Individualized prognosis in childhood immune thrombocytopenia

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Part I

INTRODUCTION

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CHILDHOOD IMMUNE THROMBOCYTOPENIA

Childhood immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by an incompletely understood pathogenesis, impaired quality of life, and very divergent clinical disease courses. The diagnosis is established in a thrombocytopenic child (platelet count $< 100x10^9$/L) with mucocutaneous bleeding symptoms after the exclusion of alternative causes of thrombocytopenia by history, physical examination, and basic laboratory tests [1]. Clinically, children with ITP appear well except for the bleeding symptoms. Thus, ITP is diagnosed as a clinical syndrome based on clinical signs; no gold-standard test is available for the diagnosis. The annual incidence is 2 - 6 per 100 000 children [2]. Although ITP occurs at any age, it is observed more frequently in children younger than six years [3]. Approximately 50-55% of children with ITP experienced a preceding infectious episode or vaccination in the previous month [4, 5]. The disease course of ITP is distinguished in three phases: newly diagnosed ($\leq$ 3 months), persistent (> 3 months), and chronic ITP ($\geq$ 12 months). Notably, childhood ITP has different disease characteristics than adult ITP, featuring more substantial cutaneous and mucous bleeding symptoms and a high rate of post-infectious and self-limiting cases [6].

1.1 MORBIDITY AND MORTALITY

ITP is categorized as a benign hematological disorder, but this classification is misleading because of the significant morbidity of the disease [7]. ITP profoundly influences the health-related quality of life of affected children and their next-of-kin [8, 9]. In a systematic review of studies that used a predefined bleeding measurement tool, severe bleeding occurred in 20% of children with ITP (defined as at least extensive mucosal bleeding in most studies, i.e., Buchanan score grade $\geq$ 3)[10]. End-organ or internal bleeding that requires medical intervention occurs in 3% of children [11, 12]. Intracranial hemorrhage (ICH) occurs in 0.5% of affected children and is the most feared complication, next to severe bleeding [3, 10]. Mortality during hospitalization is approximately 2% with any bleeding and 25% with ICH [13, 14]. Apart from bleeding, the morbidity of the disease is characterized by fatigue, fear of bleeding episodes, and disease-associated health care procedures (fear of doctor visits, venipuncture, medication). The morbidity is more severe in patients who exhibit persistent thrombocytopenia and chronic disease courses [8, 9].

1.2 DISEASE MECHANISMS

The exact cause of ITP is not known and may not be the same for all patients diagnosed with ITP. The current explanatory paradigm features:

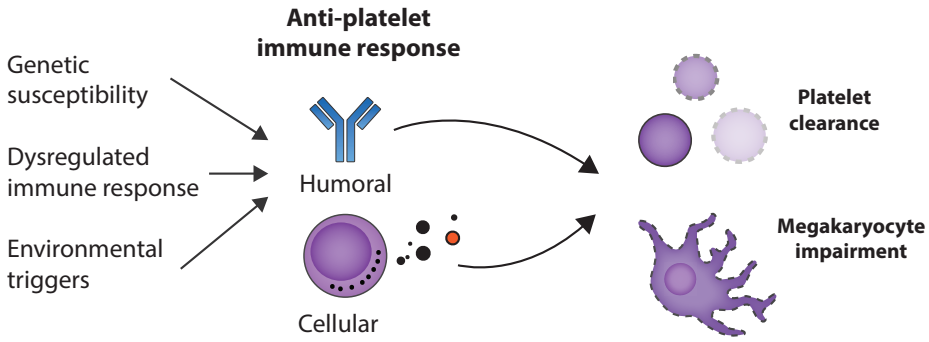

Figure 1.1: ITP disease mechanisms

1. an environmental trigger, such as an infection or vaccination, and
2. a state of immune dysbalance, which takes place in
3. a (genetically) susceptible individual (Figure 1.1).

Considering the environmental trigger, given the post-infectious nature of the disease onset, childhood ITP has been associated with many viral infections. This includes the Epstein-Barr Virus, cytomegalovirus, [15] varicella zoster virus [16], and incidentally parvovirus [17]. Moreover, recent vaccinations are associated with ITP [16]. Adult ITP is associated with H. pylori, Hepatitis C virus, and human immunodeficiency virus [16]. Together, these events precipitate

a humoral and cellular immune response with a loss of tolerance to platelet self-antigens, such as platelet glycoprotein GP IIb/IIIa (CD41/CD61), GP Ib/IX (CD42a/b/c complex), or GP V (CD42d)[18]. This loss of tolerance can be demonstrated by the presence of circulating and platelet-adsorbed antibodies to these platelet antigens[19–22], antigen-specific B cells in the circulation and spleen (adult ITP)[23–26], as well as antigen-specific CD4 [27, 28] and CD8 T cells [29] (adult ITP). It is unclear whether the loss of tolerance and platelet-specific induced adaptive immunity is a consequence of 'bystander' activation with the development of pathogen-specific immunity (activation of cells reactive to pathogen antigens that are cross-reactive with platelet antigens) [30]. Alternatively, platelet-specific B and T cells may become specifically activated (aspecific activation of cells reactive with platelet antigens, unrelated to the pathogen). Platelet autoantibodies cannot be absorbed to antigen-negative platelets, such as those from Glanzmann thrombasthenia patients [21]. Given that platelet antigens are also expressed by bone marrow megakaryocytes, they are also affected by the anti-platelet immune response.

THE MAIN IMMUNOLOGICAL CONCEPTS involved in the ITP pathogenesis as relevant to this thesis are summarized in Figure 1.2. Naive CD4 and CD8 T cells react to platelet antigens present on antigen-presenting cells in the appropriate immunological context, differentiate and proliferate into effector cells, and contribute to B cell activation and modulation of the immune response. Effector CD8 T cells may directly clear platelets (cell-mediated cytotoxicity). Innate immune cells such as polymorphonuclear cells (PMN), monocytes, and NK cells may react to immunoglobulin- or complement-

opsonized platelets. The humoral antibody-mediated immune response by B cells engages immune cells through their membrane IgG-Fc receptor (FcγR) and triggers activation of the complement system through the classical pathway. This leads to opsonization with C3b fragments and formation of the membrane attack complex (MAC). Serum IgG, including pathologic antibodies, has a long half-life because of recycling in endolysosomes through binding to the neonatal Fc receptor (FcRn). When FcRn is saturated or blocked, increased IgG turnover occurs.

DISEASE SYMPTOMS The initial immune response leads to the pathophysiological events that explain thrombocytopenia and bleeding:

1. platelet clearance by innate or adaptive immune mechanisms, including platelet opsonization by immunoglobulins and complement [31–34],
2. impaired platelet production, by inference with megakaryocytes [35–39],
3. impaired platelet function[40–42], by blocking of platelet receptors [43], and
4. platelet apoptosis [44].

During childhood, the hemostatic and immune systems continue to develop and undergo significant changes, which may affect the pathophysiology and bleeding severity in childhood ITP. Moreover, it is essential to recognize that in childhood ITP, many of the proposed

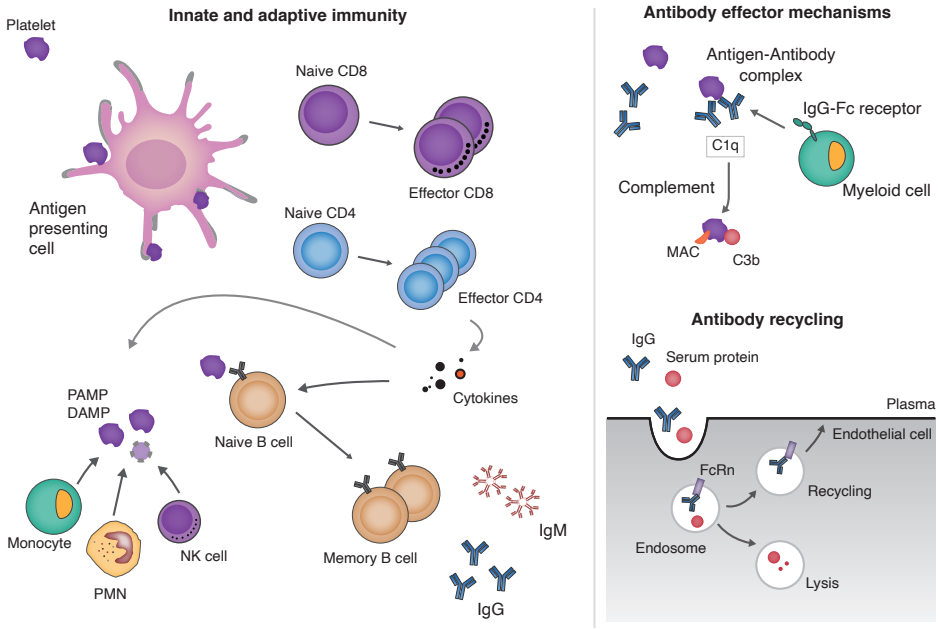

Figure 1.2: Immune mechanisms in ITP pathophysiology.

mechanisms are based on non-controlled case series with a low level of evidence and still require molecular and clinical validation (Table 1.1). Clinically, fatigue symptoms may be attributed to the ongoing inflammatory response, but there is no direct evidence for this, and it may remain challenging to prove.

Table 1.1: Newly proposed disease mechanisms of childhood ITP: level of evidence and relevance to clinical practice.

MECHANISM	FINDINGS	LIMITATIONS
Cellular anti-platelet immunity	(Adult ITP) CD8 T cells react to platelet antigens [29, 45]	Anti-CD3 stimulation included in T cell proliferation assay, TCR engagement not required. As opposed to CD8-mediated reactivity to platelet antigens, platelets of ITP patients may affect CD8 proliferation and activation by other mechanisms.
Platelet antibodies: platelet activation and desialylation	(Adult ITP) Anti-GP Ib/IX antibodies induce Fc-independent platelet activation, potentially leading to neuraminidase release and desialylation [46]. Desialylation is induced by anti-GP Ib/IX antibodies [47]	Very selected ITP sera (antibody levels extremely high, rarely observed in our laboratory). Difference of anti-GPIb/IX to anti-GP IIb/IIIa antibodies not formally shown for human sera, as both are observed to induce activation and desialylation in this study. Outliers driving effects.

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MECHANISM	FINDINGS	LIMITATIONS
Antibody profiles as disease course indicators	3/4 (n/N, 75%) of 131 newly diagnosed children with isolated anti-GP Ib/IX antibodies at diagnosis develop chronic ITP[48]	Very few positive cases. High test PPV, but overall low sensitivity for chronic ITP (3/18, 17%). Patients with both anti-GP Ib/IX and anti-GP IIb/IIIa antibodies (47%) showed no difference in disease course. Asian population (China).
	(Adult ITP) Presence of anti-GP Ib/IX antibodies is associated with non-response to IVIg with an odds ratio of 0.1 (95% CI 0.1 – 0.3) [49].	Inter-laboratory variability in anti-GP Ib/IX antibody assay; much lower prevalence than in other studies (29% here vs > 60% in others [50]). Asian population (China)
Modulation of self-immunity by regulatory T cells	CD4+ CD25+ T cells are reduced in acute childhood ITP, compared to chronic patients, remission, or controls [51]	CD4+ CD25+ definition of Treg also includes non-regulatory activated T cells; gating not reported. Confounders not considered: age (different between patients), time from diagnosis (extremely varying). Current and previous treatments not reported. African population (Egypt)

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MECHANISM	FINDINGS	LIMITATIONS
	Compared to healthy controls, CD4+ CD25+ and CD4+ CD25+ FOXP3+ cells were decreased in patients with ITP [52]. CD4+ CD25+ and CD4+ CD25+ FOXP3+ cells were increased in patients predicted to have transient disease compared to prolonged ITP [52]	Age as a confounder has not been considered. Predictive value was not compared and association with other lymphocyte subset changes (CD4, CD8, CD19) has not been assessed.

PPV, positive predictive value.

GENETIC SUSCEPTIBILITY In line with the immune paradigm, several variants in immune genes affect the susceptibility for ITP (Table 1.2). Susceptibility denotes the risk of an individual to develop ITP in comparison to the overall population at risk. A genetic susceptibility to ITP is further indicated by the clinical observation of familial cases of ITP [53–56]. It remains unclear if the investigated variants are causally related to ITP susceptibility or because of linkage disequilibrium with other causative variants. Genome-wide association studies or multi-loci multivariate analyses have not been performed. A standard for genetic susceptibility studies is to evaluate a potential variant in discovery and validation cohorts. None of the studies reaches this level of evidence (except for polymorphisms in the Fc-γ receptors, which multiple independent studies have identified). Moreover, the data must be interpreted cautiously due to incomplete reporting of relevant clinical data, ethnic diversity,

Table 1.2: Childhood ITP disease susceptibility genes.

GENE	ASSOCIATION
Chemokines and cytokines	
TNF	-308 allele associated with susceptibility [58]
IL1RN	(Adult ITP) VNTR is associated with susceptibility [59]
IL2	(Adult ITP) -330 (T>G) polymorphism associated with susceptibility[59]
IL4	VNTR in intro 3 associated with susceptibility [60]
IL10	-627 (C>A) polymorphism associated with susceptibility [60]
BAFF	-871 (T>C) polymorphism associated with susceptibility [61]
LTA	Associated with susceptibility [58]
Immune receptors	
HLA	Inconsistent results between studies [62, 63]
FCGR2A	p.H131R variant associated with susceptibility[64, 65]
FCGR2C	Open reading frame is associated with susceptibility [66]
FCGR3A	p.F158V variant associated with susceptibility [58, 64, 67]
FCGR3B	NA1/NA2 variants associated with susceptibility [58]

VNTR, variable number of tandem repeats

and a lack of correction for multiple testing. Interestingly, the HLA locus, which is strongly implicated with the development of other autoimmune diseases, has not shown a consistent association with susceptibility for ITP (Table 1.2) [57].

1.3 DIAGNOSIS

ITP is diagnosed as a clinical syndrome based on history, physical exam, and basic complete blood count, thereby excluding alternative causes of thrombocytopenia [68]. No gold-standard test is available for the diagnosis. Immune thrombocytopenia was previously named idiopathic thrombocytopenic purpura. The change to the current definition recognizes the immune pathogenesis of the disease and the fact that some patients show no bleeding symptoms despite thrombocytopenia. The immune pathogenesis is broadly interpreted from the identification of platelet autoantibodies [19, 22] and the effectivity of immune therapies (e.g., IVIg, anti-D)[69, 70].

Apart from a careful patient and family history, selected biological assays have been proposed as supportive rule-in or rule-out tests (Table 1.3). Notably, some of these tests allow the distinction between primary (idiopathic, i.e., not associated with another condition) and secondary immune-mediated thrombocytopenia (e.g., infection - viral PCR, blood counts). Other tests differentiate immune-mediated thrombocytopenia (e.g., platelet glycoprotein-specific antibodies) from non-immune-mediated thrombocytopenia (e.g., genetic - blood film, TPO).

Table 1.3: International Working Group (IWG) evaluation of laboratory tests for newly diagnosed childhood ITP.

TEST	IWG RECOMMENDATION
Complete blood count and reticulocytes	Evaluate in all cases
Platelet glycoprotein-specific antibodies	Potential utility
Peripheral blood film	Evaluate in all cases
Quantitative immunoglobulin levels	Consider to evaluate in all children
HIV, HCV	Evaluate in adults in appropriate geographic setting
EBV, CMV, Parvovirus	Potential utility
TPO levels	Unproven or unclear benefit
Immature platelet fraction	Unproven or unclear benefit
Pregnancy test (women of childbearing potential)	Potential utility
HBV	Evaluate in all cases
Bone marrow exam (only selected patients)	Potential utility

HIV, Human immunodeficiency virus; HCV, hepatitis C virus; HBV, hepatitis B virus; EBV, Eppstein Barr virus; CMV, cytomegalovirus; TPO, thrombopoietin.

IWG recommendations from [1].

CHALLENGES DUE TO THE CLINICAL DIAGNOSIS OF ITP Importantly, the diagnosis of ITP by the exclusion of other causes is a major clinical challenge, can lead to misdiagnosis [71–73], and likely results in a case mix of different pathophysiologies that present with a common clinical syndrome of thrombocytopenia [16]. Thrombo-

cytopenia may be a first presenting sign in children with syndromic disorders, some of which can be easily diagnosed (velocardiofacial syndrome [22q11 deletion], thrombocytopenia with absent radii [TAR], amegakaryocytic thrombocytopenia [CAMT]). Other genetic disorders have a more diverse phenotype, such as congenital thrombocytopenia or thrombocytopathies (e.g., grey platelet syndrome or Bernard-Soulier syndrome; MYH9-related disease[74], von Willebrand type 2b with an increased affinity of VWF to GPIb). Thrombocytopenia may present as part of immune disorders (e.g., common variable immunodeficiency [CVID] [75]; hyper IgM syndrome [76]), autoimmune lymphoproliferative syndrome (ALPS)[77], or systemic autoimmune disorders (e.g., systemic lupus erythematosus [78–80]). Some primary immunodeficiencies may present with thrombocytopenia, without signs of immunodeficiency, such as the X-linked thrombocytopenia form of Wiskott-Aldrich syndrome (WAS) or common variable immunodeficiency (CVID)[81, 82]. After a diagnosis of ITP, patients may be subjected to ITP-specific treatments, including multiple regimens of corticosteroids, IVIg, platelet transfusions, or even splenectomy, before a thrombocytopathy is later confirmed [83, 84]. Up to 14% of children initially diagnosed with ITP have a revised clinical diagnosis during ten-year follow-up [85]. Similarly, among adult ITP, registry data indicate that 12% are diagnosed with another disorder during a median two years follow-up [72]. Clinical experience suggests that in treatment-refractory cases, the diagnosis of ITP must be reconsidered [86]. Interestingly, by high-throughput sequencing, seven of 29 patients with treatment-refractory ITP are revealed to have an underlying genetic thrombocytopathy, including *ANKRD26, ETV6, ITGA2B,* and *TUBB1* variants [87]. The diagnosis

of exclusion strategy may result in the delayed diagnosis and appropriate management of the underlying disorder. It may also underestimate treatment effects because patients with varying underlying disease etiologies respond differently to the therapies. Moreover, the diagnosis imposes a conceptual problem for evaluating new diagnostic assays, as the "gold standard" represents a syndrome that includes multiple underlying molecular etiologies. Thus, a diagnostic test related to only one single etiology can only be positive in a proportion of patients (reduced sensitivity), implying that it cannot be used as a rule-out test. We suggest that diagnostic assays should be associated with a specific (longitudinal) disease prognosis, but this requires a large number of observations and significant follow-up time.

1.4 PROGNOSIS

Clinically, three ITP periods are prospectively distinguished: newly diagnosed ITP (diagnosis – 3 months), persistent ITP (> 3 months), and chronic ITP (> 12 months)[68]. A child that recovers within three months after the diagnosis is retrospectively classified as transient ITP (Figure 1.3)[88]. Different longitudinal platelet counts (platelet trajectories) one year after the diagnosis of newly diagnosed ITP for observed and IVIg-treated patients are displayed in Figure 1.3.

Childhood ITP is a self-limiting disorder in most children. Children with severe thrombocytopenia (platelet counts $\leq$ 20x 10^9/L) are significantly affected by bleeding symptoms, reduced health-related quality of life, and may represent the group where treatment is considered [89]. In this group, the multinational prospective NOPHO

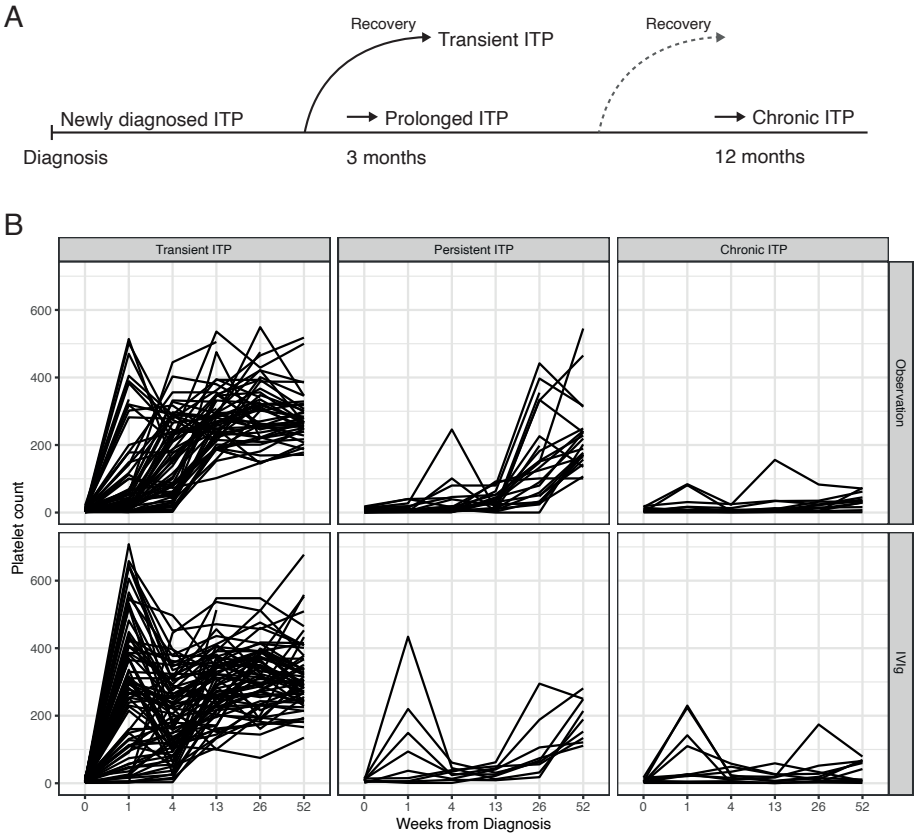

Figure 1.3: Disease courses in childhood ITP. (A) Classification of disease courses in three distinct phases. (B) Observed platelet trajectories in 200 children with newly diagnosed ITP (TIKI trial), stratified by treatment and disease course.

ITP study and the multicenter TIKI trial indicated that spontaneous recovery from ITP is observed in 20% (one week), 40% (one month), 60% (three months), 80% (six months) and 90% (twelve months) of children [5, 90]. Thus, the 10% of children still thrombocytopenic 12 months after the diagnosis are classified as chronic ITP. Registry data from the Intercontinental Childhood ITP Study Group (ICIS), where the inclusion and exclusion criteria allow children with platelet counts up to 100 or 150 x 10^9/L at diagnosis to be recruited, the chronic disease develops in 30% of children [4, 91, 92]. Notably, the disease morbidity and reduced health-related quality of life are most severe in children who do not respond to treatment or show prolonged or chronic disease [8, 9, 93, 94].

1.5 CLINICAL MANAGEMENT

"The good physician treats the disease;
the great physician treats the patient who has the disease."

Sir William Osler
1849 - 1919

For guidance on the clinical management of childhood ITP, both the American Society of Hematology evidence-based guidelines [95, 96] and the recommendations of the International Working Group on ITP [1, 97] were updated in 2019. In addition, the Joint Working Group (JWG) of the German, Austrian and Swiss hematological societies published updated recommendations in 2018 [98]. The current management paradigm is governed by a *"do not treat, unless"* strategy, implying that a child with newly diagnosed ITP should be carefully observed unless there are sufficient indications for medical treatment. The decision to treat is weighted by carefully considering symptoms, the health-related quality of life (including mental health, activities, and social participation), and the potential benefits and the side effects of treatment. Indications for treatment entail non-life-threatening mucosal bleeding, severe and life-threatening bleeding, and reduced quality of life.[96] The current ASH guidelines give no treatment recommendations for children with severe or life-threatening bleeding (where the rapid effect of IVIg may be desired). The benefits of IVIg treatment are observable within days after the administration [69, 99], and the critical phase where IVIg is effective corresponds to 1-3 months of treatment [90]. During these months, multiple randomized controlled trials showed a significantly higher recovery rate, with IVIg treatment, and IVIg has a considerably shorter time to response than corticosteroids [70, 90, 100, 101]. In children with newly diagnosed ITP and no or mild (skin) bleeding,

the current guidelines favor a restrictive treatment strategy, with careful observation and avoidance of hospital admission. A notable change in the 2019 ASH guidelines is the suggestion, based on low certainty of the evidence, that children with non-life-threatening mucosal bleeding could be treated with oral corticosteroids (prednisone 2-4mg/kg/day, max 120mg daily, over 5-7 days), as preferred over IVIg. This recommendation has been made based on the assessment of durable response (6 months platelet count) and remission (12 months platelet count), in addition to the costs, donor exposure, and logistical issues with IV drug administration.

1.6 TREATMENT OPTIONS

In most European countries, corticosteroids and IVIg are the first-line therapies for newly diagnosed ITP. Traditionally, corticosteroids are used for the treatment of many autoimmune diseases; compared to IVIg they may require more time to induce a response in platelet counts [70, 90, 100, 101], and have relevant side effects in children, particularly when given over a longer time. IVIg therapy involves possibly multiple divergent working mechanisms, and after more than 30 years of research, the mechanisms of action remain unresolved (Table 1.4). The prevailing concept is that the effect of platelet and megakaryocyte autoantibodies is modified by IVIg-mediated FcγR blocking and acceleration of IgG clearance through FcRn (Figure 1.2)[102, 103]. Only the Fc-dependent mechanism of action has been formally tested in clinical study infusing Fc-fragments of IVIg [104]. Still, the results remain challenging to interpret because no control group was recruited. A key observation in the blocking ac-

tion of IVIg was that after an ITP patient is treated with IVIg, the in vivo clearance of RhD-sensitized red blood cells is reduced [105]. Moreover, experiments in selected individuals with ITP indicated that anti-FcγRIIIa antibody treatment might be functional in the treatment of ITP [106, 107], which may indirectly suggest a role for Fc-mediated blocking of activating FcγR. Intriguingly, monoclonal IgG antibodies can be used instead to recapitulate IVIg's effects in ITP mouse models [108]. The mechanism of action of monoclonal IgG may be due to blocking of FcγR (potentially by sensitized bystander cells) or interaction of sialylated Fc portions with the regulatory lectin receptors on immune cells [109]. In vitro phagocytosis experiments suggest that IVIg effects are mediated by FcγR blockade and not by sialylated IgG-Fc [110]. Moreover, by engineering human Fc to have a higher affinity for Fc blockage, IVIg may be more effective in blocking FcγR mediated cell clearance [111]. Interestingly, ITP patients can respond to IVIg even after splenectomy, suggesting that the reticuloendothelial system in the spleen is not required for IVIg action [86]. Mouse models of ITP indicated that the inhibitory FcγRIIb is necessary for the mechanism of action of IVIg [112, 113], although there are differences between mouse strains [114]. To treat ITP in a mouse model, FcγRIIb is required for IVIg $F(ab')_2$-mediated binding to endogenous soluble antigens. However, IVIg $F(ab')_2$-mediated binding to circulating cellular antigens does not require FcγRIIb to treat ITP in this mouse model [115]. The relevance of this concept in human ITP remains largely unknown.

Table 1.4: Proposed working mechanisms of IVIg in ITP.

MECHANISMS	LEVEL OF EVIDENCE
Fc-mediated mechanisms	
Blocking of activating FcγR	Human interventional study (ITP; not controlled) [104]
FcRn saturation, accelerated clearance of pathogenic IgG	Human observational (GBS, ITP) [116, 117], Rat (ITP model) [118]
Modulation of activating FcγR expression	
Upregulation of inhibitory FcγRIIb	Human observational (CIPD) [119], Mouse (ITP model) [112]
Expansion of regulatory T cells	Mouse (EAEM model) [120]; Human in vitro [121]
Modulation of dendritic cell	Mouse (ITP model) [122]
Modulation of total serum IgG glycosylation	Human observational (GBS) [123]
$F(ab')_2$-mediated mechanisms	
Cytokine neutralization	Human in vitro [124]
Anaphylatoxin neutralization	Mouse (asthma model) [125]
Autoantibody neutralization (anti-idiotypic effects)	Human in vitro (Thyroiditis, SLE, gastritis, HLA alloimmunity) [126–128]
FAS (CD95) or FAS ligand blocking; SIGLEC9 blocking	Human in vitro (Toxic epidermal necrolysis) [129]; in vitro [130]

GBS, Guillain-Barre syndrome, CIDP; chronic inflammatory demyelinating polyneuropathy.

Extended from [102].

SECOND-LINE AND EMERGING TREATMENTS The alternative treatments danazol, sirolimus, or anti-D hypersensitized immunoglobulin are infrequently used in Europe in childhood ITP. Rituximab or tacrolimus are relatively established as second-line treatments in refractory cases. Based on an improved understanding of the ITP pathophysiology, several new molecular therapies are currently being investigated to treat ITP in children and adults (Table 1.5). The TPO-receptor agonists (TPO-Ra) romiplostim (peptibody; peptides coupled to Fc domain; subcutaneous administration) and eltrombopag (small molecule; oral administration) have been developed after the identification of the TPO gene in 1994. Eltrombopag has been established to treat persistent childhood ITP $\geq$6 months after the diagnosis in the randomized controlled PETIT and PETIT2 studies [131]. Here, eltrombopag resulted in improved platelet responses [132, 133]. Comparing the active drug to placebo, a benefit for reducing clinically significant bleeding of WHO grade 2-4 was shown in the phase 2 study PETIT, but not in the larger phase 3 study PETIT2 [132, 133]. The health-related quality of life was not improved in PETIT (even when considering only patients who responded)[133, 134], although the study may have been underpowered to show moderate effects. Of note, eltrombopag treatment is costly. In adult patients, it has been shown that after treatment with TPO-Ra, treatment could eventually be weaned and discontinued, at the risk of 10-15% of rebound thrombocytopenia [135].

Table 1.5: Emergent treatment options for ITP.

THERAPY	MECHANISM	CURRENT DEVELOPMENT
FcRn block (efgartigimod; rozanolixizumab)	Inhibition of serum IgG binding to FcRn; accelerated IgG clearance	Recruiting phase 3 study, ADVANCE (NCT04188379). Phase 2 study not yet recruiting (NCT04200456)
Complement inhibition (sutimlimab)	Inhibition of opsonization and/or complement-mediated cell lysis	Phase 1 data, Adult Chronic ITP [136]
TPO-Ra in newly diagnosed ITP (eltrombopag)	Stimulation of platelet production; immune modulation [137]	Recruiting phase 3 study, newly diagnosed ITP, ICON3 (NCT03939637)
Syk inhibitor (fostamatinib)	Inhibition of FcyR downstream signaling	FDA approved for Adult Refractory/Chronic ITP (2018); not investigated in children

Trial access through NCT number on clinicaltrials.gov

1.7 MODIFICATION OF ITP DISEASE COURSES

A crucial issue in the management of newly diagnosed ITP is whether the ITP disease courses can be modified by early (first-line) treatment [138]. Data from several non-randomized cohort studies and a meta-analysis suggested that IVIg treatment may reduce the proportion of children who develop chronic ITP [139–141]. The *Treatment with IVIg in Kids with ITP* (TIKI) randomized controlled trial showed that in 200 children with newly diagnosed ITP, compared to observation, recovery rates are increased for 3 months after IVIg treatment [90].

Still, IVIg does not affect the development of chronic disease [90]. In a health-related quality of life (HRQoL) substudy of TIKI, quality of life questionnaires were administered to children and parents. The HRQoL in ITP may be reduced due to bleeding symptoms [8, 142], although this is not consistently observed [143], possibly due to differences in patient characteristics and instruments for assessment of bleeding. In the TIKI study, contrary to what one may expect, children treated with IVIg showed no improvement of HRQoL over observation, even though the clinical effect of IVIg induced the recovery from ITP and a reduction of bleeding symptoms [9, 90]. This observation agreed with earlier cross-sectional data [144], but the results of that study were likely affected by confounding by indication. A possible explanation for this could be that children reported a reduced HRQoL after IVIg compared to observation. In contrast, parents' experience did not change (this was observed but not statistically significant [9]). Thus, the experience of intravenous cannulation and hospital admission surrounding the IVIg administration may offset benefits. Although actual comparative data are currently lacking, in line with the current ASH guidelines, oral corticosteroid treatment might be a more suitable choice that could avoid hospitalization (but potentially has more side effects). Notably, in observational studies, corticosteroid-treated patients showed a reduced quality of life, compared to no treatment [144]. This could be related to the administration of treatment to children with more severe disease (confounding by indication) or bias due to differences between the patients. Overall, the data are difficult to interpret in the absence of randomization and accounting for confounders. The randomized administration of the TPO-Ra romiplostim versus placebo to children

with chronic ITP improved the HRQoL [93], where children showed a 90% platelet response rate after romiplostim [145]. However, in the PETIT study, the health-related quality of life after TPO-Ra treatment was not improved [134]. In the TIKI study, the most significant reduction in HRQoL was observed for the group of children with persistent thrombocytopenia at six months. It was related to hospital visits, blood tests, and reduced participation in activities [9]. Children who recovered showed favorable HRQoL compared with patients who showed persistent thrombocytopenia one month after diagnosis/treatment. These results are fully supported by a second, independent longitudinal study [8]. Importantly, in TIKI, 30% of children with newly diagnosed ITP did not respond to IVIg [90], which can be expected to affect the HRQoL of children in the same way, with lower HRQoL when thrombocytopenia is persistent.

1.8 TOWARDS INDIVIDUALIZED PROGNOSIS AND TREATMENT

As presented in this chapter, a proportion of patients with newly diagnosed ITP and severe thrombocytopenia will remain thrombocytopenic for more than three months and be classified as persistent or chronic ITP. These patients often exhibit continued bleeding symptoms. Treatment at the time of diagnosis with IVIg effectively prevents bleeding episodes, but it does not lead to a resolution of thrombocytopenia in 30% of patients. In general, a key clinical challenge is identifying an individual's disease course at the time of diagnosis. We expect that personalized treatment of childhood ITP and knowledge of the expected disease course could improve disease outcomes and quality of life. This would allow to determine the optimal clinical management (start of treatment vs watchful waiting, the timing of additional diagnostic tests) and counsel families appropriately.

1.8.1 *Proposed prediction markers of ITP disease courses*

Individual patient characteristics and laboratory parameters may be used to individualize the prognosis and clinical management. Several established parameters and candidate markers have been proposed(Table 1.6, Table 1.7) [141]. Despite the wide range of published studies, no multivariate analyses or multivariate prediction models are available. Thus, we do not know how the proposed predictors interact when they are assessed simultaneously in the same individual. Nonetheless, a Bayesian prediction rule for transient ITP has been proposed[146], based on univariate weighted predictors.

Two retrospective studies have assessed this rule and indicated that differentiation of disease courses could be possible [147, 148].

Table 1.6: Established markers of childhood ITP disease courses.

MARKER	CHRONIC ITP	EFFECT (95% CI) [141]
Sex	Females	Female, OR 1.17 (1.04 - 1.31)
Age	Older	>11 years, OR 2.45 (1.94. - 3.15) > 8 years, OR 2.97 (1.42 - 6.21)
Preceding infection or vaccination	Absence of infection or vaccination	Absence, OR 3.08 (2.19 – 4.32)
Disease onset	Insidious onset	Insidious, OR 11.27 (6.27 – 20.27)
Bleeding	Absence of mucosal bleeding	Mucosal, OR 0.39 (0.28 – 0.54)
Platelet count	Higher count	Plt $\geq 10x10^9$/L, OR 2.00 (1.52 - 2.64) Plt $\geq 20x10^9$/L, OR 2.15 (1.63 - 2.83).
Leukocytes	Lower total leukocyte count	WBC $\leq 6.25x10^9$/L, OR 2.31 (1.20 – 4.44)
Lymphocytes	Lower absolute lymphocyte count	Lymphocyte count $\geq 3.05x10^9$/L, OR 3.79 (2.37 – 6.07)
Anti-nuclear antibodies (ANA)	ANA presence	Presence, OR 2.87 (1.57 – 5.24)

OR, odds ratio for association with chronic ITP. Plt, platelet count. WBC, white cell count.

Table 1.7: Candidate markers of childhood ITP disease courses.

MARKER	ASSOCIATION	LIMITATIONS
Direct antiglobulin test (DAT)	Positive DAT is associated with chronic ITP vs remission (8% vs 29%) [149]	Mean difference may be overestimated% due to inclusion of Evans syndrome [149]; association with other markers not known
IgG levels	IgG levels lower or higher than the 95% reference interval are associated with chronic ITP [150]	Does not discriminate at the diagnosis when 2.5% false positive rate among is considered because of low incidence chronic ITP; association with other markers unknown
Regulatory CD4 T cells	Regulatory T cells are increased in chronic ITP [51, 52]	Confounders not considered. Prior treatment effects not considered
Oxidative stress	Oxidative stress-related pathways and vanin-1 expression [151]	Not validated
HLA	12 HLA alleles were overrepresented in chronic ITP patients (whole exome sequencing) [152]	Not yet clear if individual loci or a combination can be predictive; linkage disequilibrium with other potentially causative variants

Continued on next page

MARKER	ASSOCIATION	LIMITATIONS
FCGR2B	p.232I/I genotype is associated with recovery from ITP vs chronic ITP (75% of 44 patients vs 25% of 16 patients) [139]	Small cohort. p.232I/I may be associated with early spontaneous recovery [90].
IL-4, IL-10	*IL-4* intron 3 RP1/RP2 genotype and *IL-10* -627 A/C genotype are associated with chronic ITP [60, 153]	Small study populations. Asian population (Taiwan). African population (Egypt).
TNF, FCGR3	*TNF* -308 1/2 and 2/2 genotype underrepresented in chronic ITP; LTA 2/2 genotype overrepresented in chronic ITP; *FCGR3B* NA1/NA1 variant overrepresented in chronic ITP, *FCGR3A* p.158V/F genotype overrepresented in chronic ITP. [58]	No correction for multiple comparisons. Small study population. No validation cohort.
IFNA17	*IFNA17* rs9298814 A>C present in 26% of chronic ITP vs. <0.01% of healthy controls [154]	
CNR2	Cannabinoid receptor type 2 Q63R variant associated with chronic ITP [155]	

PPV/NPV, positive and negative predictive value.

CURRENT CHALLENGES Several limitations to currently available data restrict their straightforward use for individualized prognosis:

1. *Quality of data.* Retrospective studies have the usual problems of reporting, information, and selection biases.

2. *Differences in the clinical setting and baseline characteristics of recruited study populations.* The predictors obtained in single-center small cohorts may not generalize well to the population at large.

3. *Questions to the integration of multiple predictors and correlation between predictors (multicollinearity).* Once there are substantive and dependable data for a predictor, a fundamental problem is how predictors should be integrated. Some clinicians may subjectively 'sum up' the observed predictors to form an opinion. However, in the absence of well-built multivariate prediction models, such predictions may be flawed. For instance, multiple predictors may correlate (e.g., a patient of twelve years of age diagnosed with ITP may be more likely to be female than male; a patient with a low patient count may be more likely to show more severe bleeding symptoms). It is thus unclear which of the predictors drives effects (i.e., multivariate effect sizes, regression coefficients). The impact of one predictor may depend on a second predictor (e.g., correlation, interaction effects).

Evidence-based medicine proposes integrating clinical expertise with clinically relevant research, in this case in the form of prognostic markers and prediction models[156]. An evidence-based solution to determine an individual's prognosis is to gather multimodal data from a study population and make predictions about an outcome by weighting the individual predictors. Weighting is done such that

the outcome is best explained by the data (multivariate regression). This requires a sufficient sample size to observe enough variance of candidate predictors and the outcome in the population [157]. With current 'omics' molecular analyses (genomics, transcriptomics, proteomics, metabolomics, immunomics), the number of candidate predictors may easily extend the number of observations ($n<p$ problem), and integrated analysis of such data requires specialized methods [158]. A further challenge is that prediction models may overfit the data, such that they do not generalize well to new populations.

1.9 STATISTICAL LEARNING

To overcome some of the shortcomings of traditional multivariate regression techniques, advanced prediction strategies have been developed that carry many advantages, such as dealing with the $n<p$ problem, collinearity, overfitting and variable selection. In *supervised statistical learning*, we are concerned with the identification of variables ("predictors") that predict a given response variable. In general, given a response Y and p predictors $X = (x_1, x_2, \ldots, x_p)^T$ of n observations, we aim to obtain an accurate function f to describe the relationship $Y = f(X) + e$, where e is an error term with mean zero. The error term captures unmeasured variance. The function $f(X)$ is estimated by obtaining solutions that come close to the actually observed response Y. In the case of simple linear regression, for the continuous response variable $Y = \beta X + e$ a solution is sought that minimizes the residual sum of squares (RSS),

$$RSS = \sum_{i=1}^{n} (y_i - \beta_0 - \sum_{j=1}^{p} \beta_j x_{ij})^2 \tag{1.1}$$

for the i-th observation and the j-th predictor.

Increasing the function's complexity may allow improved predictions of Y, given X. However, an increasingly complex model tends to incorporate error (e, noise) to the relationship between X and Y, and generalizes poorly when applied to new data. This is known as overfitting. To control model complexity and prevent overfitting, penalized regression techniques have been developed, such as

ridge regression, the least absolute shrinkage and selection operator (LASSO), and the elastic net [159–161].

Introducing a penalty to the minimization problem, for ridge regression [159], the term

$$RSS + \lambda \sum_{j=1}^{p} \beta_j^2 \tag{1.2}$$

is minimized. In the case of LASSO [160, 162]

$$RSS + \lambda \sum_{j=1}^{p} |\beta_j| \tag{1.3}$$

is minimized. In the case of elastic net [161], a combination of ridge and LASSO penalties is used,

$$RSS + \lambda \sum_{j=1}^{p} (\alpha \beta_j^2 + (1 - \alpha)|\beta_j|) \tag{1.4}$$

where the mixture of LASSO and ridge penalties is controlled by the parameter α.

The penalty terms can also be applied in the estimation of other forms of regression, such as binomial (logistic, generalized linear model), multinomial, and cox-proportional hazards models. In the classic linear regression case, when λ is zero, the resulting coefficients are the least squares estimates. In ridge regression, when λ is increased, the penalty shrinks coefficients towards zero, but never

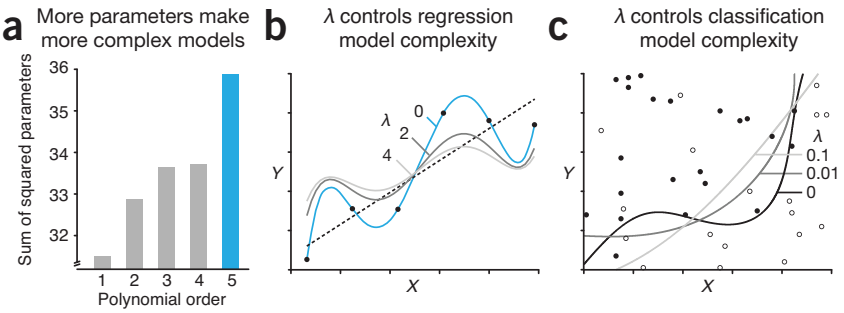

Figure 1.4: Regularization controls a model fit by restricting the magnitude of the model's parameters by the parameter λ. Reprinted by Permission from Nature [163].

exactly zero (unless λ is infinite)[164]. Thereby, the λ term controls the model complexity (Figure 1.4).

Ridge regression deals well with collinearity[165]. The penalty in ridge regression leads to the selection of a single solution, given the data. In contrast to the ridge regression penalty, the LASSO term does shrink coefficients to zero. Thus, LASSO performs variable selection. The LASSO solution is not unique, i.e., different solutions with similar error can be obtained. In elastic net, a combination of LASSO and ridge regression parameters is used, offering combined benefits of coefficient shrinkage and variable selection of both techniques (Figure 1.5).

Thus, control of the penalty term is crucial in these cases, to balance bias and variance of the prediction model. Too simple models show high bias and low variance (underfitting), too complex models

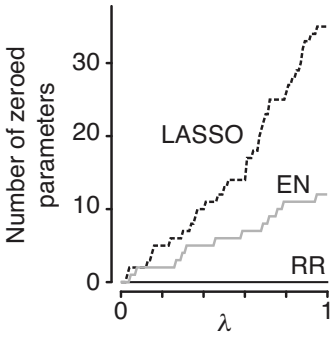

Figure 1.5: Regularization with the LASSO and elastic net (EN) selects model variables. Reprinted by Permission from Nature[163].

show a low bias and high variance (overfitting)[163, 164]. In practice, λ is usually determined by K-fold cross-validation, where a dataset is split in k folds [163]. Training and testing of the model are sequentially performed by building a model in $k-1$ folds and predicting the response the left-over fold.

1.10 RESEARCH QUESTIONS AND SCOPE OF THE THESIS

At present, the prognosis of childhood ITP cannot be predicted at the time of diagnosis. The *central thesis of this dissertation* is that clinical and molecular features can help distinguish and thus individualise the prognosis of newly diagnosed childhood ITP. Such features may reflect the biological subgroups associated with observed (IVIg) treatment responses and disease courses, i.e., transient and chronic disease.

We hypothesized that:

1. Newly diagnosed childhood ITP is a *"thrombocytopenia syndrome"* with multiple underlying etiologies (case-mix), related to the fact that it is a clinical diagnosis of exclusion. This hypothesis contrasts the currently accepted concept of a relatively homogeneous immune etiology.

2. The ITP case-mix can be dissected by a detailed clinical, molecular and cellular investigation.

3. Divergent disease courses and treatment responses can be explained by the ITP case-mix. Both causative and indirect markers of the ITP case mix are sufficient candidates for clinical prognostication.

In this dissertation, we aimed to bridge molecular and clinical data to advance the knowledge of ITP pathology, and to develop tools for clinical diagnosis and prognostication. We collected clinical and molecular data from a large multicenter randomized clinical trial in childhood ITP (TIKI trial). The clinical and molecular features

were combined by computational approaches in different prediction models to determine the ITP prognosis in a systematic, quantifiable, and comparable way. We propose that the insights and developed models can support prognostication, decision-making for clinical management, and help in counseling families of patients with newly diagnosed ITP.