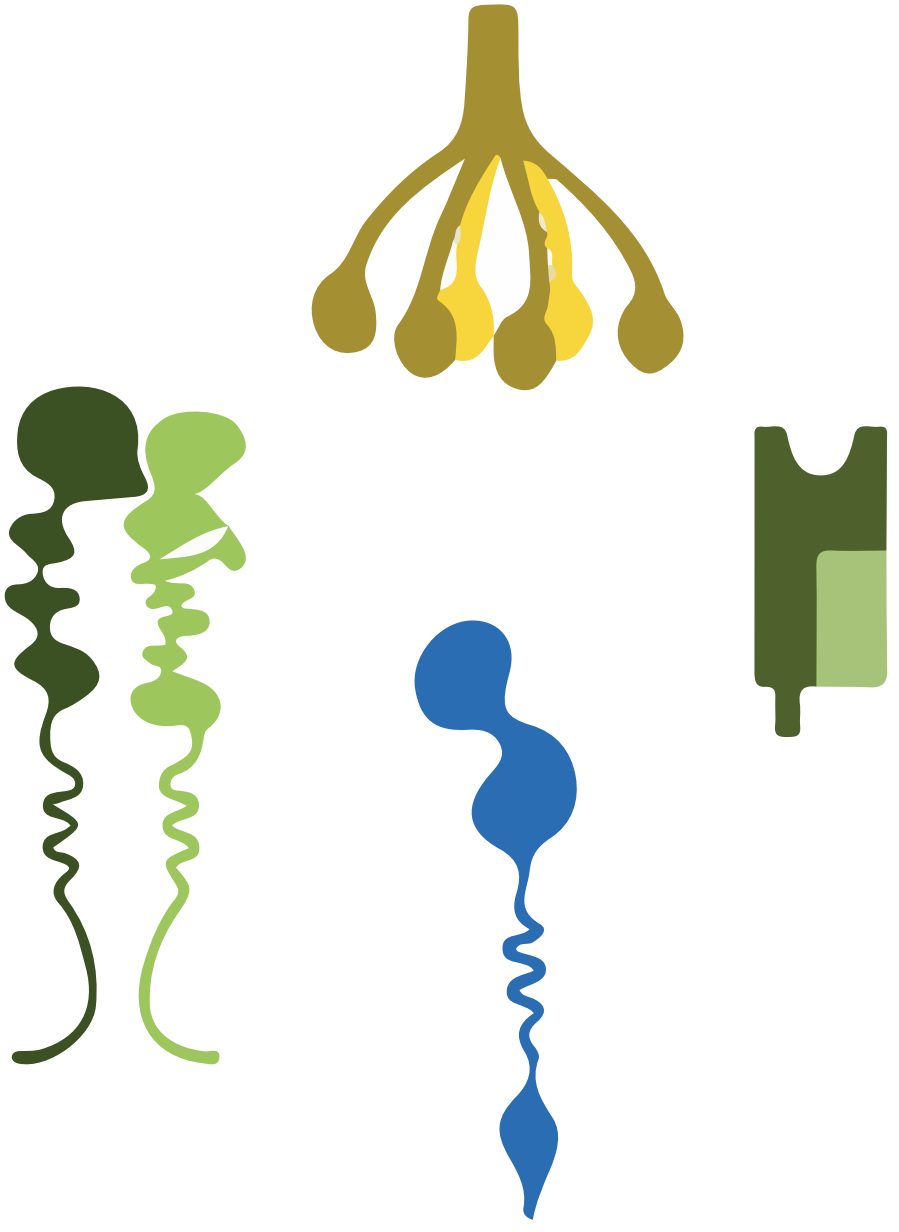
The diagnostic value of plasma thrombopoietin levels and platelet autoantibodies

Porcelijn, L.



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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

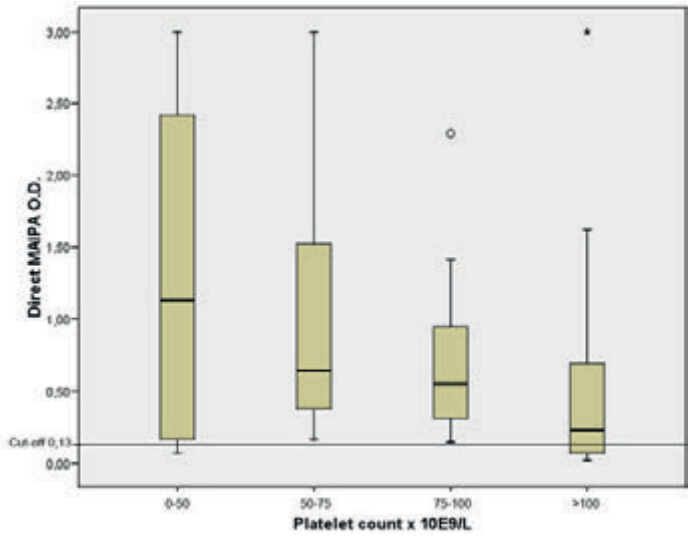
As described in this thesis, we made three observations that underlie major improvement of platelet autoantibody detection.18 First we redesigned the glycoprotein-specific monoclonal antibody immobilization of platelet antigens (MAIPA) assay for platelet autoantibody detection by validating the optimal 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



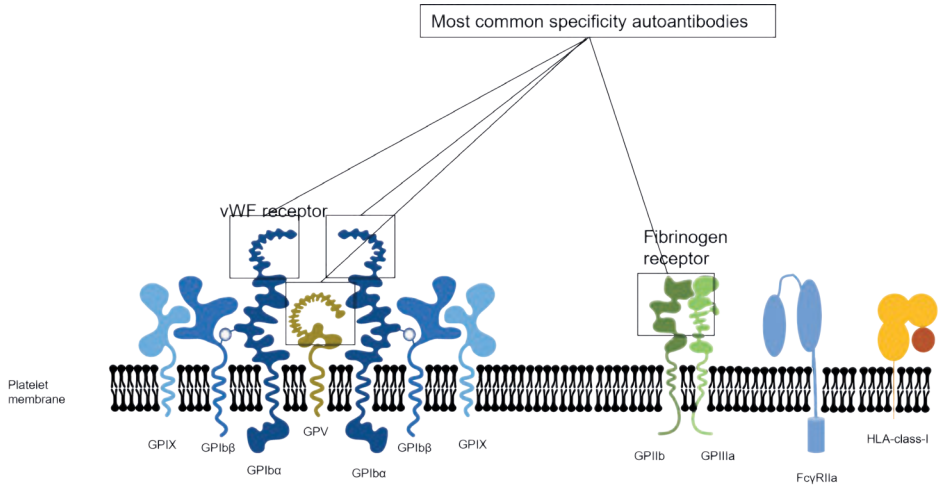
The mean direct (i.e. detecting autoantibodies bound on the patient platelets) monoclonal antibody immobilization of platelet antigens (MAIPA) results (optical densitiy) used for this figure are the highest O.D. values detected for either GPIIb/IIIa, GPIb/IX or GPV.

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.

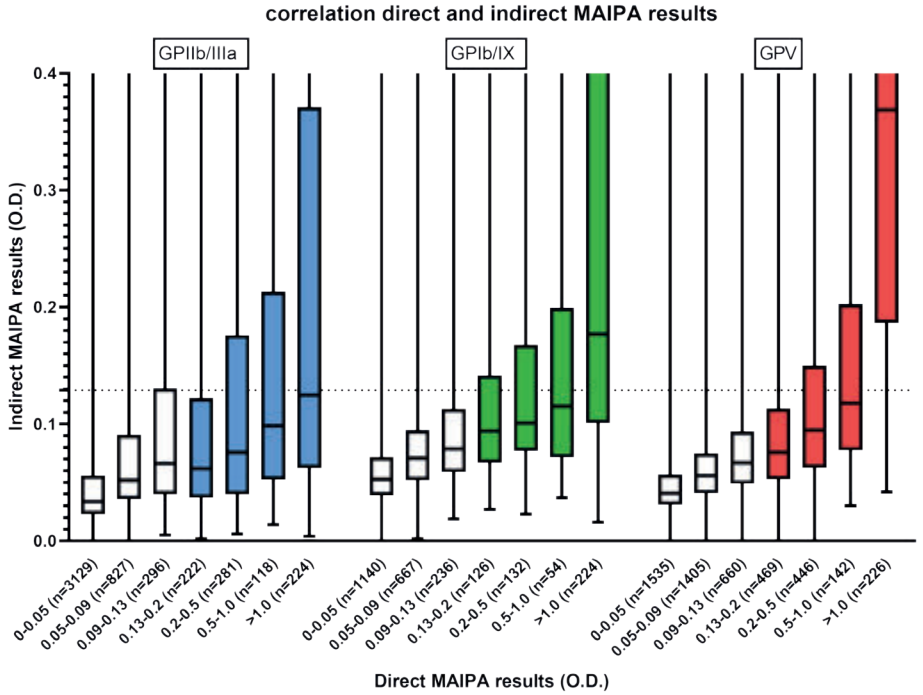
Unfortunate, but intriguing, is the fact that no antibodies can be detected in approximately 20% of patients with strongly suspected ITP. Partly, this can be explained by limitations of the tests to distinguish weak binding autoantibodies from background 'noise'.18 As shown in chapter 8, comparing the 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.

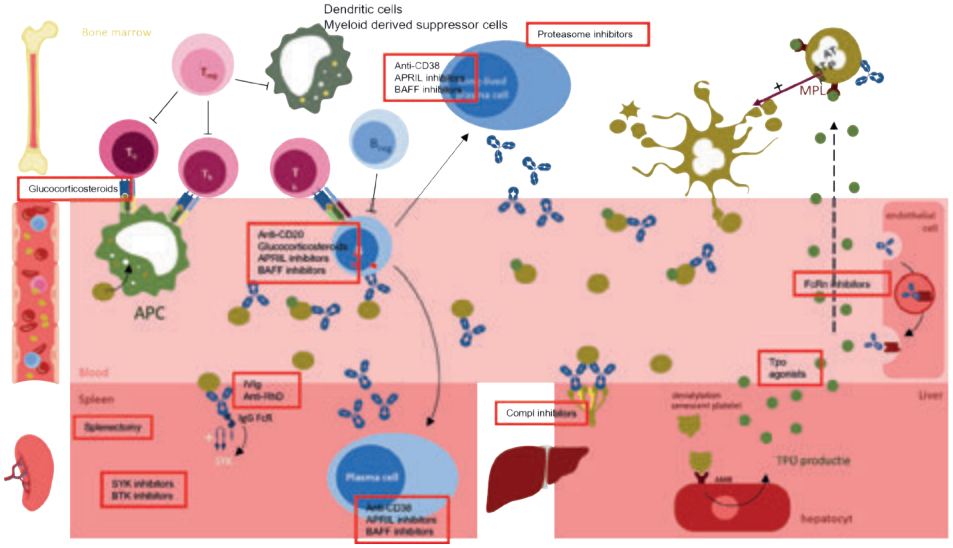
In recent decades, new therapeutic drugs, such as TPO-RA, anti-CD20 , SYK-inhibitors, FcRn-inhibitors, CTLA4-Ig fusion proteins and very recent BAFF and APRIL inhibitors (Table..) became available or are currently being investigated in clinical trials. To increase comparability between these studies, 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.

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