of thrombocytopenic neonates. Platelets. 2020;31(1). doi:10.1080/09537104.2019.1572877
15. Schmidt DE, Edslev PW, Heitink-Pollé KMJ, et al. Age at diagnosis shapes the prognosis of childhood immune thrombocytopenia. medRxiv. Published online 2020. doi:10.1101/2020.06.09.20125385
16. Schmidt DE, Lakerveld AJ, Heitink-Pollé KMJ, et al. Anti-platelet antibody immunoassays in childhood immune thrombocytopenia: a systematic review. Vox Sang. 2020;115(4). doi:10.1111/vox.12894
17. Schmidt DE, Heitink-Polle KMJ, Porcelijn L, et al. Anti-platelet antibodies in childhood immune thrombocytopenia: Prevalence and prognostic implications. Journal of Thrombosis and Haemostasis. 2020;18(5). doi:10.1111/jth.14762

18. Porcelijn L, Schmidt DE, Oldert G, et al. Evolution and Utility of Antiplatelet Autoantibody Testing in Patients with Immune Thrombocytopenia. Transfus Med Rev. 2020;34(4). doi:10.1016/j.tmrv.2020.09.003
19. Porcelijn L, Huiskes E, Oldert G, Schipperus M, Zwaginga JJ, de Haas M. Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art. Br J Haematol. 2018;182(3). doi:10.1111/bjh.15404
20. Vollenberg R, Jouni R, Norris PAA, et al. Glycoprotein V is a relevant immune target in patients with immune thrombocytopenia. Haematologica. 2019;104(6):1237-1243. doi:10.3324/haematol.2018.211086
21. Joutsi-Korhonen L, Javela K, Hormila P, Kekomaki R. Glycoprotein V-specific platelet-associated antibodies in thrombocytopenic patients. Clin Lab Haematol. 2001;23(5):307-312. doi:10.1046/j.1365-2257.2001.00405.x
22. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood. 2009;113(11):2386-2393. doi:10.1182/blood-2008-07-162503
23. Brighton TA, Evans S, Castaldi PA, Chesterman CN, Chong BH. Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. Blood. 1996;88(1):194-201. https://www.ncbi.nlm.nih.gov/pubmed/8704174
24. Warner MN, Moore JC, Warkentin TE, Santos A V, Kelton JG. A prospective study of protein-specific assays used to investigate idiopathic thrombocytopenic purpura. Br J Haematol. 1999;104(3):442-447. doi:10.1046/j.1365-2141.1999.01218.x
25. Olsson B, Andersson PO, Jernas M, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. Nat Med. 2003;9(9):1123-1124. doi:10.1038/nm921
26. Vrbensky JR, Nazy I, Clare R, Larché M, Arnold DM. T cell–mediated autoimmunity in immune thrombocytopenia. Eur J Haematol. 2022;108(1):18-27. doi:10.1111/ejh.13705
27. Malik A, Sayed AA, Han P, et al. The role of CD8+ T cell clones in immune thrombocytopenia. Blood. Published online February 7, 2023. doi:10.1182/blood.2022018380
28. Fujisawa K, Tani P, Piro L, McMillan R. The effect of therapy on platelet-associated autoantibody in chronic immune thrombocytopenic purpura. Blood. 1993;81(11):2872-2877. doi:10.1182/blood.V81.11.2872.2872
29. Aslam R, Kapur R, Segel GB, et al. The spleen dictates platelet destruction, anti-platelet antibody production, and lymphocyte distribution patterns in a murine model of immune thrombocytopenia. Exp Hematol. 2016;44(10):924-930.e1. doi:10.1016/j.exphem.2016.07.004
30. Mahévas M, Patin P, Huetz F, et al. B cell depletion in immune thrombocytopenia reveals splenic long-lived plasma cells. Journal of Clinical Investigation. 2013;123(1):432-442. doi:10.1172/JCI65689
31. Roeser A, Lazarus AH, Mahévas M. B cells and antibodies in refractory immune thrombocytopenia. Br J Haematol. 2023;203(1):43-53. doi:10.1111/bjh.18773
32. Guo L, Kapur R, Aslam R, et al. CD20+ B-cell depletion therapy suppresses murine CD8+ T-cell-mediated immune thrombocytopenia. Blood. 2016;127(6):735-738. doi:10.1182/blood-2015-06-655126
33. Nilforoushzadeh MA, Heidari N, Heidari A, et al. The role of BAFF and BAFF-R inhibitors in the treatment of immune thrombocytopenia; a focused review. Int Immunopharmacol. 2024;131:111827. doi:10.1016/j.intimp.2024.111827

34. Matthieu Mahévas, Imane Azzaoui, Etienne Crickx, et al. Efficacy, safety and immunological profile of combining rituximab with belimumab for adults with persistent or chronic immune thrombocytopenia: results from a prospective phase 2b trial. Haematologica. 2020;106(9):2449-2457. doi:10.3324/haematol.2020.259481

35. Li G, Wang S, Li N, et al. Proteasome Inhibition with Bortezomib Induces Apoptosis of Long-Lived Plasma Cells in Steroid-Resistant or Relapsed Immune Thrombocytopaenia. Thromb Haemost. 2018;118(10):1752-1764. doi:10.1055/s-0038-1669921

36. Zwaginga JJ, van der Holt B, te Boekhorst PA, et al. Multi-center randomized open label phase II trial on three rituximab dosing schemes in immune thrombocytopenia patients. Haematologica. 2015;100(3):e90-e92. doi:10.3324/haematol.2014.110213

37. Porcelijn L, Huiskes E, Schipperus M, et al. Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients. Blood. 2017;129(25):3389-3391. doi:10.1182/blood-2016-11-751719

38. Arnold DM, Vrbensky JR, Karim N, et al. The effect of rituximab on anti-platelet autoantibody levels in patients with immune thrombocytopenia. Br J Haematol. 2017;178(2):302-307. doi:10.1111/bjh.14664

39. Feng R, Liu X, Zhao Y, et al. GPIIb/IIIa autoantibody predicts better rituximab response in ITP. Br J Haematol. 2018;182(2):305-307. doi:10.1111/bjh.14782

40. Kapur R, Aslam R, Speck ER, Rebetz JM, Semple JW. Thrombopoietin receptor agonist (TPO-RA) treatment raises platelet counts and reduces anti-platelet antibody levels in mice with immune thrombocytopenia (ITP). Platelets. 2020;31(3):399-402. doi:10.1080/09537104.2019.1624709

41. Sanfilippo KM, Cuker A. TPO-RAs and ITP remission: cause or coincidence? Blood. 2023;141(23):2790-2791. doi:10.1182/blood.2023020243

42. Guillet S, Crickx E, Azzaoui I, et al. Prolonged response after TPO-RA discontinuation in primary ITP: results of a prospective multicenter study. Blood. Published online March 9, 2023. doi:10.1182/blood.2022018665

43. Schifferli A, Rüfer A, Rovo A, et al. Immunomodulation with romiplostim as a second-line strategy in primary immune thrombocytopenia: The <scp>iROM</scp> study. Br J Haematol. 2023;203(1):119-130. doi:10.1111/bjh.19074

44. Barlassina A, González-López TJ, Cooper N, Zaja F. European Delphi panel to build consensus on tapering and discontinuing thrombopoietin receptor agonists in immune thrombocytopenia. Platelets. 2023;34(1). doi:10.1080/09537104.2023.2170999

45. Cooper N, Scully M, Percy C, et al. Real-world use of thrombopoietin receptor agonists for the management of immune thrombocytopenia in adult patients in the United Kingdom: Results from the <scp>TRAIT</scp> study. Br J Haematol. Published online March 2024. doi:10.1111/bjh.19345

46. Mahévas M, Fain O, Ebbo M, et al. The temporary use of thrombopoietin-receptor agonists may induce a prolonged remission in adult chronic immune thrombocytopenia. Results of a French observational study. Br J Haematol. 2014;165(6):865-869. doi:10.1111/bjh.12888

47. González-López TJ, Pascual C, Álvarez-Román MT, et al. Successful discontinuation of eltrombopag after complete remission in patients with primary immune thrombocytopenia. Am J Hematol. 2015;90(3). doi:10.1002/ajh.23900

48. Liu X guang, Liu S, Feng Q, et al. Thrombopoietin receptor agonists shift the balance of Fcγ receptors toward inhibitory receptor IIb on monocytes in ITP. Blood. 2016;128(6):852-861. doi:10.1182/blood-2016-01-690727

49. Kapur R. Regulatory T cells are replenished in the splenic microenvironment of patients with immune thrombocytopenia by treatment with thrombopoietin receptor agonists. Br J Haematol. 2022;198(5):803-804. doi:10.1111/bjh.18332
50. Kuwana M, Okazaki Y, Kaburaki J, Kawakami Y, Ikeda Y. Spleen Is a Primary Site for Activation of Platelet-Reactive T and B Cells in Patients with Immune Thrombocytopenic Purpura. The Journal of Immunology. 2002;168(7):3675-3682. doi:10.4049/jimmunol.168.7.3675
51. Grozovsky R, Giannini S, Falet H, Hoffmeister KM. Regulating billions of blood platelets: glycans and beyond. Blood. 2015;126(16):1877-1884. doi:10.1182/blood-2015-01-569129
52. Grozovsky R, Giannini S, Falet H, Hoffmeister KM. Novel mechanisms of platelet clearance and thrombopoietin regulation. Curr Opin Hematol. 2015;22(5):445-451. doi:10.1097/MOH.0000000000000170
53. Grozovsky R, Begonja AJ, Liu K, et al. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. Nat Med. 2015;21(1):47-54. doi:10.1038/nm.3770
54. Rumjantseva V, Hoffmeister KM. Novel and unexpected clearance mechanisms for cold platelets. Transfus Apher Sci. 2010;42(1):63-70. doi:10.1016/j.transci.2009.10.008
55. Hoffmeister KM, Falet H. Platelet clearance by the hepatic Ashwell-Morrell receptor: mechanisms and biological significance. Thromb Res. 2016;141 Suppl 2:S68-72. doi:10.1016/S0049-3848(16)30370-X
56. Li R, Hoffmeister KM, Falet H. Glycans and the platelet life cycle. Platelets. 2016;27(6):505-511. doi:10.3109/09537104.2016.1171304
57. Quach ME, Dragovich MA, Chen W, et al. Fc-independent immune thrombocytopenia via mechanomolecular signaling in platelets. Blood. 2018;131(7):787-796. doi:10.1182/blood-2017-05-784975
58. Wang Y, Chen W, Zhang W, et al. Desialylation of <i>O</i>-glycans on glycoprotein Ibα drives receptor signaling and platelet clearance. Haematologica. 2020;106(1):220-229. doi:10.3324/haematol.2019.240440
59. Xu M, Li J, Neves MAD, et al. GPIbα is required for platelet-mediated hepatic thrombopoietin generation. Blood. 2018;132(6):622-634. doi:10.1182/blood-2017-12-820779
60. Tao L, Zeng Q, Li J, et al. Platelet desialylation correlates with efficacy of first-line therapies for immune thrombocytopenia. J Hematol Oncol. 2017;10(1):46. doi:10.1186/s13045-017-0413-3
61. Revilla N, Corral J, Miñano A, et al. Multirefractory primary immune thrombocytopenia; targeting the decreased sialic acid content. Platelets. 2019;30(6):743-751. doi:10.1080/09537104.2018.1513476
62. Marini I, Zlamal J, Faul C, et al. Autoantibody-mediated desialylation impairs human thrombopoiesis and platelet lifespan. Haematologica. 2019;106(1):196-207. doi:10.3324/haematol.2019.236117
63. Webster ML, Sayeh E, Crow M, et al. Relative efficacy of intravenous immunoglobulin G in ameliorating thrombocytopenia induced by antiplatelet GPIIbIIIa versus GPIbα antibodies. Blood. 2006;108(3):943-946. doi:10.1182/blood-2005-06-009761
64. Porcelijn L, Schmidt DE, Ellen van der Schoot C, Vidarsson G, de Haas M, Kapur R. Anti-glycoprotein Ibα autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients. Haematologica. 2020;105(4). doi:10.3324/haematol.2019.228908
65. Amini SN, Nelson VS, Porcelijn L, et al. The interplay between GPIb/IX antibodies, platelet hepatic sequestration, and TPO levels in patients with chronic ITP. Blood Adv. 2023;7(6). doi:10.1182/bloodadvances.2022007751

66. Amini SN, Porcelijn L, Sobels A, et al. Anti-Glycoprotein Antibodies and Sequestration Pattern of Indium Labeled Platelets in Immune Thrombocytopenia. Blood Adv. Published online 2021. doi:10.1182/bloodadvances.2021004822

67. Meier R, Porcelijn L, Hofstede-van Egmond S, et al. Laboratory approach for vaccine-induced thrombotic thrombocytopenic diagnosis in the Netherlands. submitted for publication. Published online 2024.

68. Porcelijn L, Meier R, Hofstede-van Egmond S, et al. Presence of antibodies against platelet glycoproteins in clinically-suspected VITT patients. submitted for publication. Published online 2024.

69. Cecinati V, Principi N, Brescia L, Giordano P, Esposito S. Vaccine administration and the development of immune thrombocytopenic purpura in children. Hum Vaccin Immunother. 2013;9(5):1158-1162. doi:10.4161/hv.23601

70. Gan G, Liu H, Liang Z, Zhang G, Liu X, Ma L. Vaccine-associated thrombocytopenia. Thromb Res. 2022;220:12-20. doi:10.1016/j.thromres.2022.09.017

71. Molina V, Shoenfeld Y. Infection, vaccines and other environmental triggers of autoimmunity. Autoimmunity. 2005;38(3):235-245. doi:10.1080/08916930500050277

72. Nakayama T, Onoda K. Vaccine adverse events reported in post-marketing study of the Kitasato Institute from 1994 to 2004. Vaccine. 2007;25(3):570-576. doi:10.1016/j.vaccine.2006.05.130

73. Grimaldi-Bensouda L, Michel M, Aubrun E, et al. A case-control study to assess the risk of immune thrombocytopenia associated with vaccines. Blood. 2012;120(25):4938-4944. doi:10.1182/blood-2012-05-431098

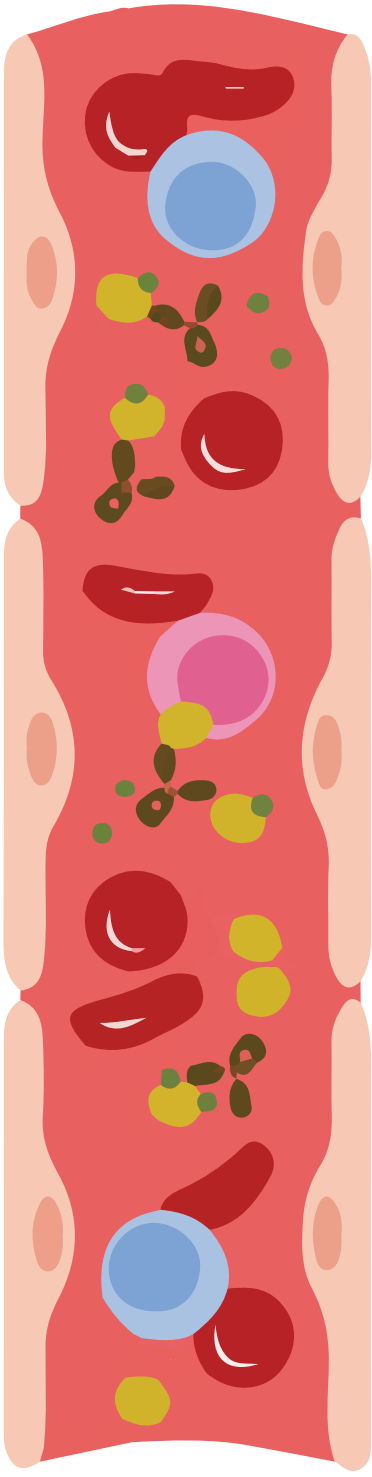
74. Elsaid M, Nune A, Brakat AM, et al. Immune thrombocytopenic purpura after influenza vaccine administration; a systematic review and meta-analysis. Trop Dis Travel Med Vaccines. 2023;9(1):22. doi:10.1186/s40794-023-00206-9

75. Talotta R. Molecular Mimicry and HLA Polymorphisms May Drive Autoimmunity in Recipients of the BNT-162b2 mRNA Vaccine: A Computational Analysis. Microorganisms. 2023;11(7):1686. doi:10.3390/microorganisms11071686

76. van Loghem JJ, Dorfmeijer H, van der Hart M, Schreuder F. Serological and Genetical Studies on a Platelet Antigen (Zw). Vox Sang. 1959;4(2):161-169. doi:10.1111/j.1423-0410.1959.tb04032.x

77. von dem Borne AE, Verheugt FW, Oosterhof F, von Riesz E, de la Riviere AB, Engelfriet CP. A simple immunofluorescence test for the detection of platelet antibodies. Br J Haematol. 1978;39(2):195-207. doi:10.1111/j.1365-2141.1978.tb01089.x

78. Linge P, Kapur R, Semple J, Porcelijn L, Bengtsson A. Platelet glycoprotein antibodies and phospholipid antibodies in SLE patients. Paper in progress. Published online 2024.



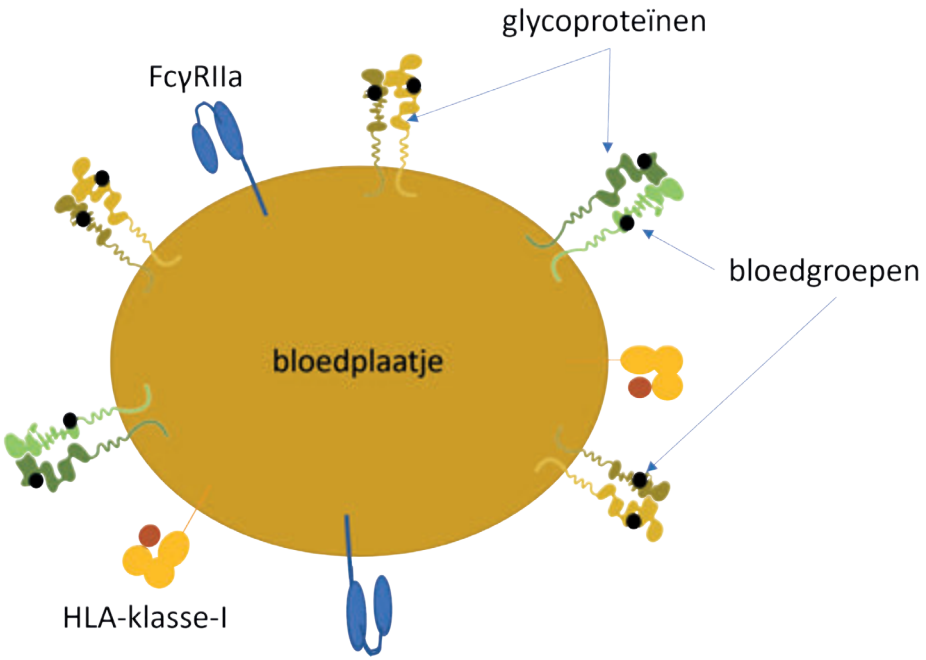
11

CHAPTER 11

Nederlandse samenvatting voor leken

Introductie

Dit proefschrift gaat over het verbeteren van de diagnostiek voor patiënten met een bloedplaatjes tekort. Bloedplaatjes, ook wel trombocyten genoemd, zijn een onderdeel van het bloed en bevinden zich naast rode bloedcellen en witte bloedcellen in het bloedplasma (een vloeistof met veel eiwitten en zouten). Bloedplaatjes zijn belangrijk voor de bloedstelping. Als er een wondje ontstaat, zullen de bloedplaatjes worden verbruikt om een stolsel te vormen waarmee het 'gat' gedicht wordt. Recent onderzoek laat zien dat bloedplaatjes ook andere functies hebben, zoals het transporteren van verschillende stofjes door het lichaam en het ondersteunen van het zogenaamde immuunsysteem dat zich bezighoudt met ons beschermen tegen infecties. Een gezonde volwassene van 70 kg heeft ongeveer 5,6 liter bloed met daarin tussen de 840 en 2240 miljard trombocyten (150 tot 400 miljard per liter). Om dit aantal op peil te houden is het nodig om iedere dag 100 tot 200 miljard trombocyten te maken die, als ze niet worden verbruikt, ongeveer 10 dagen circuleren voordat ze weer worden afgebroken.



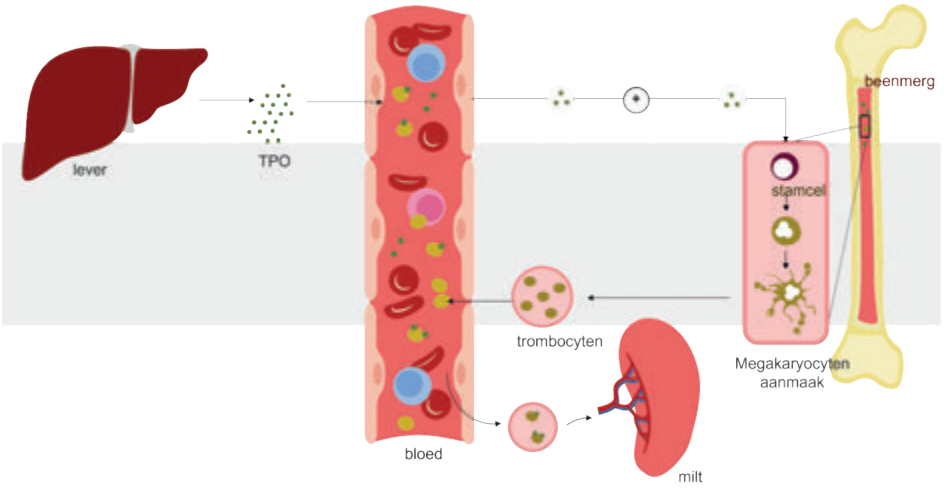
Figuur 1: Belangrijke structuren op de trombocyt.
Op trombocyten zijn verschillende structuren aanwezig, waaronder glycoproteïnen (GP), die grotendeels betrokken zijn bij het bloedstelpingsproces. Om een idee te geven, op ieder bloedplaatje zitten, alleen al van het glycoproteïne complex IIb/IIIa (groene structuren, hier slechts twee getekend) ongeveer 80.000 kopieën. Net als op rode bloedcellen (b.v. bloedgroep A en B of rhesus D) zitten er op bloedplaatjes ook bloedgroepen. Deze bloedgroepen (zwarte rondjes) worden gevormd door specifieke stukjes van de verschillende GP en zijn bekend geraakt omdat ze door andermans immuunsysteem kunnen worden herkend.

Naast de GP zijn er nog twee andere structuren afgebeeld, n.l. Fcgamma-receptor IIa (FcγRIIa) en humane leukocyten antigenen-klasse-1. Ze zijn ook betrokken bij immuunprocessen maar ook bij activatie van de bloedplaatjes. Als we het hebben over immuunprocessen dan gaat het meestal om antistoffen die binden aan plaatjes. Dit kan aan: 1) de bloedgroepen, d.w.z. dat iemand die een bloedgroep niet heeft, na transfusie of tijdens een zwangerschap, een antistof kan maken tegen deze niet-eigen bloedgroep. Dit zijn alloantistoffen, 2) het glycoproteïne zelf en dit gaat dan om autoantistoffen en 3) via de onderkant van de Y-vormige antistoffen van FcγRIIa (de naam zegt al dat het een receptor is voor de Y).

Trombopoïetine

Het eerste deel van dit proefschrift gaat over trombopoïetine (TPO), een hormoon dat de aanmaak van trombocyten stimuleert, vergelijkbaar met het u misschien wel bekende hormoon erytropoïetine (EPO) dat rode bloedcel aanmaak stimuleert. Uit zogenaamde stamcellen worden (afhankelijk van de specifieke stimulatie) bloedplaatjes, rode bloedcellen of witte bloedcellen gemaakt. Dat er precies voldoende van deze verschillende cellen zijn, komt door een wonderbaarlijk goed gecontroleerd evenwicht tussen aanmaak en afbraak. TPO wordt grotendeels gemaakt in de lever en gaat via het bloed naar het beenmerg, waar het stamcellen aanzet tot het aanmaken van trombocyten. Hiertoe worden eerst vanuit de stamcel en een aantal voorlopercellen uiteindelijk de megakaryocyten (MK) gevormd. Een MK is een zeer grote cel die kralensnoer achtige tentakels maakt en deze uitsteekt door kanaaltjes in het, om het beenmerg circulerende bloed. Vervolgens splitsen steeds kraaltjes in feite bloedplaatjes van deze tentakels af in de bloedstroom. Een MK valt uiteindelijk volledig uiteen in 2000 tot 3000 bloedplaatjes. Bloedplaatjes zijn dus geen volledige cellen, maar fragmenten van de MK.

Hoe weet het lichaam nu dat er voldoende trombocyten zijn, zodat er niet te veel of te weinig worden aangemaakt? Dat is nog steeds niet helemaal duidelijk, maar het lijkt voor een deel te worden gecontroleerd door een heel eenvoudig feedbackmechanisme. Op de voorlopercellen en op trombocyten zelf zitten receptoren die TPO kunnen binden. Dus als er voldoende trombocyten worden aangemaakt bindt het in de bloedbaan circulerende TPO aan deze receptoren en blijft de hoeveelheid TPO in het bloedplasma laag waardoor er een normale stimulatie van de trombocyten aanmaak is. Als er onvoldoende trombocyten worden aangemaakt, kan het vrije TPO niet binden aan de receptoren en stijgt de plasma TPO spiegel en daarmee de stimulatie van de aanmaak. Er is daarnaast echter nog een systeem dat de aanmaak van TPO kan beïnvloeden. Als trombocyten verouderen, verliezen ze suikers van hun oppervlak, waardoor ze herkend en gebonden worden door de zogenaamde Ashwell-Morell receptor (AMR) in de lever. Binding aan deze AMR zorgt (naast andere processen waar dit proefschrift niet over gaat) voor afbraak van verouderde trombocyten en door deze koppeling wordt tegelijkertijd ook de aanmaak van TPO in de lever positief beïnvloed.



Figuur 2: De aanmaak van bloedplaatjes (trombocyten) wordt gestimuleerd door trombopoietine. Trombopoietine (TPO) wordt aangemaakt in de lever en gaat via het bloed naar het beenmerg, waar het de aanmaak van megakaryocyten (MK) uit stamcellen stimuleert. Het vrije TPO in het bloed kan binden aan de trombocyten, zodat, als er voldoende trombocyten worden geproduceerd, de vrije TPO spiegel laag blijft.

Bloedplaatjes te kort door een fout in het immuunsysteem

Er zijn veel verschillende oorzaken voor het ontstaan van een bloedplaatjes tekort. In dit proefschrift hebben we ons met name gefocust op oorzaken waarbij het immuunsysteem zich met antistoffen richt tegen de bloedplaatjes. Het immuunsysteem houdt zich onder andere bezig met het voorkomen en herstellen van infecties. Als schadelijke micro-organismen (o.a. virussen en bacteriën) het lichaam binnen komen, herkent het immuunsysteem deze als lichaamsvreemd en wordt er (naast een algemeen bewakingssysteem) een pasklaar afweersysteem aangemaakt. Dit afweersysteem kan bestaan uit cellen die de micro-organismen direct aanvallen en vernietigen, maar meestal worden witte bloedcellen aangezet tot het maken van antistoffen tegen iets op het micro-organisme. Antistoffen kan je zien als een Y, waarbij de twee bovenste delen binden aan het micro-organisme en het onderste deel bindt aan opruim cellen. Als het antistof-micro-organisme complex bindt aan de opruim cel, wordt dit afgebroken.

Helaas vergist het immuunsysteem zich soms en richt het zich tegen iets wat lichaamseigen is. Er kunnen daardoor verschillende zogenaamde autoimmuun aandoeningen ontstaan, b.v. reuma en hypothyreoïdie (verminderde schildklierwerking).

Immuun trombocytopenie

Bij de aandoening autoimmuun trombocytopenie(ITP) richt het immuunsysteem

zich tegen de eigen bloedplaatjes, waardoor er een bloedplaatjes tekort ontstaat. Bij ITP wordt met name gedacht aan
afbraak door antistoffen, die omdat ze tegen de eigen bloedplaatjes zijn gericht autoantistoffen worden genoemd. Binding van autoantistoffen aan bloedplaatjes zorgt voor een versnelde afbraak in de milt en lever. Er zijn verschillende uitingsvormen van ITP, namelijk ITP op de kinderleeftijd en primaire en secundaire ITP bij volwassenen. ITP op de kinderleeftijd kenmerkt zich door een plotselinge ernstige daling van het bloedplaatjes aantal en een verhoogde bloedingsneiging (b.v. spontaan optredende blauwe plekken of tandvleesbloedingen). Meestal betreft het kinderen tussen de 2 en 8 jaar. Deze aandoening begint nogal eens in de herfst of winter met voorafgaand een virusinfectie (b.v. verkoudheid). 80 tot 90% van de kinderen herstelt binnen enkele weken tot een jaar, maar bij 10-20% blijft ITP aanwezig. Maar ITP zowel de primaire als de secundaire vorm die samengaat met andere ziekten wordt vooral bij volwassenen gezien. Primaire ITP is bijna altijd een chronische aandoening, waarbij therapie voor een stijging van de bloedplaatjes maar nog niet voor genezing zorgt. Er wordt steeds meer bekend over de onderliggende oorzaken voor het 'ontsporen' van het immuunsysteem. Zo denkt men dat verschillende cellen die een controlerende/regulerende rol hebben en het immuunsysteem in toom houden tekortschieten, maar helaas is het nog steeds niet helemaal duidelijk waardoor dit gebeurt. Bij gebrek aan goede diagnostiek (zie ook onder), wordt primaire ITP gediagnosticeerd door het uitsluiten van andere oorzaken voor een bloedplaatjes tekort, wat niet altijd eenvoudig is. De behandeling van ITP bestond jarenlang uit het geven van corticosteroïden (b.v. prednison), dat het immuunsysteem op allerlei manieren onderdrukt, en het verwijderen van de milt (het orgaan waar de meeste afbraak van bloedplaatjes plaatsvindt). Helaas heeft het langdurig toedienen van corticosteroïden veel bijwerkingen en is het lange termijneffect van het verwijderen van de milt zeer onzeker en kan het leiden tot ernstige infecties en meer trombose. De ontdekking en het gebruik van de eigenschappen van TPO heeft voor een grote doorbraak gezorgd. Je zou verwachten dat de verhoogde afbraak van bloedplaatjes een verhoging van het TPO-gehalte in het bloed zou veroorzaken, maar dat is niet het geval. Bij ITP-patiënten blijft het TPO-gehalte in het bloed normaal of verhoogd slechts licht. Daardoor is het toedienen van medicijnen die net als TPO de bloedplaatjes aanmaak stimuleren, zogenaamde TPO-receptor antagonisten (TPO-RA), nu een veelgebruikte ITP-behandeling. Doordat er steeds meer bekend wordt over het functioneren van het immuunsysteem komen er de laatste jaren medicijnen beschikbaar die ingrijpen op verschillende aspecten hiervan. Zo zijn er medicijnen die de antistof producerende cellen onderdrukken, medicijnen die de opruimcellen remmen en medicijnen die zorgen voor een versneld verdwijnen van antistoffen uit het bloed. Om nog onduidelijke - redenen is er nogal wat variatie in effect van de verschillende therapie keuzes bij ITP patiënten. Zo heeft slechts 70% langdurig

baat bij het verwijderen van de milt, en reageert ongeveer 40% op de medicatie die de antistof producerende cellen verwijdert. Onderzoek zal moeten uitwijzen waardoor dat komt en of het mogelijk is om een meer gerichte behandeling van ITP-patiënten mogelijk is.

Diagnostiek voor patiënten met bloedplaatjes tekort

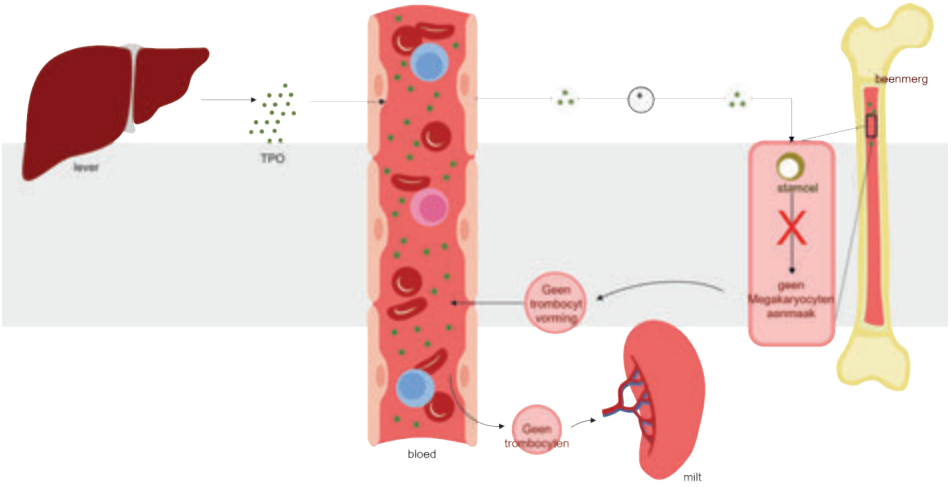
Al decennialang wordt er gezocht naar goede diagnostische tests of inderdaad de oorzaak van een bloedplaatjes tekort te wijten is aan autoantistoffen zonder dat sprake is van echt meer plaatjesaanmaak maw of het gaat om ITP. Dit proefschrift toont een deel van deze zoektocht, waarbij we ons hebben gericht op twee diagnostische tests:

1. *1) Het onderscheiden van een te geringe bloedplaatjes aanmaak door een beenmerg aandoening en een te hoge bloedplaatjes afbraak door bijvoorbeeld ITP.* Er bestaat geen eenvoudige directe methode om te kunnen zien of er in het beenmerg voldoende MK en bloedplaatjes worden aangemaakt. Om dat te kunnen zien is een beenmergpunctie noodzakelijk. Daarbij wordt een klein beetje beenmerg afgenomen en onder de microscoop onderzocht op de hoeveelheid MK. Zoals boven beschreven kan het meten van de hoeveelheid vrij in het bloedplasma circulerend TPO mogelijk wel als een indirecte maat voor de aanmaak van bloedplaatjes worden gebruikt. Als dat zo is, hebben we aan slechts een klein beetje bloed van de patiënt voldoende. Wij hebben de plasma TPO-waarden bij patiënten met verschillende oorzaken voor bloedplaatjes tekort bestudeerd en vergeleken met de waarden bij gezonde personen.
2. *2) Het aantonen van de aanwezigheid van autoantistoffen tegen bloedplaatjes in het bloed van patiënten met een verdenking op ITP.* Er bestonden al heel lang tests om antistoffen tegen bloedplaatjes aan te tonen, maar deze toonden bij slechts 60-70% van de ITP-patiënten autoantistoffen aan en hadden een specificiteit van slechts 40-50%, d.w.z. dat er in ongeveer dezelfde percentages ook autoantistoffen bij patiënten met bloedplaatjes tekort door andere oorzaken dan ITP werden aangetoond. Wij hebben gezocht naar een meer sensitieve (dus vaker positief bij ITP-patiënten) en meer specifieke (minder vaak positief bij niet-ITP patiënten) methode.

Hoofdstuk 2: Is het mogelijk om met het meten van het TPO-gehalte in plasma iets te zeggen over de oorzaak van een bloedplaatjes tekort?

Al in een studie verricht in 1998 hebben we, gebruik makend van een door collega Claudia Folman in 1997 opgezette plasma TPO meetmethode (TPO ELISA), onderzocht of we onderscheid konden maken tussen patiënten met een bloedplaatjes tekort door een verminderde trombocyten aanmaak en een verhoogde trombocyten afbraak. We hebben daarvoor groepen patiënten met een aangeboren bloedplaatjes aanmaak stoornis en met een verminderde

beenmergfunctie door b.v. chemotherapie vergeleken met patiënten met ITP. We toonden aan dat patiënten met ITP een normaal of slechts licht verhoogd plasma TPO-gehalte en patiënten met een beenmerg aandoening en daardoor een verminderde bloedplaatjes aanmaak een significant hoger TPO-gehalte hebben. Uit deze bevindingen bleek dat het meten van het TPO-gehalte in plasma van patiënten een bijdrage kan leveren aan het vinden van de onderliggende oorzaak voor een trombocytopenie.



Figuur 3: De plasma TPO spiegel stijgt als er onvoldoende trombocyten worden aangemaakt.

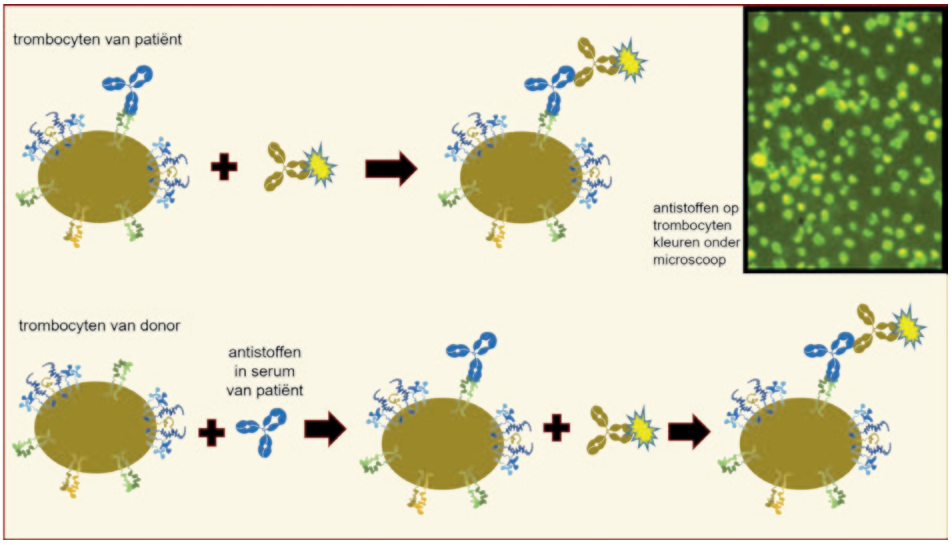
Hoofdstuk 3 en 4: Kan het meten van het TPO gehalte in het bloed van pasgeboren kinderen met een bloedplaatjes tekort helpen bij het stellen van de diagnose?

Foetale/neonatale alloimmuun trombocytopenie (FNAIT) is een aandoening waarbij het ongeboren kind een bloedplaatjes tekort krijgt doordat er antistoffen van de moeder, gericht tegen een bloedgroep op de kinderlijke bloedplaatjes, via de placenta (moederkoek) naar het kind gaan. Deze aandoening is te vergelijken met de zogenaamde 'rhesus baby's', waarbij de moeder, die de rhesus D bloedgroep niet heeft, tijdens de zwangerschap antistoffen maakt tegen de rhesus D bloedgroep van het kind. In het geval van de rhesus baby's worden de rode bloedcellen van het kind afgebroken en in het geval van de FNAIT baby's worden de bloedplaatjes afgebroken. Het tekort aan bloedplaatjes bij het nog ongeboren of pasgeboren kind kan leiden tot milde of ernstige bloedingen. Het snel kunnen vaststellen van de oorzaak voor het bloedplaatjes tekort bij de baby is dan ook belangrijk om de juiste therapie te kunnen geven. Het verrichten van een beenmerg onderzoek bij een pasgeboren kind wordt liever vermeden. Om

toch snel extra informatie te krijgen over de mogelijk onderliggende oorzaak, hebben we onderzocht of, net als bij ITP, ook bij deze vorm van versnelde afbraak van trombocyten door antistoffen het TPO-gehalte in plasma normaal of licht verhoogd bleef. We hebben daarvoor in eerste instantie het TPO-gehalte in plasma van een groep gezonde pasgeborenen gemeten en een groep kinderen met een andere aandoening zonder bloedplaatjes tekort. Daarna hebben we deze waarden vergeleken met het gehalte in kinderen met FNAIT. Inderdaad bleek ook bij deze antistof afbraak het TPO-gehalte niet of slechts licht verhoogd. In een latere studie hebben we dit nogmaals onderzocht en ook vergeleken met groepen pasgeborenen, waarbij FNAIT als oorzaak voor een trombocytopenie werd uitgesloten. De pasgeboren kinderen werden gecategoriseerd in groepen met virale of bacteriële infecties, zuurstoftekort bij de geboorte (asfyxie), het te vroeg of te klein geboren zijn, trisomie 21 en hypoglycaemie. Met behulp van het plasma TPO-gehalte konden we pasgeborenen met een trombocytopenie veroorzaakt door moederlijke antistoffen (TPO-gehalte normaal tot licht verhoogd) onderscheiden van pasgeborenen waarbij de trombocytopenie werd veroorzaakt door ernstige asfyxie, congenitale virale infecties of een aangeboren trombocyten aanmaakstoornis (amegakaryocytose) (TPO-gehalte verhoogd).

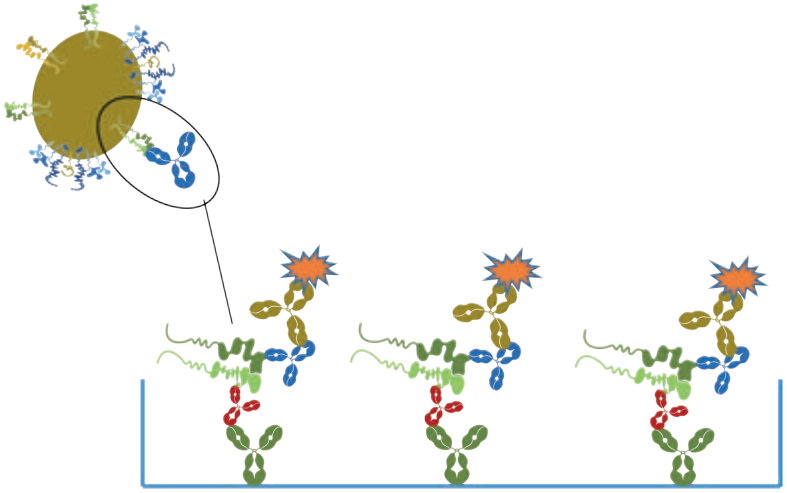
Hoofdstuk 5: Het verbeteren van de test voor het aantonen van autoantistoffen tegen trombocyten

Tussen de jaren 70 en het tweede decennium van deze eeuw, werd gebruik gemaakt van de plaatjes immunofluorescentie test (PIFT) voor het aantonen van trombocyten autoantistoffen. Dit werd gedaan door het aantonen van autoantistoffen op de trombocyten van de patiënt (directe methode) of in het plasma (indirecte methode) van de patiënt. Voor de directe methode werden de trombocyten van de patiënt uit het bloed geïsoleerd waarna het al dan niet aanwezig zijn van de autoantistoffen werd aangetoond met fluorescerende antistoffen tegen de autoantistoffen. Dus een fluorescerende Y vormige antistof die bindt aan de auto antistof op de trombocyten van de patiënt. Als deze autoantistoffen aanwezig zijn, zie je de trombocyten als het ware oplichten onder de microscoop. Voor de indirecte methode werd het plasma van de patiënt bij trombocyten van gezonde donoren gedaan. Als er in het plasma autoantistoffen aanwezig zijn binden ze aan de donor trombocyten en kan dit weer zichtbaar worden gemaakt met de fluorescerende Y. Helaas was deze methode weinig sensitief (dus in veel patienten met autoantistoffen negatief) en nog minder specifiek (erg veel fout positieve uitslagen) doordat er allerlei antistoffen aan trombocyten kunnen binden. De matige prestaties van deze methode was een van de redenen waardoor het verrichten van autoantistof detectie als diagnostiek voor ITP niet werd gebruikt.



Figuur 4: De directe (boven) en indirecte plaatjes immunofluorescentie test (PIFT)

In de jaren 90 kwam er een andere methode voor het aantonen van antistoffen tegen bloedplaatjes beschikbaar. Bij deze methode worden structuren, waarvan bekend is dat antistoffen er aan kunnen binden, geïsoleerd uit de trombocyt membraan en apart getest. Deze zogenaamde MAIPA methode leek alleen geschikt voor het aantonen van alloantistoffen, d.w.z. tegen bloedgroepen op bloedplaatjes van een bloeddonor of van het ongeboren kind (zie bij FNAIT) die niet 'eigen' zijn.



Figuur 5: De glycoproteïne specifieke methode
Het is mogelijk om een specifiek GP van de trombocyt op een microtiterplaat te binden (daarvoor wordt gebruik gemaakt van verschillende antistoffen, hier groen en rood). Als er antistoffen op het GP gebonden zijn, kan dat met een fluorescerende antistof worden aangetoond.

Deze zogenaamde glycoproteïne-specifieke methode had als voordeel dat de binding van allerlei andere antistoffen aan trombocyten niet meer tot aspecifieke (fout positieve) resultaten kon leiden en dus een hogere specificiteit had.
Wij hebben met behulp van ruim 462 bloedmonsters van gezonde personen en 268 bloedmonsters (149 bekend met ITP en 119 niet-ITP) van trombocytopenie patiënten onderzocht wat de optimale test condities van deze glycoproteïne-specifieke methode zijn voor het aantonen van autoantistoffen. We hebben daarbij aangetoond dat 1) de scheidings cut-off waarde voor positief of negatief veel lager ligt dan voor het aantonen van alloantistoffen 2) autoantistoffen met name binden aan drie verschillende GP (GPIIb/IIIa, GPIX en GPV) en 3) de sensitiviteit ook afhankelijk is van het bloedplaatjes aantal bij de patiënt.
Door het toepassen van deze bevindingen werd bij 70-80% (= de sensitiviteit van de test) van de ITP patiënten en slechts bij zeer weinig niet-ITP patiënten (specificiteit >90%) autoantistoffen aangetoond. Ons onderzoek heeft geleid tot het opnemen van deze specifieke autoantistof detectie methode, als 'rule-in' test voor ITP, in de Nederlandse ITP-richtlijn 2021.

Hoofdstuk 6: Het al dan niet aantoonbaar zijn van autoantistoffen tegen trombocyten en de respons op een medicijn voor ITP.
Bij het verrichten van autoantistofonderzoek bij ITP patiënten voor en tijdens behandeling met een medicijn (rituximab) dat zorgt voor afbraak van de witte bloedcellen die betrokken zijn bij het produceren van antistoffen zagen we een bijzonder patroon. Een deel van de patiënten liet een stijging van het aantal bloedplaatjes en vermindering van autoantistoffen zien na het starten met rituximab en bij een deel van de patiënten bleef het bloedplaatjes aantal laag en bleven de autoantistoffen sterk aantoonbaar. Opvallend was dat bij patiënten waarbij het bloedplaatjes aantal niet verbeterde significant vaker zowel voor als tijdens behandeling met rituximab geen autoantistoffen aantoonbaar waren. Dit zou kunnen betekenen dat het bloedplaatjes tekort bij deze patiënten misschien niet door antistoffen maar door een ander mechanisme (bijvoorbeeld direct door immuuncellen) wordt veroorzaakt.
Recent onderzoek laat zien dat rituximab niet altijd voldoende afbraak geeft van de antistof producerende cellen. Het niet (voldoende) stijgen van het aantal bloedplaatjes door rituximab lijkt dus deels verklaarbaar doordat er antistof producerende cellen aanwezig blijven. Er zijn nu studies gestart met medicijnen die een meer volledige afbraak van deze cellen veroorzaken en we zullen dan zien of er inderdaad patiënten zijn die nog steeds niet reageren en of er in deze groep patiënten nog steeds een significant hoger percentage is met negatieve antistof detectie.

Hoofdstuk 7: Stelt de vraag of autoantistoffen specifiek gericht tegen GPIb/IX meer afbraak van bloedplaatjes via de Ashwell-Morell receptor veroorzaken.
Zoals boven beschreven, vindt er dus afbraak van verouderde bloedplaatjes plaats door binding aan de AMR in de lever. De verouderde bloedplaatjes worden herkend door de AMR als er bepaalde suikers van de GP afbreken, waardoor onderliggende suikers herkend kunnen worden.

Er zijn veel studies verricht om te zien of door de binding van autoantistoffen ook suikers kunnen afbreken. Als dat zo is zouden medicijnen die de opruimcellen remmen minder effect kunnen hebben, want er vindt dan geen/ of niet alleen afbraak plaats doordat deze opruimcellen de antistoffen aan trombocyten binden maar doordat de beschadigde bloedplaatjes aan de AMR binden. De aandacht van deze studies gaat met name uit naar GPIb/IX, omdat dit GP veel suikers bevat. Zoals boven beschreven wordt TPO productie gestimuleerd door bloedplaatjes afbraak via de AMR. Wij hebben aan de hand van veel autoantistof en TPO uitslagen gekeken of het plasma TPO gehalte hoger is bij de groep patiënten met antistoffen tegen GPIb/IX. Dit was niet het geval. Een latere studie, waarbij door Sufia Amini meer specifiek is gekeken bij een kleine groep ITP patiënten met een ernstig bloedplaatjes tekort laat echter wel een zij het zwakke correlatie zien. De afbraak route van trombocyten bij ITP patiënten is van belang omdat het verwijderen van de milt een van de therapie keuzes kan zijn. Bij afbraak via de AMR in de lever is dat wellicht minder effectief. Verder onderzoek is nodig om deze relatie beter te onderzoeken en dan de succeskans van een miltverwijdering beter te kunnen inschatten.

Hoofdstuk 8: Geeft inzicht in de mogelijkheden van en de nog bestaande vragen over trombocyten autoantistof detectie.
In dit hoofdstuk wordt, op basis van literatuuronderzoek en eigen ervaring, ingegaan op de mogelijkheden en tekortkomingen van de voorgaande en huidige autoantistof detectie methodes.

Hoofdstuk 9: Is er een correlatie tussen vaccinatie voor het SARS-COV-2 virus en de detectie van trombocyten autoantistoffen.
De vaccinatie tegen het SARS-COV-2 (Corona) virus liet, gelukkig zeer zelden, een ernstige bijwerking zien. Deze bijwerking, vaccine induced thrombotic thrombocytopenia (VITT) genoemd, uitte zich in een dalend aantal bloedplaatjes meestal in combinatie met ernstige trombose, optredend dagen tot weken na vaccinatie. Voor het onderbouwen van het mechanisme van deze bijwerking werd van meer dan 300 patiënten met verdenking op VITT, bloed naar ons laboratorium gestuurd om een bloedplaatjes activatie test te verrichten. Gezien het optreden van de bloedplaatjes daling, hebben we ook gekeken naar de aanwezigheid van ITP achtige autoantistoffen (bij gebrek aan materiaal om

bloedplaatjes uit te isoleren) in het serum van 232 patiënten. In het serum van 44 (19%) patiënten konden autoantistoffen worden aangetoond, waarbij er geen significant verschil werd gevonden tussen uiteindelijk gediagnosticeerde VITT en niet-VITT patiënten. Opvallend was wel dat het antistofpatroon, d.w.z. tegen welke GP de autoantistoffen gericht zijn, anders was dan voor een controle groep van patiënten met verdenking ITP waarvan materiaal in een periode langer na vaccinatie was ingestuurd. Er werden meer antistoffen (41% in plaats van 25%) tegen GPIb/IX aangetoond in de, van VITT verdachte, patiënten die recent waren gevaccineerd. Vaccinatie voor SARS-COV-2 heeft tot gevolg dat er lokaal (dicht bij de plaats van vaccinatie) spike-eiwitten van het virus worden geproduceerd, waartegen (omdat het lichaamsvreemd is) het immuunsysteem antistoffen aanmaakt. De antistoffen zorgen ervoor dat bij een echte virus infectie het lichaam veel beter deze infectie kan bestrijden en minder ziek wordt. Mogelijk is er sprake van een zogenaamde kruisreactie. Waarbij de antistoffen die tegen een epitoop (= plaats waar antistof bindt) op het spike-eiwit dat door de vaccinatie aan het immuuunsysteem wordt gepresenteerd gericht zijn, ook reageren met een vergelijkbaar epitoop op het GPIb/IX. Als dit echt zo is, zou het dus kunnen wijzen op een andere bijwerking dan VITT, namelijk op een vaccinatie geïnduceerd bloedplaatjes tekort. De kruisreactie mogelijkheid is al langer bekend voor ITP op de kinderleeftijd, nogal eens optredend na een virale infectie (b.v. verkoudheid). Deze vorm van ITP gaat in 80 tot 90% van de gevallen binnen een jaar over. Verder onderzoek zal moeten uitwijzen of vaccinatie voor SARS-COV-2 en infectie met SARS-COV-2 eenzelfde soort beeld kan veroorzaken.

A

APPENDIX

List of publications

Curriculum Vitae

Dankwoord

LIST OF PUBLICATIONS

1. Huisman EJ, Holle N, Schipperus M, Cnossen MH, de Haas M, Porcelijn L, Zwaginga JJ. Should HLA and HPA-matched platelet transfusions for patients with Glanzmann Thrombasthenia or Bernard-Soulier syndrome be standardized care? A Dutch survey and recommendations. Transfusion. 2024 Apr 20. doi: 10.1111/trf.17824. Epub ahead of print. PMID: 38642032.
2. Meier RT, Porcelijn L, Hofstede-van Egmond S, Henskens YMC, Coutinho JM, Kruip MJHA, Stroobants AK, Zwaginga JJ, van der Born JG, van der Schoot CE, de Haas M, Kapur R. Laboratory approach for vaccine-induced thrombotic thrombocytopenia diagnosis in the Netherlands. Vox Sang. 2024 Apr 10. doi: 10.1111/vox.13633. Epub ahead of print. PMID: 38597072.
3. de Vos TW, Winkelhorst D, Porcelijn L, Beaufort M, Oldert G, van der Born JG, Lopriore E, Oepkes D, de Haas M, van der Schoot E. Natural history of human platelet antigen 1a-alloimmunised pregnancies: a prospective observational cohort study. Lancet Haematol. 2023 Dec;10(12):e985-e993. doi:10.1016/S2352-3026(23)00271-5. Epub 2023 Oct 27. PMID: 38407610.
4. Oosterhoff JJ, Linty F, Visser R, de Vos T, Hofstede-van Egmond S, van de Weerd M, Porcelijn L, de Haas M, van der Schoot E, Vidarsson G. Generation of human antibodies targeting human platelet antigen (HPA)-1a. Transfusion. 2024 Feb 24. doi: 10.1111/trf.17758. Epub ahead of print. PMID: 38400657.
5. Raadsen MP, Visser C, Lavell AHA, van de Munckhof AAGA, Coutinho JM, de Maat MPM, GeurtsvanKessel CH, Amsterdam Umc Covid-S/Hcw Study Group, Somers MK, Haagmans BL, van Gorp ECM, Porcelijn L, Kruip MJHA. Transient Autoreactive PF4 and Antiphospholipid Antibodies in COVID-19 Vaccine Recipients. Vaccines (Basel). 2023 Dec 14;11(12):1851. doi: 10.3390/vaccines11121851. PMID: 38140254; PMCID: PMC10747426.
6. Wang JJ, van der Neut Kolfschoten M, Rutten L, Armour B, Tan CW, Chataway T, Bos R, Koornneef A, Abeywickrema P, Kapur R, Porcelijn L, Khalifa M, Sadi A, Bouchier P, Kourkouta E, Perkasa A, Kwaks T, Zahn R, Solforosi L, Gordon TP. Characterization of reverse-engineered anti-PF4 stereotypic antibodies derived from serum of patients with VITT. Blood. 2024 Jan 25;143(4):370-374. doi: 10.1182/blood.2023021307. PMID: 37976451.
7. Szittner Z, Bentlage AEH, Temming AR, Schmidt DE, Visser R, Lissenberg Thunnissen S, Mok JY, van Esch WJE, Sonneveld ME, de Graaf EL, Wuhrer M, Porcelijn L, de Haas M, van der Schoot CE, Vidarsson G. Cellular surface plasmon resonance-based detection of anti-HPA-1 a antibody glycosylation in fetal and neonatal alloimmune thrombocytopenia. Front lmmunol. 2023 Oct 5;14:1225603. doi: 10.3389/fimmu.2023.1225603. PMID: 37868955; PMCID: PMC10585714.
8. van der Velden S, van Osch TLJ, Seghier A, Bentlage AEH, Mok JY, Geerdes DM, van Esch WJE, Pouw RB, Brouwer MC, Jongerius I, de Haas M, Porcelijn L, van der Schoot CE, Vidarsson G, Kapur R. Complement activation drives antibody-mediated transfusion-related acute lung injury via macrophage trafficking and formation of NETs. Blood. 2024 Jan 4;143(1):79-91. doi: 10.1182/blood.2023020484. PMID: 37801721.
9. Bayat B, Lowack J, Audrain M, Croisille L, Curtis B, Dangerfield R, Esmaeili B, Grabowski C, Keller M, Kim H, Kroll H, Kvanka MM, Kwok J, Moritz E, Nathalang 0, Nelson D, Nielsen KR, Pahn G, Poles A, Porcelijn L, Sachs UJ, Schanbacher M, Kormoczi GF, Kupatawintu P, Takahashi D, Uhrynowska M, Flesch B, Fung YL. World human neutrophil antigens investigation survey. Vox Sang. 2023 Sep;118(9):763-774. doi: 10.1111/vox.13500. Epub 2023 Aug 22. PMID: 37608544.
10. Kreft IC, Huisman EJ, Cnossen MH, van Alphen FPJ, van der Zwaan C, van Leeuwen K, van Spaendonk R, Porcelijn L, Veen CSB, van den Biggelaar M, de Haas M, Meijer AB, Hoogendijk AJ. Proteomic landscapes of inherited platelet disorders with different etiologies. J Thromb Haemost. 2023 Feb;21(2):359-372.e3. doi: 10.1016/j.jtha.2022.11.021. Epub 2022 Dec 22. PMID: 36700500.

11. van Osch TLJ, Pongracz T, Geerdes OM, Mok JY, van Esch WJE, Voorberg J, Kapur R, Porcelijn L, Kerkhoffs JH, van der Meer PF, van der Schoot CE, de Haas M, Wuhrer M, Vidarsson G. Altered Fe glycosylation of anti-HLA alloantibodies in hemato-oncological patients receiving platelet transfusions. J Thromb Haemost. 2022 Dec;20(12):3011-3025. doi: 10.1111/jth.15898. Epub 2022 Oct 11. PMID: 36165642; PMCID: PMC9828502.
12. de Vos TW, Winkelhorst D, Arnad6ttir V, van der Born JG, Canals Surfs C, Caram-Deelder C, Deschmann E, Haysom HE, Hverven HBC, Lazar Krivec J, McQuilten ZK, Mul"liz-Diaz E, Nogues N, Oepkes D, Porcelijn L, van der Schoot CE, Saxonhouse M, Sola-Visner M, Tiblad E, Tiller H, Wood EM, Young V, L'.eleznik M, de Haas M, Lopriore E. Postnatal treatment for children with fetal and neonatal alloimmune thrombocytopenia: a multicentre, retrospective, cohort study. Lancet Haematol. 2022 Nov;9(11):e844-e853. doi: 10.1016/S2352-3026(22)00243-5. Epub 2022 Sep 12. PMID: 36108655.
13. Vanholder IM, Ecury-Goossen GM, Admiraal J, Porcelijn L, van Spaendonk RM, Gouw SC. Congenital amegakaryocytic thrombocytopenia presenting with a new thrombopoietin receptor (MPL) pathogenic variant: An instructive neonatal case. J PaediatrChild Health. 2022 Dec;58(12):2319-2321. doi: 10.1111/jpc.16168. Epub 2022Aug 16. PMID: 35972311.
14. Amini SN, Nelson VS, Porcelijn L, Netelenbos T, Zwaginga JJ, de Haas M, Schipperus MR, Kapur R. The interplay between GPIb/IX antibodies, platelet hepatic sequestration, and TPO levels in patients with chronic ITP. Blood Adv. 2023 Mar 28;7(6):1066-1069. doi: 10.1182/bloodadvances.2022007751. PMID: 35901281; PMCID: PMC10034565.
15. Langerak T, Bakker GJ, Porcelijn L, Lauw MN, van de Laar RJ, Eefting M. Vaccine-induced immune thrombocytopenia and thrombosis after mRNA-1273 booster vaccination. Thromb Res. 2022 Jun;214:21-22. doi: 10.1016/j.thromres.2022.03.026. Epub 2022 Apr 5. PMID: 35429895; PMCID: PMC8979885.
16. Van Osch TLJ, OosterhoffJJ, BentlageAEH, Nouta J, Koeleman CAM, Geerdes OM, Mok JY, Heidt S, Mulder A, Van Esch WJE, Kapur R, Porcelijn L, Van der Schoot CE, De Haas M, Wuhrer M, Voorberg J, Vidarsson G. Fe galactosylation of anti-platelet human lgG1 alloantibodies enhances complement activation on platelets. Haematologica. 2022 Oct 1;107(10):2432-2444. doi: 10.3324/haematol.2021.280493. PMID: 35354253; PMCID: PMC9521249.
17. Nelson VS, Jolink AC, Amini SN, Zwaginga JJ, Netelenbos T, Semple JW, Porcelijn L, de Haas M, Schipperus MR, Kapur R. Platelets in ITP: Victims in Charge of Their Own Fate? Cells. 2021 Nov 19;10(11):3235. doi: 10.3390/cells10113235. PMID: 34831457; PMCID: PMC8621961.
18. Amini SN, Porcelijn L, Sobels A, Kartachova M, de Haas M, Zwaginga JJ, Schipperus MR. Anti-glycoprotein antibodies and sequestration pattern of indium labeled platelets in immune thrombocytopenia. Blood Adv. 2022 Mar 22;6(6):1797-1803. doi: 10.1182/bloodadvances.2021004822. PMID: 34654052; PMCID: PMC8941471.
19. de Vos TW, Porcelijn L, Hofstede-van Egmond S, Pajkrt E, Oepkes D, Lopriore E, van der Schoot CE, Winkelhorst D, de Haas M. Clinical characteristics of human platelet antigen (HPA)-1a and HPA-5b alloimmunised pregnancies and the association between platelet HPA-5b antibodies and symptomatic fetal neonatal alloimmune thrombocytopenia. Br J Haematol. 2021 Nov;195(4):595-603. doi: 10.1111/bjh.17731. Epub 2021 Aug 16. Erratum in: Br J Haematol. 2022 Mar;196(6):1412-1413. PMID: 34402048; PMCID: PMC9291578.
20. Jolink AC, Nelson VS, Schipperus MR, Amini SN, Vidarsson G, van der Schoot CE, Porcelijn L, de Haas M, Kapur R. Potential Diagnostic Approaches for Prediction of Therapeutic Responses in Immune Thrombocytopenia. J Clin Med. 2021 Jul 30;10(15):3403. doi: 10.3390/jcm10153403. PMID: 34362187; PMCID: PMC8347743.

21. de Vos TW, Winkelhorst D, Baelde HJ, Dijkstra KL, van Bergen ROM, van der Meeren LE, Nikkels PGJ, Porcelijn L, van der Schoot CE, Vidarsson G, Eikmans M, Kapur R, van der Keur C, Trouw LA, Oepkes D, Lopriore E, van der Hoorn MP, Bos M, de Haas M. Placental Complement Activation in Fetal and Neonatal Alloimmune Thrombocytopenia: An Observational Study. Int J Mol Sci. 2021 Jun 23;22(13):6763. doi: 10.3390/ijms22136763. PMID: 34201864; PMCID: PMC8267834.

22. Schmidt DE, Heitink-Polle KMJ, Mertens B, Porcelijn L, Kapur R, van der Schoot CE, Vidarsson G, van der Born JG, Bruin MCA, de Haas M. Biological stratification of clinical disease courses in childhood immune thrombocytopenia. J Thromb Haemost. 2021 Apr;19(4):1071-1081. doi: 10.1111/jth.15232. Epub 2021 Mar 18. PMJD: 33386662; PMCID: PMC8048469.

23. Flesch BK, Reil A, Nogues N, Canals C, Bugert P, Schulze TJ, Huiskes E, Porcelijn L, Hoglund P, Ratcliffe P, Schanbacher M, Kerchrom H, Kellershohn J, Bayat B. Multicenter Study on Differential Human Neutrophil Antigen 2 Expression and Underlying Molecular Mechanisms. Transfus Med Hemother. 2020 Oct;47(5):385-395. doi: 10.1159/000505523. Epub 2020 Jan 22. PMID: 33173457; PMCID: PMC7590758.

24. Schmidt DE, Wendtland Edslev P, Heitink-Polle KMJ, Mertens B, Bruin MCA, Kapur R, Vidarsson G, van der Schoot CE, Porcelijn L, van der Born JG, Rosth0j S, de Haas M. A clinical prediction score for transient versus persistent childhood immune thrombocytopenia. J Thromb Haemost. 2021 Jan;19(1):121-130. doi: 10.1111/jth.15125. Epub 2020 Nov 27. PMID: 33058474; PMCID: PMC7839442.

25. Porcelijn L, Schmidt DE, Oldert G, Hofstede-van Egmond S, Kapur R, Zwaginga JJ, de Haas M. Evolution and Utility of Antiplatelet Autoantibody Testing in Patients with Immune Thrombocytopenia. Transfus Med Rev. 2020 Oct;34(4):258-269. doi: 10.1016/j.tmrv.2020.09.003. Epub 2020 Sep 16. PMID: 33046350.

26. Zeeuw van der Laan EAN, van der Velden S, Porcelijn L, Semple JW, van der Schoot CE, Kapur R. Evaluation of Platelet Responses in Transfusion-Related Acute Lung Injury (TRALI). Transfus Med Rev. 2020 Oct;34(4):227-233. doi: 10.1016/j.tmrv.2020.08.002. Epub 2020 Sep 16. PMID: 33036839; PMCID: PMC7493815.

27. Zeeuw van der Laan EAN, van der Velden S, Porcelijn L, Semple JW, van der Schoot CE, Kapur R. Update on the pathophysiology of transfusion-related acute lung injury. Curr Opin Hematol. 2020 Nov;27(6):386-391. doi: 10.1097/MOH.0000000000000607. PMID: 32868671.

28. Zeeuw van der Laan EAN, van der Velden S, Bentlage AEH, Larsen MD, van Osch TLJ, Mok JY, Brasser G, Geerdes OM, Koeleman CAM, Nouta J, Semple JW, Porcelijn L, van Esch WJE, Wuhrer M, van der Schoot CE, Vidarsson G, Kapur R. Biological and structural characterization of murine TRALI antibody reveals increased Fe mediated complement activation. Blood Adv. 2020 Aug 25;4(16):3875-3885. doi: 10.1182/bloodadvances.2020002291. PMID: 32810222; PMCID: PMC7448591.

29. Winkelhorst D, de Vos TW, Kamphuis MM, Porcelijn L, Lopriore E, Oepkes D, van der Schoot CE, de Haas M. HIP (HPA-screening in pregnancy) study: protocol of a nationwide, prospective and observational study to assess incidence and natural history of fetal/neonatal alloimmune thrombocytopenia and identifying pregnancies at risk. BMJ Open. 2020 Jul 20;10(7):e034071. doi: 10.1136/bmjopen-2019-034071. PMID: 32690731; PMCID: PMC7375633.

30. Nagelkerke SQ, Porcelijn L, Geissler J, Tanck MWT, Huiskes E, van Bruggen R, van den Berg TK, de Haas M, Kuijpers TW. The association and functional relevance of genetic variation in low-to-medium-affinity Fe-gamma receptors with clinical platelet transfusion refractoriness. J Thromb Haemost. 2020 Aug;18(8):2047-2053. doi: 10.1111/jth.14892. Epub 2020 Jun 25. PMID: 32588549.

31. Schmidt DE, Lakerveld AJ, Heitink-Polle KMJ, Bruin MCA, Vidarsson G, Porcelijn L, de Haas M. Anti-platelet antibody immunoassays in childhood immune thrombocytopenia: a systematic review. Vox Sang. 2020 May;115(4):323-333. doi: 10.1111/vox.12894. Epub 2020 Feb 20. PMID: 32080872; PMCID: PMC7317748.

32. Schmidt DE, de Haan N, Sonneveld ME, Porcelijn L, van der Schoot CE, de Haas M, Zwaginga JJ, Wuhrer M, Vidarsson G. lgG-Fc glycosylation before and after rituximab treatment in immune thrombocytopenia. Sci Rep. 2020 Feb 20;10(1):3051. doi: 10.1038/s41598-020-59651-7. PMID: 32080262; PMCID: PMC7033207.
33. Schmidt DE, Heitink-Polle KMJ, Porcelijn L, van der Schoot CE, Vidarsson G, Bruin MCA, de Haas M. Anti-platelet antibodies in childhood immune thrombocytopenia: Prevalence and prognostic implications. J Thromb Haemost. 2020 May;18(5):1210-1220. doi: 10.1111/jth.14762. Epub 2020Apr 12. PMID: 32053276; PMCID: PMC7318215.
34. Porcelijn L, Huiskes E, de Haas M. Progress and development of platelet antibody detection. Transfus Apher Sci. 2020 Feb;59(1):102705. doi: 10.1016/j.transci.2019.102705. Epub 2019 Dec 31. PMID: 31911048.
35. Jongerius I, Porcelijn L, van Beek AE, Semple JW, van der Schoot CE, Vlaar APJ, Kapur R. The Role of Complement in Transfusion-Related Acute Lung Injury. Transfus Med Rev. 2019 Oct;33(4):236-242. doi: 10.1016/j.tmrv.2019.09.002. Epub 2019 Oct 18. PMID: 31676221; PMCID: PMC7127679.
36. Winkelhorst D, Porcelijn L, Muizelaar E, Oldert G, Huiskes E, van der Schoot CE. Fast and low-cost direct ELISA for high-throughput serological HPA-1a typing. Transfusion. 2019 Sep;59(9):2989-2996. doi: 10.1111/trf.15454. Epub 2019 Jul 22. PMID: 31329320.
37. Porcelijn L, Schmidt DE, van der Schoot CE, Vidarsson G, de Haas M, Kapur R. Anti-glycoprotein Iba autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients. Haematologica. 2020 Apr;105(4):e172-e174. doi: 10.3324/haematol.2019.228908. Epub 2019 Jul 11. PMID: 31296573; PMCID: PMC7109722.
38. Schmidt DE, Heitink-Polle KMJ, Laarhoven AG, Bruin MCA, Veldhuisen B, Nagelkerke SQ, Kuijpers TW, Porcelijn L, van der Schoot CE, Vidarsson G, de Haas M. Transient and chronic childhood immune thrombocytopenia are distinctly affected by Fc-γ receptor polymorphisms. Blood Adv. 2019 Jul 9;3(13):2003-2012. doi: 10.1182/bloodadvances.2019000068. PMID: 31270082; PMCID: PMC6616256.
39. Porcelijn L, de Haas M. Neonatal Alloimmune Neutropenia. Transfus Med Hemother. 2018 Oct;45(5):311-316. doi: 10.1159/000492949. Epub 2018 Sep 24. PMID: 31049048; PMCID: PMC6489100.
40. de Kruijff E, van Gammeren AJ, Porcelijn L, van Esser JWJ. Post-transfusion purpura in a woman with acute myeloid leukemia. Neth J Med. 2019 Feb;77(2):81-83. PMID: 30895931.
41. Porcelijn L, Huiskes E, Onderwater-Van Den Hoogen L, Folman CC, Zwaginga JJ, De Haas M. Plasma thrombopoietin levels as additional tool in clinical management of thrombocytopenic neonates. Platelets. 2020;31(1):62-67. doi: 10.1080/09537104.2019.1572877. Epub 2019 Feb 13. PMID: 30759044.
42. Rijkers M, Schmidt D, Lu N, Kramer CSM, Heidt S, Mulder A, Porcelijn L, Claas FHJ, Leebeek FWG, Jansen AJG, Jongerius I, Zeerleder SS, Vidarsson G, Voorberg J, de Haas M. Anti-HLA antibodies with complementary and synergistic interaction geometries promote classical complement activation on platelets. Haematologica. 2019 Feb;104(2):403-416. doi: 10.3324/haematol.2018.201665. Epub 2018 Sep 27. PMID: 30262558; PMCID: PMC6355480.
43. Heitink-Polle KMJ, Uiterwaal CSPM, Porcelijn L, Tamminga RYJ, Smiers FJ, van Woerden NL, Wesseling J, Vidarsson G, Laarhoven AG, de Haas M, Bruin MCA; TIKI Investigators. Intravenous immunoglobulin vs observation in childhood immune thrombocytopenia: a randomized controlled trial. Blood. 2018 Aug 30;132(9):883-891. doi: 10.1182/blood-2018-02-830844. Epub 2018 Jun 26. PMID: 29945954.

44. Rijkers M, Saris A, Heidt S, Mulder A, Porcelijn L, Claas FHJ, Bierings R, Leebeek FWG, Jansen AJG, Vidarsson G, Voorberg J, de Haas M. A subset of anti HLA antibodies induces FcγRIIa-dependent platelet activation. Haematologica. 2018 Oct;103(10):1741-1752. doi: 10.3324/haematol.2018.189365. Epub 2018 Jun 1. PMID: 29858387; PMCID: PMC6165798.

45. Porcelijn L, Huiskes E, Oldert G, Schipperus M, Zwaginga JJ, de Haas M.Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art. Br J Haematol. 2018 Aug;182(3):423-426. doi: 10.1111/bjh.15404. Epub 2018 May 29. PMID: 29808904.

46. Winkelhorst D, Oostweegel M, Porcelijn L, Middelburg RA, Zwaginga JJ, Oepkes D, van der Born JG, de Haas M, Lopriore E. Treatment and outcomes of fetal/neonatal alloimmune thrombocytopenia: a nationwide cohort study in newly detected cases. Br J Haematol. 2019 Mar;184(6):1026-1029. doi: 10.1111/bjh.15216. Epub 2018 Apr 16. PMID: 29682737.

47. Kapur R, Kim M, Rebetz J, Rondina MT, Porcelijn L, Semple JW. Low levels of interleukin-10 in patients with transfusion-related acute lung injury. Ann Transl Med. 2017 Aug;5(16):339. doi: 10.21037/atm.2017.04.37. PMID: 28861436; PMCID: PMC5566739.

48. Porcelijn L, Huiskes E, Schipperus M, van der Holt B, de Haas M, Zwaginga JJ; Dutch HOVON 64 Study Group. Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients. Blood.2017 Jun 22;129(25):3389-3391. doi: 10.1182/blood-2016-11-751719. Epub 2017 May 3. PMID: 28468795.

49. Kapur R, Kim M, Aslam R, McVey MJ, Tabuchi A, Luo A, Liu J, Li Y, Shanmugabhavananthan S, Speck ER, Zufferey A, Yousef G, Zhang H, Rondina MT, Weyrich AS, Porcelijn L, Kuebler WM, Slutsky AS, Semple JW. T regulatory cells and dendritic cells protect against transfusion-related acute lung injury via IL-10. Blood. 2017 May 4;129(18):2557-2569. doi: 10.1182/blood-2016-12-758185. Epub 2017 Feb 15. PMID: 28202460; PMCID: PMC5418638.

50. Middelburg RA, Vrielink H, Porcelijn L. Prevalence of granulocyte antibodies in never allo-exposed female and male donors. Eur J Haematol. 2017 Mar;98(3):250-253. doi: 10.1111/ejh.12823. Epub 2016 Nov 25. PMID: 27797412.

51. Kapur R, Kim M, Rondina MT, Porcelijn L, Semple JW. Elevation of C-reactive protein levels in patients with transfusion-related acute lung injury. Oncotarget. 2016 Nov 22;7(47):78048-78054. doi: 10.18632/oncotarget.12872. PMID: 27793007; PMCID: PMC5363642.

52. van Stein D, Beckers EA, Peters AL, Porcelijn L, Middelburg RA, Lardy NM, van Rhenen DJ, Vlaar AP. Underdiagnosing of antibody-mediated transfusion related acute lung injury: evaluation of cellular-based versus bead-based techniques. Vax Sang. 2016 Jul;111(1):71-8. doi: 10.1111/vox.12383. Epub 2016 Mar 18. PMID: 26991993.

53. Chong W, Turro E, Metcalfe P, Yusuf R, Merieux Y, Rigal D, Porcelijn L, Huiskes E, Lucas G, Bendukidze N, Green A, Fontao-Wendel R, Husebekk A, Dixey J, Guest A, Mushens R, Ouwehand WH, Navarrete CV. A multicenter validation of recombinant f33 integrin-coupled beads to detect human platelet antigen-1 alloantibodies in 498 cases of fetomaternal alloimmune thrombocytopenia. Transfusion. 2015 Nov;55(11):2742-51. doi: 10.1111/trf.13222. Epub 2015 Jul 14. PMID: 26173471.

54. Kapur R, Heitink-Polle KM, Porcelijn L, Bentlage AE, Bruin MC, Visser R, Roos D, Schasfoort RB, de Haas M, van der Schoot CE, Vidarsson G. C-reactive protein enhances IgG-mediated phagocyte responses and thrombocytopenia. Blood. 2015 Mar 12;125(11):1793-802. doi: 10.1182/blood-2014-05-579110. Epub 2014 Dec 29. PMID: 25548320.

55. van den Tooren-de Groot R, Ottink M, Huiskes E, van Rossum A, van der Voorn B, Slomp J, de Haas M, Porcelijn L. Management and outcome of 35 cases with foetal/neonatal alloimmune neutropenia. Acta Paediatr. 2014 Nov;103(11):e467-74. doi: 10.1111/apa.12741. Epub 2014 Jul 29. PMID: 25039288.

56. Veldhuisen B, Porcelijn L, Ellen van der Schoot C, de Haas M. Molecular typing of human platelet and neutrophil antigens (HPA and HNA). Transfus Apher Sci. 2014 Apr;50(2):189-99. doi: 10.1016/j.transci.2014.02.014. Epub 2014 Mar 6. PMID: 24685245.

57. Kamphuis MM, Paridaans NP, Porcelijn L, Lopriore E, Oepkes D. Incidence and consequences of neonatal alloimmune thrombocytopenia: a systematic review. Pediatrics. 2014 Apr;133(4):715-21. doi: 10.1542/peds.2013-3320. Epub 2014 Mar 3. PMID: 24590747.

58. van Bladel ER, Laarhoven AG, van der Heijden LB, Heitink-Polle KM, Porcelijn L, van der Schoot CE, de Haas M, Roest M, Vidarsson G, de Groot PG, Bruin MC. Functional platelet defects in children with severe chronic ITP as tested with 2 novel assays applicable for low platelet counts. Blood. 2014 Mar 6;123(10):1556-63. doi: 10.1182/blood-2013-08-519686. Epub 2014 Jan 2. PMID: 24385537.

59. Porcelijn L, Huiskes E, Comijs-van Osselen I, Chhatta A, Rathore V, Meyers M, de Haas M. A new bead-based human platelet antigen antibodies detection assay versus the monoclonal antibody immobilization of platelet antigens assay. Transfusion. 2014 Jun;54(6):1486-92. doi: 10.1111/trf.12509. Epub 2013 Dec 3. PMID: 24299453.

60. Kapur R, Kustiawan I, Vestrheim A, Koeleman CA, Visser R, Einarsdottir HK, Porcelijn L, Jackson D, Kumpel B, Deelder AM, Blank D, Skogen B, Killie MK, Michaelsen TE, de Haas M, Rispens T, van der Schoot CE, Wuhrer M, Vidarsson G. A prominent lack of IgG1-Fc fucosylation of platelet alloantibodies in pregnancy. Blood. 2014 Jan 23;123(4):471-80. doi: 10.1182/blood-2013-09-527978. Epub 2013 Nov 15. PMID: 24243971; PMCID: PMC3901064.

61. Lucas G, Porcelijn L, Fung YL, Green F, Reil A, Hopkins M, Schuller R, Green A, de Haas M, Bux J. External quality assessment of human neutrophil antigen (HNA)-specific antibody detection and HNA genotyping from 2000 to 2012. Vax Sang. 2013 Oct;105(3):259-69. doi: 10.1111/vox.12041. Epub 2013 May 11. PMID: 23663230.

62. De Cuyper IM, Meinders M, van de Vijver E, de Korte D, Porcelijn L, de Haas M, Eble JA, Seeger K, Rutella S, Pagliara D, Kuijpers TW, Verhoeven AJ, van den Berg TK, Gutierrez L. A novel flow cytometry-based platelet aggregation assay. Blood. 2013 Mar 7;121(10):e70-80. doi: 10.1182/blood-2012-06-437723. Epub 2013 Jan 9. PMID: 23303822.

63. Korterink JJ, Boersma B, Schoorl M, Porcelijn L, Bartels PC. Pseudothrombocytopenia in a neonate due to mother? Eur J Pediatr. 2013 Jul;172(7):987-9. doi: 10.1007/s00431-012-1892-4. Epub 2012 Nov 16. PMID: 23229187.

64. Madani K, Kamphuis MM, Lopriore E, Porcelijn L, Oepkes D. Delayed diagnosis of fetal and neonatal alloimmune thrombocytopenia: a cause of perinatal mortality and morbidity. BJOG. 2012 Dec;119(13):1612-6. doi: 10.1111/j.1471-0528.2012.03503.x. Epub 2012 Oct 19. PMID: 23078538.

65. Albersen A, Porcelijn L, Schilders J, Zuetenhorst H, Njo T, Hamberg P. Sunitinib-associated pseudothrombocytopenia induced by IgM antibody. Platelets. 2013;24(7):566-70. doi: 10.3109/09537104.2012.731545. Epub 2012 Oct 15. PMID: 23066976.

66. MOiier MC, Porcelijn L, Vlaar AP. Prevention of immune-mediated transfusion related acute lung injury; from bloodbank to patient. Curr Pharm Des. 2012;18(22):3241-8. doi: 10.2174/1381612811209023241. PMID: 22621273.

67. Middelburg RA, Beckers EA, Porcelijn L, Lardy N, Wiersum-Osselton JC, Schipperus MR, Vrielink H, Briet E, van der Born JG. Alla-exposure status and leucocyte antibody positivity of blood donors show a similar relation with TRALI. Transfus Med. 2012 Apr;22(2):128-32. doi: 10.1111/j.1365-3148.2012.01140.x. Epub 2012 Mar 1. PMID: 22380759.

68. Achterbergh R, Vermeer HJ, Curtis BR, Porcelijn L, Aster RH, Deenik W, Daemen-Gubbels C. Thrombocytopenia in a nutshell. Lancet. 2012 Feb 25;379(9817):776. doi: 10.1016/S0140-6736(11)61643-7. PMID: 22364760; PMCID: PMC4357488.

69. Middelburg RA, van Stein D, Atsma F, Wiersum-Osselton JC, Porcelijn L, Beckers EA, Briet E, van der Born JG. Alloexposed blood donors and transfusion related acute lung injury: a case-referent study. Transfusion. 2011 Oct;51(10):2111-7. doi: 10.1111/j.1537-2995.2011.03118.x. Epub 2011 Mar 24. PMID: 21985044.

70. Vlaar AP, Cornet AD, Hofstra JJ, Porcelijn L, Beishuizen A, Kulik W, Vroom MB, Schultz MJ, Groeneveld AB, Juffermans NP. The effect of blood transfusion on pulmonary permeability in cardiac surgery patients: a prospective multicenter cohort study. Transfusion. 2012 Jan;52(1):82-90. doi: 10.1111/j.1537-2995.2011.03231.x. Epub 2011 Jul 11. PMID: 21745211.

71. Vlaar AP, Hofstra JJ, Determann RM, Veelo DP, Paulus F, Kulik W, Korevaar J, de Mal BA, Koopman MM, Porcelijn L, Binnekade JM, Vroom MB, Schultz MJ, Juffermans NP. The incidence, risk factors, and outcome of transfusion-related acute lung injury in a cohort of cardiac surgery patients: a prospective nested case-control study. Blood. 2011 Apr 21;117(16):4218-25. doi: 10.1182/blood-2010-10-313973. Epub 2011 Feb 16. PMID: 21325598.

72. Middelburg RA, Porcelijn L, Lardy N, Briet E, Vrielink H. Prevalence of leucocyte antibodies in the Dutch donor population. Vax Sang. 2011 Apr;100(3):327-35. doi: 10.1111/j.1423-0410.2010.01420.x. Epub 2010 Oct 15. PMID: 20946548.

73. Vlaar AP, Porcelijn L, van Rooijen-Schreurs IH, Lardy NM, Kersten MJ, Juffermans NP. The divergent clinical presentations of transfusion-related acute lung injury illustrated by two case reports. Med Sci Monit. 2010 Oct;16(1O):CS129-34. PMID: 20885357.

74. Porcelijn L, Abbink F, Terraneo L, Onderwater-vd Hoogen L, Huiskes E, de Haas M. Neonatal alloimmune neutropenia due to immunoglobulin G antibodies against human neutrophil antigen-5a. Transfusion. 2011 Mar;51(3):574-7. doi: 10.1111/j.1537-2995.2010.02858.x. Epub 2010Aug 23. PMID: 20735765.

75. Kamphuis MM, Paridaans N, Porcelijn L, De Haas M, Van Der Schoot CE, Brand A, Bonsel GJ, Oepkes D. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. BJOG. 2010 Oct;117(11):1335-43. doi: 10.1111/j.1471-0528.2010.02657.x. PMID: 20618318.

76. van Stein D, Beckers EA, Sintnicolaas K, Porcelijn L, Danovic F, Wollersheim JA, Brand A, van Rhenen DJ. Transfusion-related acute lung injury reports in the Netherlands: an observational study. Transfusion. 2010 Jan;50(1):213-20. doi: 10.1111/j.1537-2995.2009.02345.x. Epub 2009 Aug 18. PMID: 19694998.

77. Pereboom IT, de Boer MT, Haagsma EB, van der Heide F, Porcelijn L, Lisman T, Porte RJ. Transmission of idiopathic thrombocytopenic purpura during orthotopic liver transplantation. Transpl Int. 2010 Feb;23(2):236-8. doi: 10.1111/j.1432-2277.2009.00936.x. Epub 2009 Aug 18. PMID: 19691662.

78. Ghevaert C, Rankin A, Huiskes E, Porcelijn L, Javela K, Kekomaki R, Bakchoul T, Santoso S, Nutland S, Smyth DJ, Smith GA, McBride S, Watkins NA, Ouwehand WH. Alloantibodies against low-frequency human platelet antigens do not account for a significant proportion of cases of fetomaternal alloimmune thrombocytopenia: evidence from 1054 cases. Transfusion. 2009 Oct;49(10):2084-9. doi: 10.1111/j.1537-2995.2009.02246.x. Epub 2009 Jun 4. PMID: 19500319.

79. ISBT Working Party on Granulocyte lmmunobiology; Bierling P, Bux J, Curtis B, Flesch B, Fung L, Lucas G, Macek M, Muniz-Diaz E, Porcelijn L, Reil A, Sachs U, Schuller R, Tsuno N, Uhrynowska M, Urbaniak S, Valentin N, Wikman A, Zupanska B. Recommendations of the ISBT Working Party on Granulocyte lmmunobiology for leucocyte antibody screening in the investigation and prevention of antibody- mediated transfusion-related acute lung injury. Vax Sang. 2009 Apr;96(3):266-9. doi: 10.1111/j.1423-0410.2008.01144.x. Epub 2008 Dec 29. PMID: 19207164.

80. Porcelijn L, Huiskes E, Maatman R, de Kreuk A, de Haas M. Acquired Glanzmann's thrombasthenia caused by glycoprotein lib/IIIa autoantibodies of the immunoglobulin G1 (lgG1), lgG2 or lgG4 subclass: a study in six cases. Vax Sang. 2008 Nov;95(4):324-30. doi: 10.1111/j.1423-0410.2008.01093.x. PMID: 19138263.

81. van Gils JM, Stutterheim J, van Duijn TJ, Zwaginga JJ, Porcelijn L, de Haas M, Hordijk PL. HPA-1a alloantibodies reduce endothelial cell spreading and monolayer integrity. Mol Immunol. 2009 Jan;46(3):406-15. doi: 10.1016/j.molimm.2008.10.015. Epub 2008 Nov 26. PMID: 19038456.

82. Wuhrer M, Porcelijn L, Kapur R, Koeleman CA, Deelder A, de Haas M, Vidarsson G. Regulated glycosylation patterns of lgG during alloimmune responses against human platelet antigens. J Proteome Res. 2009 Feb;8(2):450-6. doi: 10.1021/pr800651j. PMID: 18942870.

83. Wiersum-Osselton JC, Porcelijn L, van Stein D, Vlaar AP, Beckers EA, Schipperus MR. Transfusiegerelateerde acute longbeschadiging (TRALI) in Nederland in 2002-2005 [Transfusion-related acute lung injury (TRALI) in the Netherlands in 2002-2005]. Ned Tijdschr Geneeskd. 2008 Aug 9;152(32):1784-8. Dutch. PMID: 18754313.

84. Porcelijn L, van Beers W, Gratama JW, van't Veer M, De Smet A, Sintnicolaas K. External quality assessment of platelet serology and human platelet antigen genotyping: a 10-year review. Transfusion. 2008 Aug;48(8):1699-706. doi: 10.1111/j.1537-2995.2008.01748.x. Epub 2008 May 14. PMID: 18482186.

85. Porcelijn L, Van den Akker ES, Oepkes D. Fetal thrombocytopenia. Semin Fetal Neonatal Med. 2008Aug;13(4):223-30. doi: 10.1016/j.siny.2008.02.008. Epub 2008 Apr 8. PMID: 18400574.

86. Serrarens-Janssen VM, Semmekrot BA, Novotny VM, Porcelijn L, Lotgering FK, Delemarre FM, Steegers EA. Fetal/neonatal allo-immune thrombocytopenia (FNAIT): past, present, and future. Obstet Gynecol Surv. 2008 Apr;63(4):239-52. doi: 10.1097/OGX.0b013e31816412d3. PMID: 18348738.

87. Kanhai HH, Porcelijn L, Engelfriet CP, Reesink HW, Panzer S, Ulm B, Goldman M, Bonacossa I, Richard L, David M, Taaning E, Hedegaard M, Kaplan C, Kiefel V, Meyer 0, Salama A, Morelati F, Greppi N, Marconi M, Tassis B, Tsuno NH, Takahashi K, Oepkes D, Porcelijn L, Kanhai H, Osnes LT, HusebekkA, Killie MK, Kjeldsen-Kragh J, Zupanska B, Muniz-Diaz E, Nogues N, Parra J, Urbaniak SJ, Cameron A. Management of alloimmune thrombocytopenia. Vax Sang. 2007 Nov;93(4):370-85. doi: 10.1111/j.1423-0410.2007.00980.x. PMID: 18070283.

88. Berry J, Allen D, Porcelijn L, de Haas M, Kekomaki R, Kaplan C, Ouwehand WH, Metcalfe P. Collaborative studies to establish the first World Health Organization International Standard for detection of human antibody against human platelet antigen-3a. Vax Sang. 2007 Nov;93(4):309-15. doi: 10.1111/j.1423-0410.2007.00899.x. PMID: 18070275.

89. de Haan TR, van den Akker ES, Porcelijn L, Oepkes D, Kroes AC, Walther FJ. Thrombocytopenia in hydropic fetuses with parvovirus 819 infection: incidence, treatment and correlation with fetal B19 viral load. BJOG. 2008 Jan;115(1):76-81. doi: 10.1111/j.1471-0528.2007.01555.x. PMID: 18053103.

90. Wester JP, Leyte A, Oudemans-van Straaten HM, Bosman RJ, van der Spoel JI, Haak EA, Porcelijn L, Zandstra OF. Low-dose fondaparinux in suspected heparin induced thrombocytopenia in the critically ill. Neth J Med. 2007 Mar;65(3): 101-8. PMID: 17387236.

91. Kaplan C, Porcelijn L, Vanlieferinghen P, Julien E, Bianchi F, Martageix C, Bertrand G, Jallu V. Anti-HPA-9bw (Maxa) fetomaternal alloimmunization, a clinically severe neonatal thrombocytopenia: difficulties in diagnosis and therapy and report on eight families. Transfusion. 2005 Nov;45(11):1799-803. doi: 10.1111/j.1537-2995.2005.00606.x. PMID: 16271107.

92. Porcelijn L, Folman CC, de Haas M, Kanhai HH, Murphy MF, van dem Borne AE, Busse! JB. Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia. Pediatr Res. 2002 Jul;52(1):105-8. doi: 10.1203/00006450-200207000-00019. PMID: 12084855.

93. Lucas G, Rogers S, de Haas M, Porcelijn L, Bux J. Report on the Fourth International Granulocyte Immunology Workshop: progress toward quality assessment. Transfusion. 2002 Apr;42(4):462-8. doi: 10.1046/j.1525-1438.2002.00053.x. PMID: 12076294.

94. Glade-Bender J, McFarland JG, Kaplan C, Porcelijn L, Bussel JB. Anti-HPA-3A induces severe neonatal alloimmune thrombocytopenia. J Pediatr. 2001 Jun;138(6):862-7. doi: 10.1067/mpd.2001.114029. PMID: 11391330.

95. Engelfriet CP, Reesink HW, Garratty G, Knight R, de Silva M, Contreras M, Overbeeke MA, Porcelijn L, Lin M, So CC, Schmidt-Melbye AC, Heier HE, Sokol RJ, Booker DJ, Stamps R, Barbolla L, Zamora C, Hernandez-Jodra M, Zupar'lskaB, Goldman M, Long A, Decary F. The detection of alloantibodies against red cells in patients with warm-type autoimmune haemolytic anaemia. Vox Sang. 2000;78(3):200-7. doi: 10.1159/000031181. PMID: 10838523.

96. de Haas M, Muniz-Diaz E, Alonso LG, van der Kolk K, Kos M, Buddelmeijer L, Porcelijn L, von dem Borne AE. Neutrophil antigen 5b is carried by a protein, migrating from 70 to 95 kDa, and may be involved in neonatal alloimmune neutropenia. Transfusion. 2000 Feb;40(2):222-7. doi: 10.1046/j.1537-2995.2000.40020222.x. PMID: 10686007.

97. van dem Borne AE, Folman C, Linthorst GE, Porcelijn L, van den Oudenrijn S, van der Schoot E, de Haas M. Thrombopoietin: its role in platelet disorders and as a new drug in clinical medicine. Baillieres Clin Haematol. 1998 Jun;11(2):427-45. doi: 10.1016/s0950-3536(98)80059-0. PMID: 10097818.

98. van dem Borne AE, Folman C, Linthorst GE, Porcelijn L, van den Oudenrijn S, van der Schoot E, de Haas M. Thrombopoietin and its receptor: structure, function and role in the regulation of platelet production. Baillieres Clin Haematol. 1998 Jun;11(2):409-26. doi: 10.1016/s0950-3536(98)80058-9. PMID: 10097817.

99. Porcelijn L, van dem Borne AE. Immune-mediated thrombocytopenias: basic and immunological aspects. Baillieres Clin Haematol. 1998 Jun;11(2):331-41. doi: 10.1016/s0950-3536(98)80052-8. PMID: 10097811.

100. Porcelijn L, Folman CC, Bossers B, Huiskes E, Overbeeke MA, v d Schoot CE, de Haas M, van dem Borne AE. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. Thromb Haemost. 1998 Jun;79(6):1101-5. PMID: 9657431.

101. Porcelijn L, Kanhai HH. Fetal thrombocytopenia. Curr Opin Obstet Gynecol. 1998Apr;10(2):117-22. doi: 10.1097/00001703-199804000-00007. PMID: 9551306.

102. Westman P, Hashemi-Tavoularis S, Blanchette V, Kekomaki S, Laes M, Porcelijn L, Kekomaki R. Maternal DRB1*1501, DQA1*0102, DQB1*0602 haplotype in fetomaternal alloimmunization against human platelet alloantigen HPA-6b (GPIIIa Gln489). Tissue Antigens. 1997 Aug;50(2):113-8. doi: 10.1111/j.1399-0039.1997.tb02849.x. PMID: 9271820.

103. Vlasveld LT, de Haas M, Ermens AA, Porcelijn L, van Marion-Kievit JA, van dem Borne AE. G-CSF-induced decrease of the anti-granulocyte autoantibody levels in a patient with autoimmune granulocytopenia. Ann Hematol. 1997 Jul Aug;75(1-2):59-64. doi: 10.1007/s002770050313. PMID: 9322685.

104. Kanhai HH, Porcelijn L, van Zoeren D, Klumper F, Vietor H, Meerman RH, Brand A. Antenatal care in pregnancies at risk of alloimmune thrombocytopenia: report of 19 cases in 16 families. Eur J Obstet Gynecol Reprod Biol. 1996 Sep;68(1-2):67-73. doi: 10.1016/0301-2115(96)02485-2. PMID: 8886684.

105. Beckers EA, Porcelijn L, Ligthart P, Vermey H, Von dem Borne AE, Overbeeke MA, van Rhenen DJ. The RoHar antigenic complex is associated with a limited number of D epitopes and alloanti-D production: a study of three unrelated persons and their families. Transfusion. 1996 Feb;36(2):104-8. doi: 10.1046/j.1537-2995.1996.36296181919.x. PMID: 8614957.

106. Overbeeke MA, Kooyman PJ, Porcelijn L, von dem Borne AE. Assessment of monoclonal antibodies by the monocyte-mediated ADCC assay. Transfus Clin Biol. 1996;3(6):473-4. doi: 10.1016/s1246-7820(96)80065-6. PMID: 9018810.

107. Overbeeke MA, Van Rhenen DJ, Ligthart PC, Vermey J, de Ruiter JA, Kruit GV, Langendam M, Porcelijn L, von dem Borne AE. Reactions of anti-D monoclonal antibodies with rhesus D variant cells. Transfus Clin Biol. 1996;3(6):397-9. doi: 10.1016/s1246-7820(96)80052-8. PMID: 9018797.

108. Noris P, Simsek S, de Bruijne-Admiraal LG, Porcelijn L, Huiskes E, van der Vlist GJ, van Leeuwen EF, van der Schoot CE, von dem Borne AE. Max(a), a new low-frequency platelet-specific antigen localized on glycoprotein IIb, is associated with neonatal alloimmune thrombocytopenia. Blood. 1995 Aug 1;86(3):1019-26. PMID: 7620155.

109. Porcelijn L, Maat-Kievit JA, van Haeringen A. Dysmaturitas als symptoom van het ectrodactylie-ectodermale dysplasie-clefting (EEC) syndroom [Dysmaturity as symptom of the ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome]. Tijdschr Kindergeneeskd. 1993 Jun;61(3):96-9. Dutch. PMID: 8211943.

CURRICULUM VITAE

Leendert Porcelijn, author of this thesis was born in Hillegom on September 19th 1959. Having a sister and brother, he is the youngest son of Salomon Porcelijn and Jopie Porcelijn-van Kampen. Living close to the sea and dunes, between the bulb fields and half an hour's bike ride from Haarlem and Leiden, secondary school education was completed at the St Bernardus MAVO in Hillegom and the HAVO and Atheneum at the Cornhert lyceum in Haarlem.

He than moved to Amsterdam to study Medicine at the University of Amsterdam. After graduation he worked as Senior House Officer, Accident and Emergency Department, West Wales General Hospital, Carmarthen, South Wales, as Assistant Paediatrician, VU Medical Centre, Amsterdam and as Assistant Neonatologist, Leiden University Medical Centre, Leiden.

In 1993 he started working as Head Platelet/Leukocyte Serology and staff member of the Immunohematology Diagnostics department at the Central Laboratory of the Blood Transfusion (CLB), that was later merged with the blood banks to form Sanquin Blood supply. During his time at CLB/Sanquin, he graduated as Transfusion Physician, was president of the Dutch society of blood transfusion (NVB), presented numerous lectures, completed several scientific and managerial courses and was (co)author of more than 100 scientific papers.

Leendert is married to Ineke Witteman and they have two daughters,
Iris and Lisa.
He enjoys running, golfing and playing guitar.

DANKWOORD

Uiteindelijk dan toch. Vaak gedacht dat het samenstellen van een proefschrift wel mooi zou zijn en dat ik misschien wel genoeg artikelen geschreven had om dit te realiseren. Het heeft echter de bezieling en motivatie van Masja en Jaap Jan nodig gehad om van een wens realiteit te maken. Masja de Haas ik bedank je natuurlijk voor dit proefschrift, voor al je inzicht (een half woord was genoeg), je enorme kennis en voor de jaren van fijne en leuke samenwerking. Jaap Jan, van jouw positieve houding heb ik altijd kunnen genieten en het heeft mij echt over een drempel geholpen. Mijn bewondering voor je kritische klinische blik. Ik vergeet niet je opmerking: 'leuk dat je uitgebreid ingaat op de laboratorium kant, maar waar staat nu wat dit voor de kliniek betekent'. Daarbij moet ook nog genoemd worden dat de snelheid waarmee jij de concept artikelen nakijkt, legendarisch is. Beste Rick, het is een waar genoegen om met jou over studies na te denken en congressen te bezoeken. Mooi om te zien hoe je een prachtig internationaal netwerk met veel onderzoekers hebt opgebouwd. Dit is voor Sanquin en voor ons laboratorium van grote waarde. Natuurlijk moet ik Marijke Overbeeke bedanken, alleen al omdat zij het gedurfd heeft om mij aan te nemen, maar meer omdat ik door haar heb kunnen zien hoe je een goed team kan opbouwen en motiveren. Elly Huiskes, jouw waarde voor het trombocyten-leukocyten laboratorium en voor mij persoonlijk is enorm geweest. Je organisatorische talent en geweldige geheugen heeft het voor mij (voor ons) allemaal een stuk eenvoudiger gemaakt. Suzanne, dat jij Elly hebt willen opvolgen heeft veel goeds gebracht. Iedere keer weer heb je mij op het goede praktische spoor kunnen zetten. Dit natuurlijk in nauwe samenwerking met alle collega's binnen het Trombo-leuko team, nu bestaande uit Ilona, Gonda, Michelle, Laura, Michelle, Roxanne, Jill en Marrie. Ik kan er kort over zijn, dat ons lab al jaren een nationaal en internationaal top referentie laboratorium is, komt door jullie. Claudia Folman, speciale dank aan jou, voor het verder professionaliseren van de afdeling Immunohematologie Diagnostiek en voor de hele leuke tijd die we hier hebben gehad. Jessie en Anne, de 'paarse gang' boft maar met zulke fijne collega's. Never a dull moment, waarvoor dank. Ik moet nog veel anderen bedanken, waaronder Peer, Piet, Margriet, Daan, Conny, Junior, Ellen, Gestur, Martin, Romy, David, enz. enz. Teveel om op te noemen, maar weet dat ik aan jullie denk.

Lieve Ineke, zonder jou had dit niet gekund. Ik hou van je en van onze prachtige dochters Iris en Lisa.