Pediatric immune thrombocytopenia: Catching platelets
Laarhoven, Annemieke

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General Introduction
ITP

Primary immune thrombocytopenia (ITP), formerly known as idiopathic thrombocytopenic purpura, is an acquired autoimmune bleeding disorder. In children, the disease has an incidence of 1.9 to 6.4 cases per 100,000 a year, compared to 3.3 cases per 100,000 a year in adults.\(^1\) Pediatric ITP has a peak incidence between 2 and 5 years of age\(^2\), with no difference\(^3\) or a slight predominance of males.\(^2\)\(^4\)

ITP usually presents itself as a sudden increase in bleeding tendency, often consisting of petechiae, (spontaneous) hematomas, gingival bleeding and epistaxis. Although rare, serious bleeding, such as intracranial hemorrhage, does occur. Due to the severe, potentially fatal, consequences of serious bleeding it is important to aim research at foreseeing, treat and eventually prevent this condition. The disease is characterized by an isolated thrombocytopenia, recently redefined as a platelet count below 100*10^9/L, in the absence of an obvious underlying cause or illness.\(^4\)\(^5\)

Classification and nomenclature in ITP

The classification of ITP has been revised recently, and is complicated by the fact that primary ITP is a diagnosis of exclusion.\(^5\) Overall, a careful distinction has to be made between ITP and thrombocytopenia, which is either drug-induced, a side effect of lymphoproliferative disorders, caused by infectious disease or malnutrition, or the result of one of the rare disorders affecting platelet production or destruction, such as X-linked thrombocytopenia (XLT), Wiskott-Aldrich syndrome (WAS), Bernard-Soulier or giant platelet disorders. If the diagnosis ITP is considered, a further specification is made by distinguishing between primary and secondary ITP, in which primary ITP refers to an isolated thrombocytopenia without a clear underlying cause or illness.\(^3\) Secondary ITP encompasses all other immune-mediated thrombocytopenias due to a concurrent underlying autoimmune disease, such as Evans syndrome, Systemic Lupus Erythematosus (SLE), Common Variable Immune Deficiency (CVID), anti-phospholipid antibody syndrome or autoimmune lympho-proliferative syndrome (ALPS).\(^6\)

In addition, a division is made between pediatric and adult ITP, based on the age at disease onset, in which individuals under the age of 16 are considered children. This distinction is relevant since there are strong indications that the
onset of childhood and adult ITP differ, in both the trigger to disease onset as well as platelet numbers at presentation. Moreover, they differ in course of disease and probably even underlying disease pathophysiology. Furthermore, ITP is classified based on course of disease, which is either transient or persistent. Previously, ITP was subdivided in an acute and a chronic variant, in which acute ITP was defined as all patients with a platelet count below 150*10⁹/L and disease duration lasting less than six months, after which it automatically was defined as chronic. To date, the threshold as well as the time intervals and nomenclature have been modified. First, the threshold for the definition of thrombocytopenia in ITP was lowered to 100*10⁹/L (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Historical consensus criteria</th>
<th>International working group criteria (2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count threshold for diagnosis ITP</td>
<td>150*10⁹/L</td>
<td>100*10⁹/L</td>
</tr>
<tr>
<td>Phases of disease</td>
<td>Acute (&lt;6 months)</td>
<td>Newly diagnosed (&lt;3 months)</td>
</tr>
<tr>
<td></td>
<td>Chronic (&gt;6 months)</td>
<td>Persistent (3-12 months)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chronic (&gt;12 months)</td>
</tr>
<tr>
<td>Mild versus severe ITP</td>
<td>Severe: platelet count &lt;10<em>10⁹/L or &lt;20</em>10⁹/L#</td>
<td>Severe: those with bleeding that requires treatment at diagnosis, or those who require increased therapeutic intervention during course of disease</td>
</tr>
<tr>
<td>Refractory ITP</td>
<td>Those who do not respond (or no longer) to medication</td>
<td>Those with severe ITP who failed splenectomy and do not respond (or no longer) to medication</td>
</tr>
</tbody>
</table>

#No consensus was reached on the appropriate threshold

Based on Grace RF, Pediatr Blood Cancer. 2012; 216-20

The adjustment was made after Stasi et al. performed a 10 year follow up study in a large cohort of ITP patients with stable platelet counts between 100 and 150*10⁹/L, who had been diagnosed by coincidence, showing that they remained asymptomatic and had a 10 year probability of only 6.9% for developing symptomatic ITP. Besides, it became apparent that the range of normal platelet counts differs depending on ethnical background; as individuals from African or Arabic origin tend to have lower platelet counts than Caucasians. Furthermore, the time interval describing the stage of disease was altered, along with its nomenclature. Instead of acute and chronic, ITP is now classified as newly diagnosed (ND) (i.e. resolved within 3 months), persistent (i.e. resolved between 3 and 12 months) or chronic (i.e. lasting beyond one year). By means of
this new classification it has become possible to separate the classic pediatric ITP patients with a very sudden onset and a quick recovery, accounting for 37-60% of pediatric ITP patients, from the more subtle long lasting ITP.3,5,14 Furthermore, the diagnosis of chronic ITP is deferred, because there is still a reasonable chance of reaching complete remission after 6 months, both for childhood and adult ITP. 16-25% of pediatric patients having ongoing ITP 6 months after diagnosis will reach complete remission within the next 6 months,14,15 compared to 9% of adult patients reaching complete remission within 6 months to 3 years after diagnosis.10,16

This recently accepted delay in classification of chronic ITP is of great importance as it affects treatment of this disease. International consensus treatment guidelines advise for example splenectomy once the diagnosis chronic ITP is set and spontaneous or drug-induced remission is unlikely. These guidelines are generally accepted in adult ITP, whereas in children the decision to perform a splenectomy is usually deferred until reaching adulthood. Thus, the new classification postpones definite treatment options accordingly, which is especially relevant in adult ITP.10,16

Assessment of disease severity

Scoring of disease severity is an important tool in classification, not only to monitor disease activity, but also as a guideline to determine the need of medical intervention. Previously, disease severity was solely correlated with actual platelet numbers. However, since platelet count is not a reliable tool to assess or predict the amount of bleeding, this has been abandoned. Today, disease severity is categorized based on bleeding tendency only.17 The degree of thrombocytopenia is only used in clinical situations where preventive medication needs to be considered to anticipate on situations were bleeding is to be expected, such as before surgery or dental extractions. A commonly used tool in assessing bleeding tendency is the modified Buchanan hemorrhage grading scale,18,19 which enables categorization of bleeding tendency ranging from none (score 0) to life-threatening/fatal (score 5) (Table 2). The scale is a simplified version of the more elaborate hemorrhage grading scale developed by Buchanan, which also distinguishes between the most prevalent bleeding problems in ITP; skin, oral bleeding and epistaxis, and provides an overall bleeding assessment (Appendix 1).18 A new ITP bleeding assessment tool (ITP-BAT) has been proposed in 2013.17
Table 2. Grading of hemorrhage in children with ITP

<table>
<thead>
<tr>
<th>Grade</th>
<th>Overall bleeding severity</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>No new hemorrhage of any kind</td>
</tr>
<tr>
<td>1</td>
<td>Minor</td>
<td>Few petechiae (≤ 100 total) and/or ≤ 5 small bruises (≤ 3cm diameter); no mucosal bleeding</td>
</tr>
<tr>
<td>2</td>
<td>Mild</td>
<td>Many petechiae (&gt; 100 total) and/or &gt; 5 large bruises (&gt; 3 cm diameter); no mucosal bleeding</td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>Overt mucosal bleeding (epistaxis, gum bleeding, oropharyngeal blood blisters, menorrhagia, gastrointestinal bleeding, others) that does not require immediate medical attention or intervention</td>
</tr>
<tr>
<td>4</td>
<td>Severe</td>
<td>Mucosal bleeding or suspected internal hemorrhage (in the brain, lung, muscle, joint, elsewhere) that requires immediate medical attention or intervention</td>
</tr>
<tr>
<td>5</td>
<td>Life-threatening / fatal</td>
<td>Documented intracranial hemorrhage or life-threatening or fatal hemorrhage in any site</td>
</tr>
</tbody>
</table>

Based on Bennett CM et al. Blood 2006: 107(7)

Despite the fact that the ITP-BAT is much more detailed than the Buchanan score, the main conclusions a clinician can draw from both scales are rather similar. The ITP-BAT categorizes bleeding tendency according to the SMOG system, which encompasses three domains, Skin, visible Mucosae and Organs, that are Graded on severity. All domains are subdivided by the different modes of bleeding within each domain, e.g. skin: petechiae, ecchymoses, subcutaneous hematomas and wounds, which are in turn scored on severity. Grades range from 0 to 5, in which 0-2 is considered mild, 3 and 4 severe and 5 life threatening. Within each domain the same severity score is assigned to bleeding manifestations of similar impact, ensuring a representative overall scoring.¹⁷

Course of disease and prognosis

Disease onset in pediatric ITP is associated with a viral illness a few weeks prior to appearance of symptoms in 68% of the cases.³⁴;⁸;¹⁰;²⁰;²¹ In addition, disease onset has been associated with the measles-mumps-rubella vaccination.²² The peak incidence of childhood ITP lies between 2-5 years of age.³;²⁰ Unfortunately, some big cohort long-term prospective studies, e.g. the Intercontinental Cooperative ITP Study Group (ICIS) started before the definition and classification of ITP changed, thereby still using the old definitions described above.
By these definitions, pediatric ITP will become chronic in 18-30% of the cases.\textsuperscript{2,14,23,24} Nevertheless, Neunert et al. showed that about 27% of >700 pediatric ITP patients did not reach platelet counts above $100\times10^9/L$ after 12 months, indicating chronic disease according to the current criteria.\textsuperscript{14} Grace et al. classified >500 pediatric ITP patients retrospectively according to the old and new criteria. They found that 60% of pediatric ITP patients will recover rapidly within three months, 15% will become persistent and recover between 3 to 12 months, while the remaining 25% will develop chronic ITP, defined as lasting beyond one year (Table 3).\textsuperscript{3}

Table 3 Baseline characteristics in pediatric ITP based on the current consensus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline characteristics</strong></td>
<td></td>
</tr>
<tr>
<td>Peak incidence</td>
<td>5.5(3-16) years</td>
</tr>
<tr>
<td>Male:Female</td>
<td>0.85:1</td>
</tr>
<tr>
<td>Vaccination or infection prior to disease onset</td>
<td>67.7</td>
</tr>
<tr>
<td><strong>International working group criteria</strong></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>66.3</td>
</tr>
<tr>
<td>Severe</td>
<td>33.7</td>
</tr>
<tr>
<td>Newly diagnosed</td>
<td>61.2</td>
</tr>
<tr>
<td>Persistent</td>
<td>15.3</td>
</tr>
<tr>
<td>Chronic</td>
<td>24.5</td>
</tr>
<tr>
<td>Refractory</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Historic criteria</strong></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>60.6</td>
</tr>
<tr>
<td>Chronic</td>
<td>39.4</td>
</tr>
</tbody>
</table>

Data is represented as mean(range) or percentage

Based on Grace RF, Pediatr Blood Cancer. 2012; 216-20

Thus, independent of which definition is chosen, approximately one quarter of children with newly diagnosed ITP will develop chronic ITP. In contrast, adult ITP usually has a subtle onset, is not associated with preceding infections or vaccinations, and the majority of cases results in chronic disease.\textsuperscript{3,8-10}

Determining the percentage of patients suffering from severe ITP is difficult due to the rather vague definition of severe ITP: “patients who have clinically relevant bleeding, defined by such bleeding symptoms at presentation that treatment is mandatory, or by occurrence of new bleeding symptoms requiring additional therapeutic intervention”.\textsuperscript{5} Grace et al. retrospectively identified 34% of all pediatric ITP patients as severe.\textsuperscript{3} Neunert et al. though classified 19% of newly diagnosed ITP patients as severe based on a platelet count <$20\times10^9/L$, of which 41% had none or only one bleeding site.\textsuperscript{14} A minority of 1-3% of patients
CHAPTER 1

suffers from life-threatening bleedings. In general, pediatric ITP can be adequately controlled with or without medication. Only 1% develops refractory ITP, defined as non-responder to all medical treatment or intervention, including splenectomy. Note that the strict definition might lead to an underestimation of refractory patients in pediatric ITP, since splenectomy is usually suspended until adulthood.

Pathophysiology of ITP

The pathophysiology underlying ITP is complex and largely unknown. Moreover, it is thought that there is not only one disease model, but that the disease is highly heterogeneous, and has even been suggested to differ between onset at childhood and at adult age. Two generally accepted theories describe the mechanisms responsible for platelet, and possibly platelet precursor, destruction in ITP. The first theory is known as the classical ITP pathogenesis model and depends on the presence of autoantibodies in ITP, whereas the second theory describes cytotoxic T cell mediated platelet lysis. The classical ITP pathogenesis model is based on the theory of molecular mimicry, stating that environmental antigenic structures present on infectious agents resemble self-antigens. This does not only trigger an adequate immune response against the invading pathogen, but it also initiates an autoimmune response against similar structures. The role of autoantibodies in ITP was first described indirectly by dr. Harrington, who injected himself with plasma obtained from an ITP patient and developed a transient thrombocytopenia within hours, indicating that a factor in plasma was responsible for the onset of disease.

Indeed, in the majority of ITP patients autoantibodies have been found, mainly directed against the surface glycoproteins IIbIIIa (GPIIbIIIa) and/or IbIX (GPIbIX) present on platelets and megakaryocytes. These antibodies are held responsible for Fc-gamma receptor (FcγR) mediated platelet destruction by phagocytosis, which mainly occurs in the spleen. Besides, they may also cause destruction or inhibition of megakaryocytes, the platelet-precursor cells residing in the bone marrow. In this disease model, platelets are opsonized by autoantibodies, leading to activation of FcγR bearing phagocytes and antigen presenting cells (APC), potentially resulting in recognition of autoantigen specific T cells. These T cells in turn interact with B cells, thereby modulating, perhaps boosting, autoantibody production, both closing the continuous pathogenic loop and strengthening it due to the process of somatic
hypermutilation. In case of molecular mimicry, where antibodies directed against an invading pathogen cross-react with epitopes on glycoproteins of platelets, the T cells are likely to recognize pathogen-specific peptides, explaining the apparent dominance of antibody-mediated pathologies in ITP (Figure 1). However, this mechanism of disease fails to account for all ITP patients. Already in Harrington’s experiment there were some ITP patients whose plasma did not evoke an ITP in the healthy recipient, indicating the involvement of cells rather than factors present in plasma. Indeed, the second immune mediated mechanism of platelet breakdown identified in ITP consists of exclusively T cell mediated platelet lyses. There is evidence that CD8+ cytotoxic T cells might induce ITP in both mice and humans. Olssen et al. demonstrated increased platelet lysis in presence of CD8+ cytotoxic T cells in active ITP patients versus controls or patients in remission, whereas platelet lysis failed when donor platelets where used. In addition, these patients exhibited an increased expression of several cytotoxic- and Th1 related genes such as IFNγ and IL-2, while members of the killer cell immunoglobulin-like receptor (KIR) were downregulated in patients with active ITP in comparison with controls and patients in remission. The latter potentially sensitizes autoreactive T cells toward destruction of self as KIR are associated with downregulation of cytotoxic T cells. Both findings underline CD8+ cytotoxic T cell involvement in ITP. Interestingly, Zhao et al. confirms the increased platelet lysis in presence of CD8+ cytotoxic T cells in ITP patients in comparison to controls. Moreover, they found that this mechanism of action is found in the majority of ‘autoantibody-negative’ ITP patients (80%) in contrast to half of ‘autoantibody-positive’ ITP patients. 

In addition, when examining the proposed ITP disease model (Figure 1) in more detail, all immune cells involved might be prone to dysregulation or malfunction, resulting in the actual onset of ITP. Determining the exact disease mechanism might be impossible due to its complexity and heterogeneous origin. A wide variety of theories and different perspectives have been proposed, focusing on T cells, B cells, APCs and FcγRs.

It is well known that the balance between T cell subsets influences the outcome of the immune response significantly, thereby affecting the onset of autoimmunity or autoinflammation and course of disease. In this regard, a dominance of Th1, which is among others responsible for enhanced APC and cytotoxic T cell activity, is associated with several autoimmune diseases including ITP. ITP patients have shown to possess elevated Th1 cytokine
levels, increased Th1 gene expression profiles and even augmented Th1 associated chemokine receptor expression in the spleen has been identified. In addition to a T cell subset skewing to a more proinflammatory environment in general, T cell involvement in ITP has been observed on a more antigen specific level as well. Specific autoreactive T cells directed against platelet GPIIIa epitopes have been identified in ITP patients. Interestingly, these cells were also found in healthy individuals, apparently not leading to disease per se, due to adequate downregulation of their activity, thereby suggesting loss of tolerance in ITP. Because FOXP3 regulatory T cells (Treg) are key players in maintaining peripheral tolerance, possible Treg defects might play a principal role in the development of ITP. Indeed, several studies suggest that Treg defects, such as low numbers and dysfunction, can underlie ITP pathology in both human cases of ITP and experimental animal models.

Since B cells are responsible for the autoantibody production, they are an obvious candidate in ITP research aimed to unravel the pathogenesis of the diseases. Indeed, elevated plasma levels of B cell activating factor (BAFF) have been described in autoimmune diseases, such as ITP, suggesting a possible role for B cells. Another indication of B cell involvement in pathology underlying ITP is the initial beneficial effect of anti-CD20 monoclonal antibody (mAb) therapies, such as Rituximab, which exerts a response in 30-60% of ITP patients. Anti-CD20 mAb therapy results in total B cell depletion, except pro-B cells and plasma cells. However, Audia et al. demonstrated that the immediate B cell elimination itself does not completely account for the beneficial effect of Rituximab. Both Rituximab responders and non-responders showed complete B cell elimination, but non-responders exhibited an increased ratio of effector T cells (Teff) to Treg, indicating that the ultimate effect of Rituximab may be influencing the T cell compartment rather than B cell elimination. The elimination of B cells probably influences immune regulation by depletion of regulatory B cells (Breg) (see below) or by depleting B cells as a source of antigen-presentation. Nonetheless, B cells remain an interesting target in ITP research. An unexplored but probably crucial point in disease development into chronic ITP might be the formation of long-living plasma cells.
Based on Cines NEJM 2002; 346 (13) 995-1008

**Figure 1 Pathogenesis model of ITP.** Molecular mimicry, antibodies against a viral epitope are formed which also interact with glycoproteins on the platelets’ surface (1). These antibodies opsonize platelets leading to increased uptake by FcγR bearing cells that can function as antigen presenting cells (APC) (2). This results in an augmented platelet antigen presentation (3). Autoantigen-specific T cells can interact with these antigens, and become activated. In turn, activated autoantigen specific T cells can stimulate B cells, which will continue producing antibodies and likely even undergo somatic hypermutation (4).
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Whereas the formation of memory plasma cells might account for the cycling patterns of thrombocytopenic episodes associated with an increase of autoantibody titers, long-living plasma cells might result in chronic disease. Radbruch et al. demonstrated the existence of long-living plasma cells which function as immunological memory and can reside hidden in the bone marrow, secondary lymphoid organs or inflamed tissue for years or even decades. These cells have been shown to survive life-long in an SLE mice model, and have also been identified in patients with Sjögrens Syndrome (SS) and Reumatoid Arthritis (RA).

In addition, a new player in human immune regulation was suggested recently; the regulatory B cell (Breg). These cells were first identified in a colitis ulcerosa (CU) mice model in which mice lacking B cells developed a more aggressive CU. This depended partly on the lack of Interleukin 10 (IL10) producing B cells which suppressed autoimmunity. Although quite established in several mouse models, the existence of Breg in humans has not yet been unequivocally proven. B cells that express regulatory function by reducing Teff and monocyte activation, both at least partially mediated via Interleukin 10 (IL10) secretion, have been identified. Impaired Breg function has been suggested in both SLE and chronic adult ITP patients. However, they seem to have a more ambiguous character as they produce both inhibitory IL 10 and activating IL 6 in different ratios depending on their activating signal.

Next to T and B cells, there might be a prominent role for APCs, such as dendritic cells (DC), B cells and macrophages, in ITP pathology. APCs are the pivotal cells in initiating an adaptive immune response, possibly steering autoreactive T cells toward cytotoxicity and providing T cell help to autoreactive B cells establishing a continuous pathogenic loop (Figure 1). The milieu (e.g. pro- or anti-inflammatory cytokine environment) in which such an antigen is presented might determine if autoreactive T cells become activated. Indeed, Kuwana et al. showed that splenic macrophages of ITP patients are responsible for the uptake of IgG-opsonised platelets via FcγRI, which elicited autoreactive T cell activation. Catani et al. demonstrated that DC of ITP patients show increased capacity to present apoptotic platelets to T cells. Moreover, they identified impairment in DC function resulting in reduced Treg activation and decreased Treg function in ITP patients. Zhang et al. exhibited the crucial role of antigen primed DC as a mediator in antigen-specific Teff suppression in ITP patients in vitro. GPIIbIIIa specific Tregs could only suppress GPIIbIIIa specific Teff adequately in presence of DC primed with GPIIbIIIa.
Finally, an important line of research encompasses genetic variations underlying ITP. Among others, associations are known between genes influencing cytokines and/or its receptors involved in evoking a pro-inflammatory response, including T cell activation.\(^{72}\) In the scope of this thesis we will focus on Fc\(\gamma\)R, as a dysbalance in Fc\(\gamma\)R subsets or presence of certain Fc\(\gamma\)R polymorphisms may be involved in the pathogenesis of ITP.\(^{73-77}\) The family of Fc\(\gamma\)R show cell-type specific expression patterns and consists of several members with either a high-affinity for monomeric IgG (Fc\(\gamma\)RI) or with a low to medium affinity for monomeric IgG (Fc\(\gamma\)RII, Fc\(\gamma\)RIII). The latter have a high affinity for IgG opsonized on a target due to the increased avidity by the multivalent interaction of the complexed IgG. These low/medium affinity receptors are thought to be most important in clearance of IgG-opsonised platelets. Crosslinking Fc\(\gamma\)R after engagement of IgG-opsonized targets leads to activation of phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC), with the exception of Fc\(\gamma\)RIIb, which leads to inhibition of the response initiated by the activating Fc\(\gamma\)R. If expressed by macrophages, Fc\(\gamma\)RIIb can inhibit phagocytosis and might lead to B cell apoptosis if cross-linked together with the B cell receptor. Genetic variation within the Fc\(\gamma\)RII and Fc\(\gamma\)RIII genes leads to subtypes with higher affinity (e.g. Fc\(\gamma\)RIIa-H131, Fc\(\gamma\)RIIa-V158, Fc\(\gamma\)RIIib-HNA1a), functional defects (Fc\(\gamma\)RIIb-T232) or to increased expression levels (Fc\(\gamma\)RIIic-C-ORF) and copy number variations (CNV). Thus, the balance and function of Fc\(\gamma\)R might determine the means of processing and presentation of opsonised platelets in ITP, which may influence antigen-presentation in such a way that it results in autoreactive T cell activation. Since Fc\(\gamma\)Rs are also pivotal in the destruction of IgG opsonised platelets, it may also be that patients with an Fc\(\gamma\)R profile leading to efficient platelet destruction will be the first showing an increased bleeding tendency and/or lower response to treatment. Several studies describe a disturbed balance of the low-affinity activating Fc\(\gamma\)R (IIa, IIIa, IIIb and IIc) and the inhibitory Fc\(\gamma\)RIIb in autoimmune diseases, including ITP, which can be restored by therapy.\(^{78-81}\) Furthermore, a variety of single-nucleotide polymorphisms (SNP) and CNV have been identified in the low-affinity Fc\(\gamma\)R genes which are associated with receptor function modification.\(^{73,74,82-85}\) Some of these SNPs have been associated with ITP, such as a higher incidence of the Fc\(\gamma\)RIIa-V158 variant, which has an enhanced affinity for IgG1, IgG3 and IgG4.\(^{74,76,82-86,87}\) This also holds true for increased frequency of the Fc\(\gamma\)RIIc classical open reading frame (C-ORF), leading to an extra activating Fc\(\gamma\)RIIc receptor which is expressed on natural killer (NK) cells, B cells and
monocytes. In addition, the FcγRIIb-T232, which is associated with impaired inhibitory FcγRIIb function, and its heterozygous FcγRIIb-I/T232 genotype was shown to predict chronic disease outcome in pediatric ITP (Table 4). Thus, genetic variation within the FcγR genes is associated with ITP, but whether this reflects a role of FcγR in pathogenesis or in the clinical course of ITP is not yet known.

Management of pediatric ITP

In general, whether or not initial treatment is prescribed depends on the individual patients' characteristics. In the Netherlands, a 'watch and wait' strategy is followed in pediatric ITP, in which patients have regular doctors' visits to monitor platelet counts and bleeding tendency. In the majority of cases, children have no symptoms of significant bleeding unless injured, despite frequent low platelet counts (20-30*10^9/L). Therefore, no treatment is prescribed unless clinically required by a high bleeding tendency, defined as ≥grade 3 on the modified scale of Buchanan, or in a preventive setting for those at increased risk of bleeding, such as before surgery, or dental extractions. Intriguingly, platelet count does not necessarily correlate to bleeding tendency in ITP, as some patients with platelet counts <20*10^9/L rarely bleed whereas others with counts above 100*10^9/L suffer from bleeding regularly.

First line treatment

First line treatment in pediatric ITP is aimed at raising platelet count to prevent or treat moderate or severe bleeding. Intravenous immunoglobulin (IVIg), prednisolone, or intravenous anti-D immunoglobulin (IV anti-D) is recommended, although the latter is scarcely used. IVIg consists of pooled human IgG from over thousands of healthy donors (the actual number varying between pharmaceutical companies), and is usually prescribed as a single high-dose per treatment. Intriguingly, the exact mechanism of action of IVIg remains elusive. Classical theories describe accelerated clearance of autoantibodies due to saturation of the neonatal FcR receptor (FcRn), the presence of anti-idiotype antibodies in IVIg, which bind autoantibodies thereby neutralizing them, blockage of FcγR, thereby preventing platelet destruction or, as observed in mice studies, upregulation of the inhibitory FcγRIIb.
Table 4 Main Characteristics of FcγR SNPs and CNVs

<table>
<thead>
<tr>
<th>Activating/Inhibiting</th>
<th>Receptor</th>
<th>Cell type</th>
<th>SNP/CNV</th>
<th>Special characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activating</td>
<td>FcγRIIa</td>
<td>myeloid cells, monocytes, macrophages, platelets</td>
<td>H/R, CNV=2</td>
<td>H is associated with an increased affinity for IgG1, IgG3 and binds IgG2</td>
</tr>
<tr>
<td>Inhibiting</td>
<td>FcγRIIb</td>
<td>B cell, monocytes, macrophages, follicular dendritic cells</td>
<td>I/T, CNV=2</td>
<td>T is associated with impaired inhibitory function</td>
</tr>
<tr>
<td>Activating</td>
<td>FcγRIIc</td>
<td>NK-cells, neutrophils, monocytes, B cell</td>
<td>Stop/C-ORF/NC-ORF, CNV 1-3</td>
<td>C-ORF is the only variant that results in a low affinity activating FcγR</td>
</tr>
<tr>
<td>Activating</td>
<td>FcγRIIIa</td>
<td>NK-cells, monocytes, macrophages, T cell subsets</td>
<td>V/F, CNV 1-3</td>
<td>V is associated with a higher affinity for IgG1 and IgG3</td>
</tr>
<tr>
<td>Activating</td>
<td>FcγRIIIb</td>
<td>neutrophils</td>
<td>HNA1a/HNA1b/SH, CNV 0,2-3</td>
<td>HNA1a is associated with enhanced internalisation of IgG1 or IgG3 coated particles</td>
</tr>
</tbody>
</table>

Indeed, several studies report blockage of FcγRII an FcγRIII by IVIg, leading to increased platelet counts or prevention of ITP.99;104;105 Also, a 60% increase was observed of FcγRIIb expression on macrophages in response to IVIg in mice.104 In contrast, Aubin et al. demonstrated that IVIg successfully inhibited antigen-specific T cell responses mediated via APC in an ovalbumin immunized (OVA) mice model independent of FcγRIIb. Siragam et al. partially confirmed this finding by showing that beneficial IVIg treatment of Ab-induced ITP depended on the interaction of IVIg with activating FcγRs on DCs in mice. These ITP mice recovered upon adoptive transfer of IVIg primed DCs, in which the presence of FcγRIIb in the recipient was necessary, but not on the DC itself.107 In addition to all FcγR related theories, research revealed that Treg were essential for IVIg in preventing experimental autoimmune encephalomyelitis in mice,108 and that IVIg treatment leads to an increase in Treg, both quantitatively and qualitatively, in humans.109

Prednisolone is a corticosteroid, which has a general anti-inflammatory and immunosuppressive effect, and is as such widely used in autoimmune diseases. In ITP it is usually prescribed in a conventional low dose for 5 to 14 days maximum, or as an intensive high-dose treatment for 3-4 days. An increase in platelet counts is observed in approximately 66% of patients, however duration of response varies highly.9 Although corticosteroid treatment is an adequate and
CHAPTER 1

cheap treatment with a high response rate, IVIg, despite its costs, is also regarded as useful due to fewer side effects.

Anti-D consists of IgG obtained from Rhesus (Rh)-D negative donors who are immunized to the D antigen. Since 1983 it has been used in treatment of ITP, thereby achieving a rise of platelets in 50-77% of patients, lasting for approximately 3 weeks.\(^9\) The therapeutic effect is assigned to possible anti-idiotypic antibody function, similar to IVIg,\(^105\) but more importantly, through blockage of the mononuclear phagocytic system (MPS), which are responsible for platelet clearance. This occurs because the fraction of anti-D IgG antibodies will bind to the patients RhD-antigen on red blood cells (RBC), opsonizing them for destruction in the liver and spleen and thereby saturating the phagocyte FcγRs. Hence, anti-D is only used in RhD-positive ITP patients, and given in such a dose that it results only in a low level of sensitization of the RBCs, too low to induce major hemolysis and anemia. In agreement with this hypothesis, Song \textit{et al.} displayed the down regulation of FcγRIII on splenic macrophages in response to IV anti-D in mice.\(^110\) Also in support of this, IV anti-D treatment fails in the majority of splenectomised patients, underlining the possible mode of action via the MPS.\(^105\)

**Secondary treatment**

If first line therapy fails or evokes serious side effects, secondary treatment options should be considered. Second-line therapy includes dexamethasone, high-dose methylprednisolone, Rituximab and thrombopoeitin (TPO)-receptor agonists, next to combination regimens.\(^9\)

Dexamethasone and methylprednisolone both belong to the family of corticosteroids. Although response rates are high (±60-100%), side effects are usually severe. High-dose dexamethasone has shown to shift the balance between inhibitory and activating FcγR expressed by monocytes in favor of FcγRIIb. Furthermore, a down-regulation was observed of FcγRI and overall phagocytic activity.\(^79\) In addition, Ling \textit{et al.} described low Treg levels in ITP patients, which restored following high-dose dexamethasone treatment.\(^48,79\)

Rituximab is an anti-CD20 mAb which binds to cells from the B cell lineage, except precursor-B cells and plasma cells, and results in complete elimination of these cells, as is described earlier. Rituximab therapy evokes a response in 30-60% of ITP patients and might even lead to a sustained increased platelet count for up to 6-30 months. The positive effect of Rituximab treatment in ITP is somewhat peculiar, since the plasma cells who are responsible for the
autoantibody production escape elimination. However, evidence for Rituximab effect in ITP mediated via T cells is also accumulating.\textsuperscript{53,58,112} Stasi et al. exhibited low Treg percentages and declined Treg function in ITP patients which restores upon Rituximab treatment.\textsuperscript{52} Furthermore, they observed an increased T1/T2 ratio in ITP patients which normalized in those responding to Rituximab, whereas the non-responders did not.\textsuperscript{58,112}

Thrombopoietin (TPO) is the cytokine that is driving the proliferation of megakaryocytes and is also important for their maturation and platelet production. TPO is produced by hepatocytes of the liver, and to a lesser extent by bone marrow stromal cells.\textsuperscript{113} The production of TPO is relatively constant, whereas its plasma levels are regulated by uptake of TPO by megakaryocytes and platelets. Thus, during thrombocytosis almost no free TPO is circulating in the plasma providing a negative feedback, while during thrombocytopenia its levels are high, thereby stimulating platelet production.\textsuperscript{114-116} In ITP, megakaryopoiesis is in most cases not disturbed and platelets are produced, which leads to a normal uptake of TPO, which is rapidly destructed together with the opsonised platelets. Although only a few ITP patients show impaired megakaryocytopoiesis and increased TPO plasma levels, it has been shown that the use of TPO receptor agonists increases the production of megakaryocytes and platelets in ITP patients.\textsuperscript{117} Although these drugs are not yet approved in pediatric ITP, they are increasingly used in adult ITP. A response is reached in 59-80\%, though transiently, resulting in increased platelet counts and less bleeding.\textsuperscript{118}

**Treatment with or without IVIg in Kids with acute ITP: nationwide TIKI study**

In 2008, the TIKI study (Treatment with or without IVIg in Kids with acute ITP (NTR1563)) has been initiated (principle investigators dr M. Bruin (WKZ, UMCU) and dr M. de Haas (Sanquin). The TIKI study is a multi-center randomized controlled clinical trial in which 200 newly diagnosed ITP patients are to be included. The study aims to elucidate whether children with newly diagnosed ITP benefit from early treatment with IVIg to prevent the development of chronic disease (primary outcome). In addition, we asked ourselves some more fundamental questions regarding the role of Treg and FcyR polymorphisms in the development and natural course of disease in ITP. Simultaneously, we were interested in the underlying working mechanism of
IVIg in disease development, and if certain gene-profiles involved in immune and inflammatory responses, e.g. FcγR polymorphisms, affected the effect of IVIg (secondary outcomes).

Inclusion criteria are defined as; children aged 3 months-16 years presenting to a clinician with newly diagnosed ITP, a platelet count below $20 \times 10^9/L$ at diagnosis and a bleeding tendency $<4$ on the modified scale of Buchanan.\textsuperscript{18,19} Exclusion criteria consist of; severe or life-threatening bleeding at presentation, defined as score 4 or higher on the scale of Buchanan, the presence of clinical features that are not compatible with the diagnosis of newly diagnosed ITP and the use of immunomodulating therapy within 4 weeks prior to diagnosis. Patients included are randomly assigned to a single high-dose IVIg treatment ($0.8\text{g/kg}$ bodyweight) within 72 hours after diagnosis, or pure observation (Figure 2).

![Figure 2](image-url)

**Figure 2** Tiki study

At several fixed time points during the study, at diagnosis, one week (6-8 days after inclusion), one month (28-33 days after inclusion), three months (12-14 weeks after inclusion), six months (24-28 weeks after inclusion) and one year (48-56 weeks after inclusion), blood is sampled for regular blood counts and research purposes. Samples are used for instant testing and cryopreservation of peripheral blood mononuclear cells (PBMC), as well as for extraction of DNA.
At diagnosis the presence of platelet autoantibodies is determined by indirect platelet immunofluorescence tests (PIFT) and indirect monoclonal antibody immobilization of platelet antigens (MAIPA). Furthermore, a flow-cytometry based analysis is performed to assess numbers and relative distribution of lymphocytes, divided by B cells, CD4+ T cells and CD8+ cytotoxic T cells, and to determine the absolute numbers and percentage of regulatory T cells (Treg). Finally, TPO measurements are performed, to analyze whether a platelet production defect is present. During all following time points PIFT and MAIPA assays were repeated.

In addition, at each time point patients and parents are asked to complete the Health Related Quality of Life (HRQoL) questionnaire about the influence of ITP on their quality of life. Questionnaires are filled out by the patients themselves, their parents, and in a proxy setting, where parents are asked to complete a questionnaire the way they assumed their child would fill it out. In summary, the TIKI study has the following study outcomes:

**Primary study outcome:**
- Whether or not early IVIg treatment prevents the development of chronic ITP

**Secondary study outcomes:**
- Investigation of the effect of IVIg on clinical parameters during course of disease, such as platelet counts, autoantibodies and bleeding symptoms
- Investigation of the effect of IVIg on number, phenotype and function of Treg in comparison with the observation group.
- Whether or not certain FcγR polymorphisms are associated with a clinical response to IVIg.
- Whether or not certain FcγR polymorphisms are associated with both the development of ITP and the course of disease in the observation group.
- Comparison between the HRQoL in parents and patients with or without IVIg treatment and with or without the development of chronic ITP (not included in this thesis)

In the studies described in this thesis we attempted to answer some of the more fundamental research questions focusing on Treg function and FcγR polymorphisms in pediatric ITP for which we made use of some of the clinical samples obtained in the TIKI study. The TIKI study will continue until 200
pediatric patients with newly diagnosed ITP are included, which is estimated to be completed in 2015.

**Chronic ITP in Children in the Netherlands: CINKID study.**

In 2010 the CINKID study (Chronic ITP in Children in the Netherlands) was initiated. A multi-center observational study in which 40 chronic pediatric ITP patients were included. This research project aimed primarily at investigating platelet function in relation to bleeding tendency of the patients. Secondly, we questioned whether bleeding tendency is associated with HRQoL, the presence of antiplatelet-antibodies, TPO level, FcγR polymorphisms or response to treatment. Finally, the CINKID was founded to be able to compare chronic pediatric ITP patients to our newly diagnosed pediatric ITP patients participating in the TIKI trial.

**Figure 3 CINKID study**

Inclusion criteria consisted of children aged 2-16 years with chronic ITP, defined as a platelet count below $100\times10^9$/L for over a year. Patients with clinical features that were not compatible with the diagnosis of primary chronic ITP were excluded (Figure 3). Patients were classified as mild or severe based on their bleeding tendency. Patients were defined as severe if they suffered from at least two score 4-5 on the modified scale of Buchanan\textsuperscript{18,19} bleeding events during the course of ITP.
Data on duration of the ITP, bleeding tendency and medication were collected by a questionnaire and from the patient’s medical files. Peripheral blood was collected to test platelet function, cryopreserve PBMC, extract DNA, determine the TPO levels, and to test for the presence of platelet autoantibodies by indirect PIFT and MAIPA testing. Furthermore, patients were asked to complete the HRQoL questionnaires.

**Scope of this thesis**

The research presented in this thesis focuses on pathophysiological mechanisms underlying pediatric newly diagnosed ITP, thereby concentrating on the role of regulatory T cells and FcγR polymorphisms. Besides, one study was dedicated to explore actual platelet function in chronic pediatric ITP. To this end, diagnostic tests were developed, applicable for low platelet counts. Treg play a crucial role in maintaining peripheral tolerance, as such they are an important subject of research in the field of autoimmune diseases in which loss of tolerance is suspected. In that perspective they are an intriguing topic in ITP research in which the presence of platelet specific autoreactive T cells has been demonstrated, whereas these cells are tolerogenic when found in healthy individuals. Although the current literature is highly contradicting and almost solely based on research performed in adult chronic ITP patients, several studies suggested that Treg defects might underlie ITP pathology, describing decreased Treg count and dysfunction. Therefore, we explored the role of Treg in pediatric ITP in a homogeneous population; children with newly diagnosed ITP, who did not receive treatment and were followed for one year. (Chapter 2).

One of the key players in ITP pathology are the platelet specific autoantibodies. They opsonize platelets and as such, are held responsible for accelerated platelet destruction by FcγR bearing phagocytes, particularly in the spleen. A selection of these FcγR bearing cells, including macrophages, DCs and B cells, can function as APCs and therefore might play a role in determining disease outcome. Besides playing a prominent role in current ITP disease models, the importance of FcγR is underlined by the success rate of IVIg treatment and splenectomy. In addition, it is known that the low affinity FcγRs are subject to a variety of SNPs and CNVs, which modify FcγR expression and function, some of them have been associated with ITP (Table 4). However, due
to small group sizes, group variations and clustering of different disease outcomes, findings in this field have been contradicting.\textsuperscript{74,75,86,125-127} In addition, the techniques required for SNP and CNV analysis have become increasingly powerful and the number of detectable SNPs has been enhanced the past decade.\textsuperscript{74,88,128} The TIKI study provides the unique opportunity to study a well characterized cohort of children with newly diagnosed ITP, not biased to include high numbers of cases with chronic ITP as in previous studies. In this cohort we studied the possible role of FcγR polymorphisms and CNVs in disease pathology. Moreover, association studies which link FcγR profiles of ITP patients to response to immunomodulating therapy with IVIG are lacking. Therefore, we investigated in the patients included in the TIKI study, whom were randomized to IVIg treatment or observation, whether FcR profiles correlated with a response to IVIg (Chapter 3).

Investigating chronic ITP patients in a cross-sectional study is a real challenge. They consist of a heterogeneous group of patients, subjected to different treatment regimens, and matters are complicated further by its retrospective component. Nevertheless, we attempted to relate quality of life to mild or severe bleeding tendency by use of the HRQoL questionnaire. In addition several laboratory measures were performed, such as the presence of antiplatelet antibodies, TPO levels and FcγR polymorphisms to explore whether these are associated with the patients’ bleeding tendency. In the end also the different treatment regimens in both patient groups were analyzed (Chapter 4).

Historically, severity of ITP, as well as the decision whether a patient needs to be treated or not, was determined by the degree of thrombocytopenia. Over time, investigators realized that platelet counts do not necessarily predict bleeding. Although rare, cases with either low platelet counts without bleeding, or relatively high platelet counts with severe bleeding do occur. We hypothesized that variation in platelet function might account for the differences observed in bleeding phenotype, possibly due to antiplatelet autoantibodies that block platelet function rather than, or in addition to, opsonization resulting in accelerated platelet clearance. Unfortunately, today’s methods to assess platelet function require platelet counts above 50*10^9/L to be reliable and are consequently unsuitable for patients with low platelet counts.\textsuperscript{129,130} Therefore, we developed two new functional assays, which assess actual platelet aggregation and platelet reactivity in response to an agonist, and are applicable in patients
with low platelet counts. By using this assay we determined platelet function in a cohort of chronic pediatric ITP patients (Chapter 5).

In conclusion, the research presented in this thesis focuses on pathophysiological mechanisms underlying pediatric ITP, both newly diagnosed and chronic, thereby concentrating on the role of regulatory T cells and FcγR polymorphisms. Besides, one study was dedicated to explore actual platelet function in pediatric ITP, to which end two diagnostic tests were developed, applicable to low platelet counts.
## Appendix 1

### Buchanans bleeding assessment scale in pediatric ITP; Grading of hemorrhage in children with ITP

<table>
<thead>
<tr>
<th>Grades</th>
<th>Score</th>
<th>Category</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall bleeding</td>
<td>0</td>
<td>None</td>
<td>no new hemorrhage from any kind</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Minor</td>
<td>few petechiae (≤100 total) and/or ≤5 small bruises (≤3 cm Ø)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild</td>
<td>many petechiae (&gt;100 total) and/or &gt;5 large bruises (&gt;3 cm Ø), no mucosal bleeding</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moderate</td>
<td>overt mucosal bleeding (epistaxis, gingival bleeding, oropharyngeal blood blisters, menorrhagia, gastrointestinal bleeding, etc) that does not require immediate medical attention or intervention</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Severe</td>
<td>mucosal bleeding or suspected internal hemorrhage (in the brain, lung, muscle, joint, etc) that requires immediate medical attention or intervention.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Life-threatening or fatal</td>
<td>documented intracranial hemorrhage or life-threatening or fatal hemorrhage in any site.</td>
</tr>
<tr>
<td>epistaxis</td>
<td>0</td>
<td>None</td>
<td>spotting on sheet or pillow and/or blood noted in nares, no active bleeding or need to apply pressure</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Minor</td>
<td>active bleeding on 1 or more occasions with need to apply pressure for &lt;15 minutes.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild</td>
<td>active bleeding on 1 or more occasions with need to apply pressure for at least 15 minutes.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moderate</td>
<td>repeated, continuous and/or profuse bleeding</td>
</tr>
<tr>
<td>oral bleeding</td>
<td>0</td>
<td>None</td>
<td>petechiae on palate or buccal mucosae</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Minor</td>
<td>one or more buccal blood blisters (hemorrhagic bullae or infiltrates) with or without petechiae, no active bleeding,</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild</td>
<td>intermittent active bleeding from gums, lips, buccal mucosae or posterior oropharynx</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moderate</td>
<td>continuous bleeding from gums, lips, buccal mucosae or posterior oropharynx</td>
</tr>
<tr>
<td>skin bleeding</td>
<td>0</td>
<td>None</td>
<td>no new cutaneous bleeding</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Minor</td>
<td>possibly a few new petechiae (≤100 total)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild</td>
<td>definitely a few new petechiae (≤100 total) and/or ≤5 small bruises (≤3 cm Ø)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moderate</td>
<td>numerous new petechiae (&gt;100 total) and/or &gt;5 large bruises (&gt;3 cm Ø)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Severe</td>
<td>extensive (hundreds of) petechiae and &gt;5 large bruises (&gt;3cm Ø)</td>
</tr>
</tbody>
</table>

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CHAPTER 1


