The diagnostic value of plasma thrombopoietin levels and platelet autoantibodies

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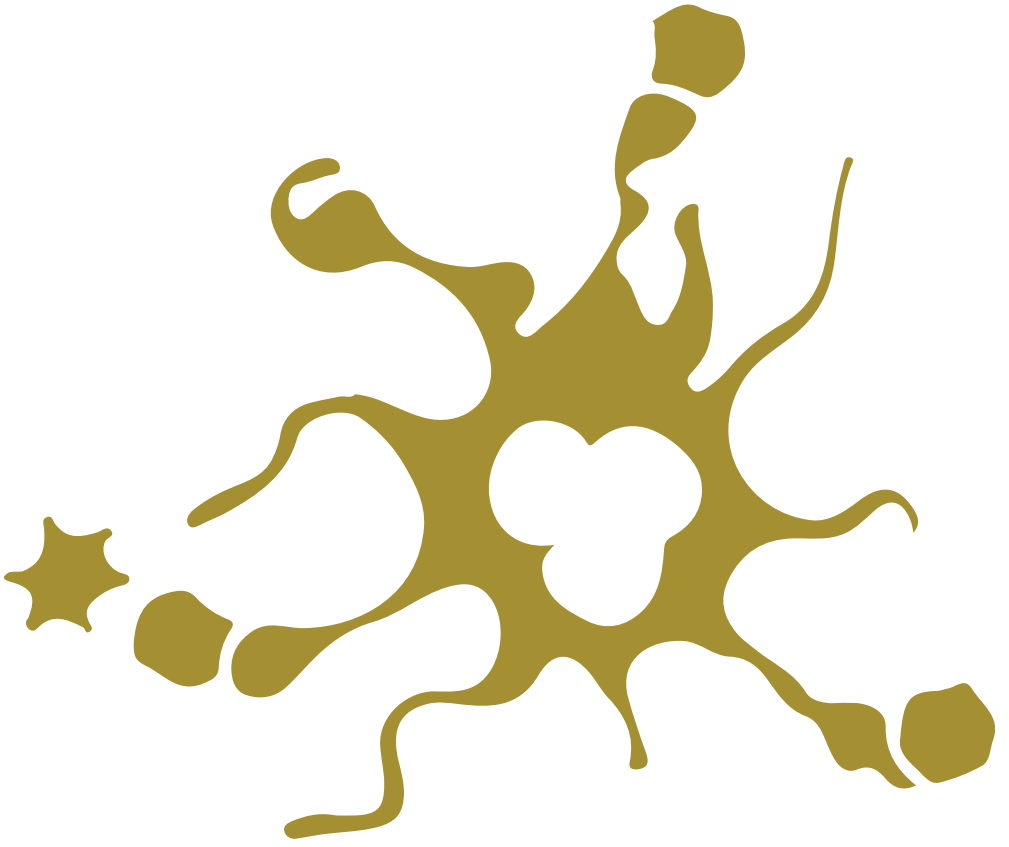

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CHAPTER 1

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.

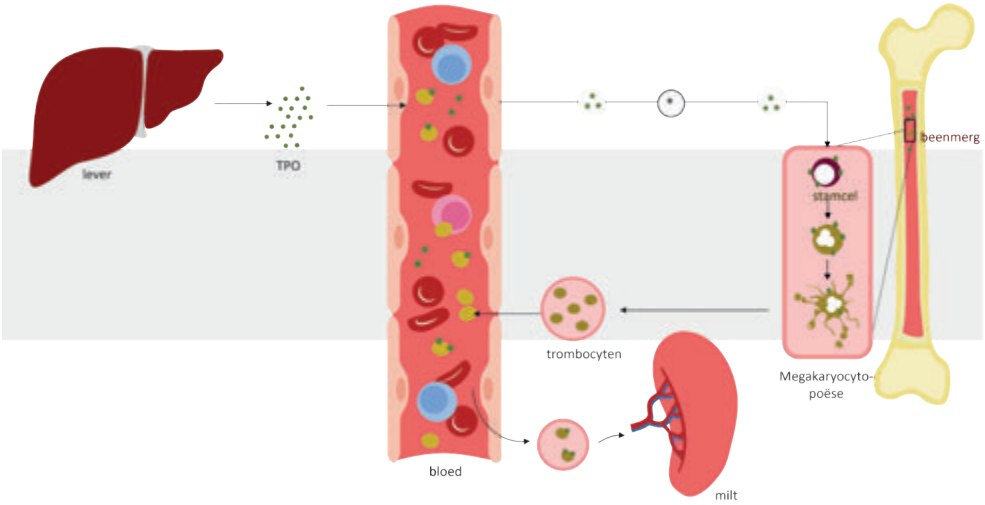
Platelet production

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

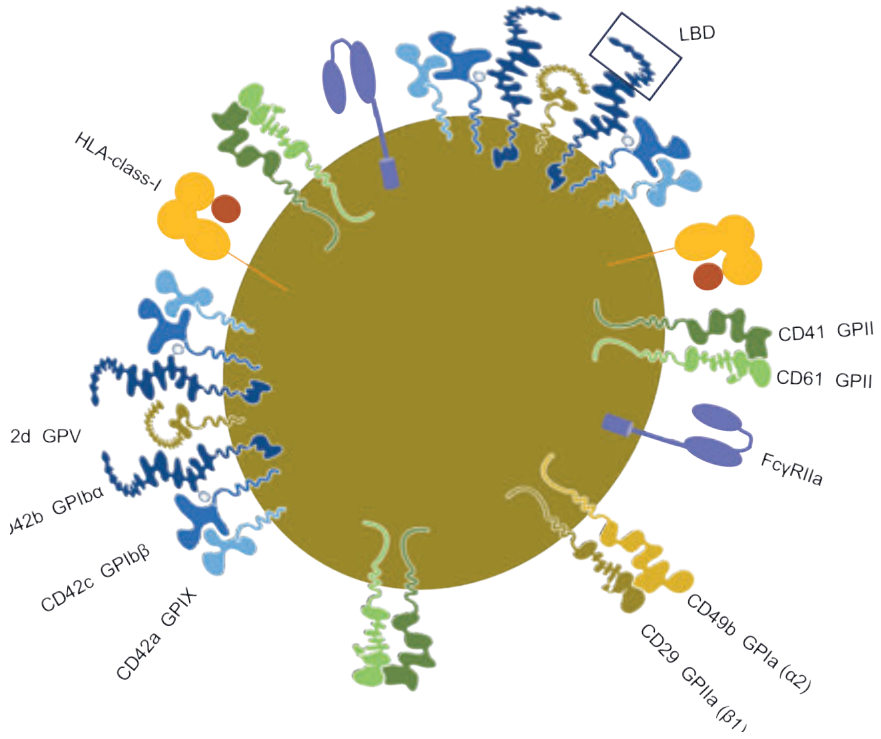
The life span of produced platelets is dependent on the rates of their clearance from the circulation and subsequent destruction in the reticuloendothelial system in spleen and liver where macrophages target platelets. The obligatory physiologic 'disappearance' of 'old platelets' in this respect is intriguing. 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]

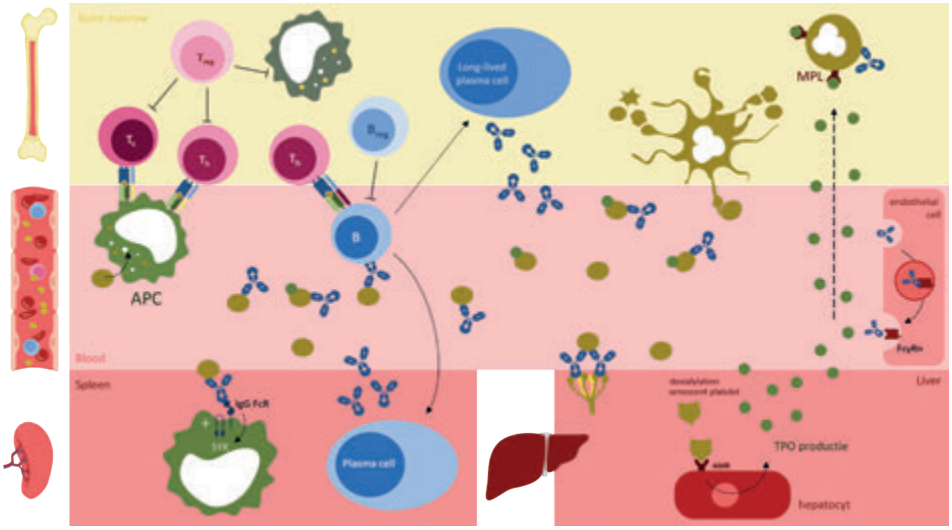
Autoantibody mediated platelet clearance and destruction.
ITP is an isolated thrombocytopenia and results from increased platelet clearance and probably also some decrease in platelet production. The underlying cause is not yet fully understood although increased platelet destruction via the reticulo-endothelial 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.

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