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# Platelet count, previous infection and *FCGR2B* genotype predict development of chronic disease in newly diagnosed idiopathic thrombocytopenia in childhood: results of a prospective study

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## Summary

About 25–30% of children with acute idiopathic thrombocytopenia (ITP) develop chronic disease. It is not well known which patient characteristics influence the course of the ITP. A prospective study in 60 children with newly diagnosed ITP was performed. The aim of the study was to identify patient characteristics at the onset of thrombocytopenia that predicts the progression to chronic ITP. Clinical data and blood samples were collected at several time points during the first 6 months of the disease. Variables predicting chronic disease, as calculated in a multivariate logistic regression analysis, were a platelet count $>10 \times 10^9$/l at the onset [odds ratio (OR) 1·1, 95% confidence interval (CI) 1·01–1·14], the absence of infection shortly before the onset of the disease (OR 4·8, CI 1·16–19·57) and *FGR2B-232I/T* genotype (OR 7·9, CI 0·96–65·27). The latter may point at an immune-modulating role of FcγRIIb in ITP. Although only three patients had serious bleeds, 35 patients received immune-modulating treatment for low platelet counts only. Seventeen patients were treated with intravenous immunoglobulin (IVIG) and 18 patients received corticosteroids. Patient variables did not differ between these treatment groups. However, patients receiving IVIG had significantly lower risk for chronic disease.

**Keywords:** idiopathic thrombocytopenia, childhood, FCGR2B.

Acute idiopathic thrombocytopenia (ITP) in childhood is characterized by a typical history of acute development of purpura and bruising in an otherwise healthy child. Laboratory investigations show an isolated thrombocytopenia (platelet count $<150 \times 10^9$/l), normal haemoglobin concentration, normal white blood cell count and normal peripheral blood smear; no signs of underlying autoimmune disease, no viral infections, such as human immunodeficiency virus and no signs of malignancy are shown. Various clinical dilemmas persist in newly diagnosed ITP, as recently described by Kuhne (2003). These include an estimation of the bleeding risk and the need for treatment in the individual patient and the inability to predict the disease course for the individual patient at the time of diagnosis. Most children with newly diagnosed ITP will not suffer from serious bleeding and will recover within 6 months, but a group of about 20–30% of the patients will remain thrombocytopenic after 6 months and thus develop chronic ITP (Blanchette & Price, 2003). It is currently not possible to predict which patients will develop chronic ITP.

We have performed a prospective study in 60 patients with newly diagnosed ITP. In our protocol, we advised to treat patients with newly diagnosed ITP only in case of severe (≥grade 3) bleeds, regardless of platelet count. Severe bleeds are rare (3%) (Bolton-Maggs, 2003), so, only a few patients in our study should have been treated. The aim of the study was to find, at the onset of newly diagnosed ITP, patient characteristics that predict the progression to chronic ITP. These variables could be used for future treatment strategies to prevent chronic disease.

We assumed the existence of infection-induced, antibody-mediated destruction of platelets in acute ITP. In Addition, because of the sudden onset of the thrombocytopenia, we postulated that there exists a temporary depression of megak-

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aryocytopoiesis. Increased levels of thrombopoietin (Tpo) and decreased levels of glycocalicin (GC) could be the markers of such an insufficient megakaryopoiesis, and therefore Tpo and GC levels were measured.

Previous studies have shown that leucocyte IgG-Fc receptors, such as FcγRIIa and FcγRIIIa, play a central role in the phagocytosis of auto-antibody coated platelets (Crow & Lazarus, 2003). Carcao *et al* (2003) recently showed an overrepresentation of high-affinity receptor variants *FCGR2A-131H* and *FCGR3A-158V* in childhood ITP. In their retrospective study, no difference was found in these Fcγ-receptor gene polymorphisms between patients with acute and chronic ITP. Foster *et al* (2001) studied Fcγ receptor gene polymorphisms in children with chronic ITP and found associations of *FCGR3A* and *FCGR3B* polymorphisms and chronic ITP (Foster *et al*, 2001). The role of the inhibitory receptor FcγRIIb in human ITP has not yet been well defined. In mice, the FcRIIb receptor mediates the induced down regulation of the immune response (Crow & Lazarus, 2003). Because of the potential influences of Fcγ-receptor gene polymorphisms on the severity and clinical course, we analysed polymorphisms of *FCGR2A, FCGR2B, FCGR3A* and *FCGR3B*.

## Materials and methods

### *Patients*

All paediatricians in the northern and central regions of the Netherlands were invited to participate in a prospective study of children with newly diagnosed ITP during the period January 1999–December 2003. A coordinator in each region was responsible for collecting data. The study protocol was approved by the ethical committees of each participating hospital and written informed consent was obtained from parents and patients older than 12 years. Children aged 0–16 years with the typical clinical picture of acute ITP, i.e. sudden onset (within 2 weeks) of purpura and bruising, an isolated thrombocytopenia with a platelet count below $50 \times 10^9$/l and no signs of additional disease, were included. For each newly diagnosed patient, a registration form and a completed questionnaire was sent to the coordinator within 2 weeks. Six months after diagnosis, a second questionnaire was submitted. Registration was anonymous, using case numbers. Besides clinical data, blood samples of each included patient were collected at the time of diagnosis and after 1 week, 4 weeks, 3 months and 6 months. In case of incomplete data, the referring paediatrician was asked for further information and if needed, patient charts were studied. Our study protocol offers guidelines on diagnosis and treatment of childhood ITP based on the guidelines of the American Society of Hematology (George *et al*, 1996) and the UK practice for management of acute childhood ITP (Eden & Lilleyman, 1992). The guidelines regarding the grading of bleeding tendency and treatment advice are shown in Table I. It was recommended that the platelet count should not be used as a trigger for treatment choice. At the time of diagnosis, the following data were recorded: age, gender, date of diagnosis, signs of infection (defined as an episode with fever lasting for two or more days) occurring within 3 weeks before diagnosis, bleeding tendency, bone marrow examination, hospital admission and initial treatment. Six months after diagnosis, recorded data included platelet count after 1 week, 4 weeks, 3 months and 6 months. Furthermore, data were collected on bleeding tendency and additional treatment after the acute phase of the disease. Development of other diseases, especially autoimmune diseases, was included in the questionnaire.

Laboratory tests at different times included full blood cell count, platelet autoantibodies and Tpo and GC levels in the plasma. At diagnosis, DNA was isolated from peripheral blood mononuclear cells, to analyse Fcγ-receptor gene polymorphisms.

### *Detection of platelet autoantibodies*

The sera was tested by the indirect platelet immunofluorescence test as described by Von dem Borne *et al* (1978).

### *Thrombopoietin and glycocalicin measurements*

For the measurement of Tpo and GC plasma levels, etylenediaminetetraacetic acid-anticoagulated blood was collected. Tpo levels were measured with a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (Folman *et al*, 1997). Normal Tpo levels ranged

**Table I.** Guidelines for scoring bleeding tendency and therapy. Dutch national paediatric ITP protocol.

| Grade | Bleeding symptoms | Therapy advice |
|---|---|---|
| 0 | None | No treatment |
| 1 | Purpura <3 cm, sporadic petechiae | No treatment |
| 2 | Purpura >3 cm, mucosal bleedings and/or epistaxis without decrease in haemoglobin level | No treatment |
| 3 | Persisting mucosal bleeds and/or haemoglobin decrease of >1·6 g/dl | Steroids (2 mg/kg) or IVIG (1 g/kg) |
| 4 | Life-threatening bleeds | Methylprednisolone 30 mg/kg + IVIG + platelet transfusions |

from 4 to 34 arbitrary units (AU)/ml. GC plasma levels were measured with an ELISA as described by Porcelijn *et al* (1998). Normal GC values, as determined in 95 healthy adult individuals, were between 144 and 444 AU/ml. In a former study on different causes of congenital thrombocytopenia (van den Oudenrijn *et al*, 2002), Tpo and GC plasma levels were measured in an age-related control group composed of 56 children. The mean Tpo and GC plasma levels in this group were in the same range as found in healthy adults.

### *Fcγ receptor genotyping assays*

DNA was extracted from peripheral blood mononuclear cells by standard procedures. FcγR genotyping was performed with polymerase chain reaction (PCR)-amplified genomic DNA and allele-specific amplification *(FCGR2A)* (de Haas *et al*, 1995) and for *FCGR3A*, the restriction fragment-length polymorphism analysis as described by Koene *et al* (1997). For *FCGR3B* genotyping, the PCR-based allele-specific amplification assay developed by de Haas *et al* (1995) was used. *FCGR2B* genotyping was performed by direct sequencing of an *FCGR2B*-gene-specific fragment of 1570 bp representing exon 5, intron 5 and exon 6. This fragment was obtained via amplification with *FCGR2B*-sense: 5′TGGGACAAGGAGAG-TACTGCCTGTC-3′ and *FCGR2B*-antisense: 5′CCCTCCCTG-GCTCCCAGCTGAAGTT-3′ annealing to sequences in intron 4 and 6 respectively. Subsequently, this *FCGR2B*-gene fragment was sequenced with the BigDyeTerminator cycle sequencing ready-reaction kit (Applied Biosystems, Warrington, UK) with *FCGR2B*-exon 5 (sense): 5′GAATGTGTATCTAGCCC-AAAGAGAG-3′ and *FCGR2B*-exon 5 (antisense): 5′TGGGA-CAAGGAGAGTACTGCCTGTC-3′ and was analysed with the ABI-377XL-automated DNA sequencer (Applied Biosystems). The control group consisted of laboratory personnel and blood donors (after informed consent was given).

To define the factors that influence the course of the disease, univariate logistic regression analysis was first performed, using all variables that might predict the outcome of chronic disease. Statistically significantly associated variables ($P < 0{\cdot}10$) were subsequently selected for multivariate logistic modelling in order to examine their independent predictive value.

## Results

### *Patient characteristics*

From December 1999 to April 2003, 63 newly diagnosed patients were enrolled in the study. The initial diagnosis of acute ITP was revised in three children because one child proved to have dysmegakaryocytosis, one child had familial chronic thrombocytopenia and one child was seen only once and was then lost to follow up. Sixty patients were thus eligible for further analysis. Table II shows patient characteristics at diagnosis. There was only a slight predominance of girls in our study and the mean age of the girls was 7·5 years against 6·8 years in the boys.

**Table II.** Patient characteristics ($n = 60$).

| Characteristics | No. of patients (%) |
|---|---|
| Boys | 27 (45) |
| 0–6 years | 15 |
| 7–16 years | 12 |
| Girls | 33 (55) |
| 0–6 years | 18 |
| 7–16 years | 15 |
| Platelet count at diagnosis | |
| $<10 \times 10^9$/l | 36 (60) |
| $10–20 \times 10^9$/l | 14 (23) |
| $20–50 \times 10^9$/l | 10 (17) |
| Bleeding manifestations | |
| Grade 1 | 12 (20) |
| Grade 2 | 45 (75) |
| Grade 3 | 3 (5) |
| Preceding infections | |
| Yes | 36 (60) |
| No | 24 (40) |

Most cases presented with a very low platelet count of $<10 \times 10^9$/l (60%), but only three patients had a grade 3 bleed. One of them, with a platelet count of $2 \times 10^9$/l, had a gastrointestinal bleed. One patient, with a platelet count of $3 \times 10^9$/l had serious menorrhagia. The third patient, with a platelet count of $10 \times 10^9$/l, had persistent nose bleeds. No red-cell transfusion was needed in these three patients. Grade 4 bleeding did not occur in our patients.

In 60% of the patients, an infection had occurred within 3 weeks before the onset of bleeding symptoms.

### *Laboratory evaluation on platelet autoantibodies, Tpo, GC and FcγR gene polymorphisms*

Platelet autoantibodies were found in 60% of the tested patients. No difference in positive platelet antibody tests was found between patients who did or did not develop chronic ITP. No significant difference in mean Tpo levels between patients with chronic and acute ITP was detected. Almost all the patients had GC levels within the normal range and no correlation was found between the GC levels and the course of ITP.

Table III shows the results of the Fcγ receptor polymorphism frequencies in the patient groups. For *FCGR2A* and *FCGR3B*, no difference in genotype frequencies was found between the total patient group and the patients who developed chronic ITP. There was a difference in frequency of *FCGR2B* genotypes, with a higher percentage of *FCGR2B-232I/T* in the patients who developed chronic ITP. Table III also shows differences in genotype frequencies of *FCGR2B* and *FCGR3A* genotypes between the total patient group and the group of healthy individuals.

**Table III.** Fcγ receptor genotype frequencies in patients and healthy controls.

| Genotype frequency | All patients no. (%) | Acute ITP no. (%) | Chronic ITP no. (%) | Controls no. (%) |
|---|---|---|---|---|
| *FCGR2A* | | | | |
| 131R/R | 12 (23) | 8 (22) | 4 (25) | 42 (27) |
| 131R/H | 26 (50) | 19 (53) | 7 (44) | 80 (52) |
| 131H/H | 14 (27) | 9 (25) | 5 (31) | 32 (21) |
| *FCGR2B* | | | | |
| 232I/I | 49 (89) | 37 (95) | 12 (75) | 118 (80) |
| 232I/T | 6 (11) | 2 (5) | 4 (25) | 29 (20) |
| 232T/T | 0 | 0 | 0 | 1 (1) |
| *FCGR3A* | | | | |
| 158F/F | 12 (23) | 8 (21) | 4 (27) | 66 (43) |
| 158V/F | 27 (51) | 19 (50) | 8 (54) | 73 (47) |
| 158V/V | 14 (26) | 11 (29) | 3 (19) | 15 (10) |
| *FCGR3B* | | | | |
| HNA1a/1a | 7 (13) | 5 (12·5) | 2 (14) | 27 (18) |
| HNA1a/1b | 20 (37) | 15 (37·5) | 5 (36) | 66 (43) |
| HNA1b/1b | 27 (50) | 20 (50) | 7 (50) | 61 (40) |

### *Course of the disease*

Table IV shows the possibly important variables for the prediction of chronic disease, resulting from univariate logistic regression analysis. Girls had a more than three times higher risk for chronic disease than boys. Age, at presentation, did not influence the outcome. Not having a preceding infection at presentation yielded a 3·6 times higher risk for chronicity. The degree of bleeding was not related to the development of chronic disease in this group of patients. A higher platelet count at diagnosis leads, with a 1·06 times higher risk per $1 \times 10^9$/l platelets, to development of chronic disease. The genotype *FCGR2B-232I/T* showed a more than six times higher risk for chronic ITP compared with genotype *FCGR2B-232I/I*.

### *Treatment and course of the disease*

In contrast to the recommendations, 38 patients received treatment, of whom only three (5%) had grade 3 bleeding symptoms, while 22 patients did not receive any treatment. Three patients received only tranexaminic acid. Seventeen patients were treated with intravenous immunoglobulin (IVIG) and eighteen patients received corticosteroids (CS). Patients not receiving treatment had a slightly higher chance of progression to chronic ITP. Patients receiving IVIG treatment had a significantly lower chance of developing chronic ITP. CS treatment did not benefit the outcome. The three patients treated with tranexaminic acid only were classified in the non-treatment group. However, 35 patients received immune-modulating treatment that might have influenced the natural course of ITP and the indication for this treatment might also be associated with the outcome.

As univariate variables might not be independently associated with outcome, we performed a multivariate logistic regression analysis, initially without including treatment. Table V shows that three variables independently predicted chronic disease: platelet count at diagnosis, previous infection and genotype *FCGR2B-232I/T*.

**Table IV.** Possibly important variables in the total patient group and the distribution in the acute and chronic patient group, univariate logistic regression analysis.

| | Total [($n = 60$) no.] | Not chronic [($n = 44$) no. (%)] | Chronic [($n = 16$) no. (%)] | OR (95% CI) | *P*-value |
|---|---|---|---|---|---|
| Gender (girls *versus* boys) | | | | 3·3 (0·9–11·8) | 0·07 |
| Age (years) | | | | 1·00 (0·99–1·01) | 0·54 |
| Preceding infection | | | | | |
| Yes | 36 | 30 (83) | 6 (17) | Reference | |
| No | 24 | 14 (58) | 10 (42) | 3·6 (1·1–11·8) | 0·04 |
| Bleeding manifestations | | | | | |
| Grade 1 | 12 | 9 (75) | 3 (25) | Reference | |
| Grade 2 | 45 | 33 (73) | 12 (27) | 1·1 (0·3–4·7) | 0·91 |
| Grade 3 | 3 | 2 (67) | 1 (33) | 1·5 (0·1–23·1) | 0·77 |
| Platelet count at diagnosis (per $1 \times 10^9$/l) | | | | 1·06 (1·01–1·12) | 0·03 |
| *FCGR2B* | | | | | |
| 232I/I | 49 | 37 (75) | 12 (25) | Reference | |
| 232I/T | 6 | 2 (33) | 4 (67) | 6·2 (1·0–38·0) | 0·05 |
| Treatment | | | | | |
| None | 22 | 13 (59) | 9 (41) | Reference | |
| IVIG | 17 | 16 (94) | 1 (6) | 0·09 (0·01–0·81) | 0·03 |
| CS | 18 | 12 (67) | 6 (33) | 0·7 (0·2–2·6) | 0·62 |
| Other | 3 | 3 (100) | 0 | 0·0 (–) | |

IVIG, intravenous immunoglobulin; CS, corticosteroid therapy.


 

**Table V.** Variables in the total patient group that predicted chronic disease, as calculated in a multivariate logistic regression analysis.

| | Total [($n = 60$) no. (%)] | Not chronic [($n = 44$) no. (%)] | Chronic [($n = 16$) no. (%)] | OR (95% CI) | *P*-value |
|---|---|---|---|---|---|
| Gender | | | | 2·6 (0·9–11·8) | 0·20 |
| Preceding infection | | | | | |
| Yes | 36 | 30 (83) | 6 (17) | Reference | |
| No | 24 | 14 (58) | 10 (42) | 4·8 (1·2–19·6) | 0·03 |
| Platelet count at diagnosis (per $1 \times 10^9$/l) | | | | 1·06 (1·00–1·13) | 0·02 |
| *FCGR2B* | | | | | |
| 232I/I | 49 (89) | 37 (75) | 12 (25) | Reference | |
| 232I/T | 6 (11) | 2 (33) | 4 (67) | 7·9 (1·0–65·3) | 0·05 |

Next, we analysed the relationship between treatment and the above-mentioned variables. Age and gender did not differ significantly between the three treatment groups. The only variable associated with treatment choice was platelet count at diagnosis. There was a significant difference between platelet count in the group without treatment (mean $17{\cdot}7 \times 10^9$/l) and the IVIG-treated group (mean $4{\cdot}8 \times 10^9$/l, $P = 0{\cdot}02$) and the CS-treated group (mean $6{\cdot}1 \times 10^9$/l, $P = 0{\cdot}01$). The difference in mean platelet count between the two treatment groups was not significant. Thus, platelet count and not bleeding tendency determined the decision to treat patients. In our protocol, a bone-marrow aspiration was compulsory before starting CS therapy. Practical reasons, such as availability of skilled persons to perform and judge the bone marrow aspirates, determined the treatment choice for either IVIG or CS.

Subsequently, we determined the value of the factor platelet count on the outcome in relation to the different treatment strategies. In a bivariate logistic regression analysis, CS treatment was not associated with chronic disease (OR = 0·94, 95% CI 0·50–1·78) and addition to the platelet count as a variable did not change this (OR = 1·2, 95% CI 0·89–1·11). Similarly, a bivariate logistic regression analysis with IVIG treatment showed a highly protective effect against chronicity (OR = 0·11, 95% CI 0·01–0·98), which remained statistically significant after further inclusion of platelet count in the model (OR = 0·18, 95% CI 0·02–1·86).

## Discussion

This prospective study aimed to obtain insight in the factors influencing the natural course of newly diagnosed acute ITP in childhood and determining the variables that predict the development of chronic disease. Only patients with an acute onset of the disease were included, thereby avoiding inclusion of patients with an insidious onset of thrombocytopenia and a known risk of developing chronic disease. Three independent variables were found to influence the course of the disease: initial platelet count, previous infection and *FCGR2B-232I/T* genotype. Previous infection correlated with resolving disease, irrespective of platelet count. An explanation might be found in the theory of molecular mimicry, which proposes that similarities between pathogen and platelet antigens act as mechanisms for the transient platelet-antibody production. In case of a balanced immune reaction, the B cells involved in antibody production are inhibited by normal control mechanisms. Only in cases of disturbed immune control, whether or not influenced by Fcγ receptor polymorphisms, will chronic disease develop.

We found a difference in *FCGR2B* genotypes between patients with acute and chronic disease, with a low frequency of *FCGR2B-232I/T* genotype in patients with acute disease. This shift towards *FCGR2B-232 I/I* genotype in patients with acute ITP has not been described previously. This finding fits with the possibility proposed by Kyogoku *et al* (2002) that the *FCGR2B-232I/T* polymorphism may alter apoptotic signalling, allowing the survival of B cells that produce autoantibodies, leading to chronic autoimmune disease.

Low initial platelet count is a variable predicting favourable, non-chronic outcome. The results of our study clearly show that treatment is triggered by very low platelet counts and not by the severity of bleeding. Hence, 32 patients suffering from grade 2 bleeding tendency were treated with either IVIG or CS. We analysed whether this influenced the course of the disease. Of our patients, 27% developed chronic disease, which is comparable with other prospective studies (Kuhne *et al*, 2001; Blanchette, 2002; Rosthoj *et al*, 2003). Our data confirm the findings of Kuhne *et al* (2003) that the initial platelet count is higher in patients who develop chronic disease. Rosthoj *et al* (2003), in their prospective study in the Nordic countries, did not find any influence of treatment on the risk for a chronic course, but in their study protocol, no recommendations were made about treatment and they did not mention a possible link between low platelet count and starting therapy. There was no difference in patient variables in the treatment group, but we observed a difference in the outcome within the treatment group between patients treated with IVIG and CS. Patients receiving IVIG developed chronic disease less frequently compared with patients receiving CS. The question to be answered is whether IVIG treatment protects against chronic disease or CS treatment predisposes to chronic disease. From the bivariate logistic regression analysis, one can conclude that IVIG treatment is an independent factor, but the validity of this analysis can be hampered by confounding indication. The two proposed therapeutic actions of IVIG, i.e. blocking of the

FcγR-dependent reticoendothelial system (RES) function and neutralizing anti-idiotypic interactions (Crow & Lazarus, 2003), can both explain the rapid recovery of platelets in ITP patients. Imbach (1991) reported that IVIG treatment in children may also reduce the number of patients with chronic disease (Imbach, 1991). To understand the influence of IVIG in preventing the development of chronic disease, one can speculate on the results of recent studies on the role of FcγRIIb and the influence of IVIG (Samuelsson *et al*, 2001; Crow & Lazarus, 2003). In mice, IVIG can increase FcγRIIb levels. FcγRIIb plays a role as a negative regulator of B cells (Ravetch & Lanier, 2000) and FcγRIIb possibly modulates the risk of autoimmunity (Kyogoku *et al*, 2002). Li *et al* (2003) reported a difference between FcγIIb isoforms in CD19 dephosphorylation, which is involved in B-cell receptor signalling.

In conclusion, in 60 children with newly diagnosed ITP, the majority of the patients was treated with IVIG or CS, because of very low platelet counts ($<10 \times 10^9$/l) and not because of a serious bleeding tendency. A low platelet count and an infection shortly before the development of ITP protected against chronic disease. Furthermore, patients treated with IVIG developed chronic disease less frequently; thus, IVIG treatment possibly protects against the development of chronic disease. To confirm this possible protective effect of IVIG, a future study, which randomizes patients to no treatment or IVIG treatment, is needed. In acute ITP patients, *FCGR2B-232I/T* genotype is seen less frequently than in chronic ITP patients and healthy controls. Although this difference was statistically significant, it pertained to small numbers of patients as reflected by the very wide confidence intervals. Therefore, to confirm the contribution of FcγRIIb in the course of the disease, further genetic studies of the *FCGR2B* polymorphisms and functional studies of the different alleles are probably needed.

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