Pediatric immune thrombocytopenia: Catching platelets

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IgG Fc-receptors determine susceptibility for childhood immune thrombocytopenia, recovery and response to intravenous immunoglobulins

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\#/* These authors contributed equally to this article
CHAPTER 3

Abstract

Immune thrombocytopenia (ITP) is most frequently caused by platelet specific autoantibodies leading to accelerated platelet clearance by Fc-gamma receptor (FcγR) bearing phagocytes. Single-nucleotide polymorphisms (SNP) and copy-number variations (CNV) in the FCGR gene cluster affect FcγR function through either, altered affinity for IgG or altered FcγR expression levels, respectively. The high affinity allele FCGR3A*V158 and FCGR2C*C-ORF, encoding for functional FcγRIIc (otherwise a pseudogene) have previously been associated with ITP, but are in high linkage disequilibrium. We now evaluated FcγR profiles in children with newly diagnosed ITP also in relation to recovery and response to intravenous immunoglobulins (IVIg) in the first week of disease. Our results suggest that only FCGR2C*C-ORF is a true risk factor for developing pediatric ITP. Both FCGR2C*C-ORF, and the 2B.4-promoter haplotype of FCGR2B associated with high expression of the inhibitory FcγRIIb, were overrepresented in pediatric ITP. Those with 2B.4-FCGR2B and homozygous for FCGR2B*I232, thus with high expression and maximal capacity to downmodulate function of activating FcγR, were more likely to recover quickly even without IVIg. Individuals homozygous for the FCGR2B*T232 genotype, predicting impaired FcγRIIb function, responded less to IVIg. Taken together, FcγR polymorphisms play a significant role in ITP susceptibility, fast recovery and immediate response to IVIg.
**Introduction**

Pediatric Immune Thrombocytopenia (ITP) is a bleeding disorder with an incidence of about 5 in 100,000 children.\textsuperscript{1,2} Three types of the disease are currently recognized; newly diagnosed, persistent, and chronic ITP.\textsuperscript{3} Newly diagnosed ITP is characterized by a sudden onset of bleeding and thrombocytopenia as the only abnormality. In the majority of patients, the symptoms resolve spontaneously within three months, otherwise the disease is referred to as persistent. Approximately 25\% of pediatric ITP patients will eventually develop chronic ITP,\textsuperscript{1,4} which has recently been defined as a platelet count below 100*10\(^9\)/L for over one year.\textsuperscript{3}

Although the pathogenesis is regarded as heterogeneous and not fully understood, IgG autoantibodies directed at platelet glycoproteins are at least in part thought to be responsible for accelerated platelet destruction through Fc\(\gamma\)R-bearing cells.\textsuperscript{5} Various classes of Fc\(\gamma\)R exist, which can be discriminated by their affinity for monomeric IgG (high-affinity Fc\(\gamma\)RIa versus low to medium-affinity Fc\(\gamma\)RII and Fc\(\gamma\)RIII) and by their functional activity, leading to activation or inhibition of cellular responses. Activating Fc\(\gamma\)Rs stimulate the cell through Immunoreceptor Tyrosine Activation Motifs (ITAM). In contrast, Fc\(\gamma\)RIIb contains an Immunoreceptor Tyrosine Inhibition Motif (ITIM) capable of down modulate ITAM-mediated signaling.\textsuperscript{6} Further functional differentiation within the Fc\(\gamma\)R family is introduced by genetic variation leading to amino acid polymorphisms between individuals affecting their Fc\(\gamma\)R expression levels and affinity for IgG.\textsuperscript{7-14} These include the Fc\(\gamma\)RIIa-H/R131 variation, where the FCGR2A*H131 allotype encodes for a Fc\(\gamma\)RIIa with enhanced binding capacity for IgG2,\textsuperscript{14,15} the Fc\(\gamma\)RIIa-V/F158 variation where the Fc\(\gamma\)RIIa-V158 variant has increased affinity for IgG,\textsuperscript{10,16,17} and the Fc\(\gamma\)RIIb-HNA1a/HNA1b/HNA1c variations, with augmented binding affinity and phagocytosis of IgG1 and IgG3 coated particles through Fc\(\gamma\)RIIb-HNA1a.\textsuperscript{16,18,19} The FCGR2B*I/T232 transmembrane polymorphism in the inhibitory Fc\(\gamma\)RIIb affects translocation of Fc\(\gamma\)RIIb into lipid rafts, resulting in declined capacity of the Fc\(\gamma\)RIIb*T323 variant to down regulate ITAM signaling of the activating Fc\(\gamma\)R.\textsuperscript{9,11,20} Single nucleotide (SNP) variation within exon 3 of FCGR2C, a pseudogene in most individuals, results in an open reading frame (ORF)\textsuperscript{13,21,22} and functional expression in 11\% of Western Europeans of Dutch background, and is referred to as classical ORF (C-ORF).\textsuperscript{13,23} Additionally, an FCGR2C allele has been found
in 5% of people of European origin encoding for a non-classical ORF (NC-ORF)\textsuperscript{23}. The distinction between a C-ORF and NC-ORF is important as the latter is non-functional. This is due to alternative splicing which results in loss of an exon and introduction of a premature stop-codon, thereby preventing expression of FcγRIIC.\textsuperscript{13} In addition, the haplotypic variation -386C-120A, known as promoter haplotype 2B.4, in the promoter region of FCGR2B is associated with an 1.5 fold increased expression of its receptor.\textsuperscript{12}

Finally, an added layer of complexity in the FcγR polymorphism is caused by copy number variation (CNV) of the genomic region encompassing FCGR2C, FCGR3A and FCGR3B, affecting the expression level of the receptors encoded by these genes.\textsuperscript{2,4} Since all the FCGR2 and FCGR3 genes are found within a 0.5MB region on the long arm of chromosome 1, their inheritance is linked depending on their proximity.\textsuperscript{23} In general, it is thought that the balance between activating and inhibiting regulatory elements in FcγRs is important in regulation of the immune response. Phagocytes, such as monocytes and macrophages, and also dendritic cells are known to express both activating FcγRs and FcγRIIC. Recently, it became apparent that B cells, known to express FcγRIIB, also express FcγRIIC in FCGR2C*ORF individuals.\textsuperscript{25}

It has been postulated that the FcγR profile may be a risk factor to develop pediatric ITP, in particular in individuals expressing the higher-affinity activating FcγR alleles. Most, if not all, studies to date have found an overrepresentation of the FCGR3A*V158 allele in ITP patients, while conflicting data have been reported for the other polymorphisms. An increase in homozygous FCGR2A*H131,\textsuperscript{26} homozygous FCGR2A*R131,\textsuperscript{27} homozygous FCGR3B*HNA1a,\textsuperscript{28} heterozygous FCGR2B*I/T232\textsuperscript{29} and the combination of homozygous FCGR2A*H131 and heterozygous FCGR3A*V/F158\textsuperscript{26,30} has been described in ITP, whereas others found no differences in these genotypes.\textsuperscript{21,29,31} The controversy may be due to the sample size studied, which ranges from n=27 to n=116, heterogeneous inclusion of patients, e.g. pediatric patients with mostly transient ITP and adult patients with mostly chronic ITP, mixed ethnical background and last, because linkage of FcγR alleles has not been taken into account. In addition, most studies focused solely on FcγRIIA and FcγRIIa polymorphisms, thereby ignoring the other genetic variations present at the FcγR locus. To our knowledge, only one study, of our group, has investigated FcγRIIC-polyorphisms in ITP, which showed an overrepresentation of FCGR2C*ORF in ITP patients.\textsuperscript{21} However, this study presented an
overestimation of a truly FcγRIIc-encoding gene as the distinction between C-ORF and NC-ORF was unknown at that time.\textsuperscript{13}

IVIg and corticosteroids are the most widely used treatments in newly diagnosed pediatric ITP.\textsuperscript{4,32} Treatment with IVIg has been introduced since it works fast: platelet counts usually rise considerably within two days after administration, and because of its few side effects. Unfortunately, approximately 20-30\% of the patients do not respond to IVIg. Although the exact working mechanism of IVIg is unknown, the quick rise in platelet counts might be caused by instant blockage of the low affinity FcγR receptors of the splenic macrophages,\textsuperscript{33,34} activation and/or upregulation of the inhibitory FcγRIIb,\textsuperscript{35-37} or accelerated clearance of autoantibodies due to saturation of the neonatal IgG-Fc-receptor (FcRn).\textsuperscript{38-40}

In this study, we determined FcγR polymorphisms in 138 children with newly diagnosed ITP, using the multiplex ligation-dependent probe amplification (MPLA) assay, capable of determining all possible clinically relevant genetic FcγR variations known to date. FcγR profiles of all patients were compared to that of 199 ethnically matched healthy controls to evaluate the role of the FcγR profile in the pathogenesis of pediatric ITP. Platelet counts were measured one week after diagnosis used to evaluate whether FcγR profiles were related to either spontaneous recovery (as evaluated in 59 children) or response to a single treatment with 0.8 g/kg of IVIg (n=79).

Materials and Methods

Patients

We included 138 children aged 3 months to 16 years with newly diagnosed acute ITP that were participating in the multicenter randomized study: Treatment with or without IVIG in Kids with acute ITP (TIKI). Newly diagnosed ITP was defined as an isolated thrombocytopenia with a platelet count below 20*10\(^9\)/L at presentation. Exclusion criteria consisted of severe or life threatening bleeding at presentation (defined as score 4 or higher on the modified scale of Buchanan),\textsuperscript{41,42} the presence of clinical features that were not compatible with the diagnosis of newly diagnosed ITP, and the use of immunomodulating therapy within 4 weeks prior to diagnosis. Patients were randomized to receive a single dose of 0.8 g/kg bodyweight IVIg (Nanogam, Sanquin, Amsterdam, The Netherlands) or
to be observed carefully and only receive medical treatment in case of severe bleeding. A good response to IVIg therapy was defined as a rise in platelet count >100*10^9/L after one week. Peripheral blood for DNA analysis was collected at diagnosis. Platelet counts at diagnosis and at T=1 week were used for this study. DNA from 199, ethnically matched, healthy volunteers was available as a control. Parents and patients aged 12 years and older gave written informed consent. All patients and controls were of West European background, as was indicated by short tandem repeat (STR) analysis. The study was registered in the Dutch Trial register (www.trialregister.nl; study ID number NTR TC1563) and approved by the Institutional Review Board of the University Medical Center Utrecht and performed in accordance with the Declaration of Helsinki.

**DNA isolation and Multiplex ligation-dependent probe amplification (MLPA)**

Genomic DNA was isolated from whole blood with a DNA extraction kit (QIAamp DNA blood mini kit, Qiagen Benelux, Venlo, The Netherlands). MLPA, specifically designed for genetic variations within the FCGR locus, was then performed as described previously^8;13;21;43 according to the manufacturers’ instructions (MRC Holland, Amsterdam, The Netherlands). This MLPA identifies SNPs and CNV in the low affinity FcγR genes, namely FCGR2A (encoding for FcγRIIa with two possible allelic variants 131H/R), FCGR2B (232T/I), FCGR2C (Stop/ORF), FCGR3A (158V/F) and FCGR3B (HNA1a/HNA1b/HNA1c). In addition, the FCCR2B and FCGR2C promoter haplotype (-386G/C and -120A/T) were determined. In short, 5 μl DNA (10 ng/μl) in AE buffer was denatured and 1.5 μl FcγR probe mix together with 0.5 μl synthetic probe mix and 1.5 μl SALSA MLPA buffer were added at room temperature. After hybridization at 60°C for 18 hours, SALSA ligase-65 was added with included buffer mixes and incubated at 54°C followed for 15 minutes. The reaction was inactivated by incubation at 98°C for 5 minutes. Polymerase chain reaction (PCR) was started after addition of polymerase mix for 36 cycles according to the standard PCR protocols suggested by the manufacturer. 1 μl of this sample was then mixed with 0.5 μl CXR 60-400 (Promega, Madison, WI) and 8.5 μl deionized formamide and incubated for 10 minutes at 90°C. The product was then separated by electrophoresis on an ABI-3130XL (Applied Biosystems, Foster City, CA) Data was analyzed with Genemarker, version 1.30. (Soft Genetics, State College, PA) and data assessed in
relation to three reference samples representing all known allotypic variants with predetermined CNV. STR tests and analysis were performed on all samples by the laboratory of Paternity testing of Sanquin Diagnostic Services (Amsterdam, The Netherlands) to confirm the West European origin of the patients and their controls, as described before.²³

Statistics
Statistical analysis was performed using IBM SPSS Statistics 20.0.0 for Windows (International Business Machines Corporation, New York, U.S.A.). Data are shown as median with interquartile range (IQR), or mean with percentage, unless otherwise indicated. Genotypic analysis was performed by chi square test or, when cell numbers were low, by Fisher’s exact test. Logistic regression analysis was performed to analyze genotype combinations with high linkage disequilibria. P-values <.05 were considered to be statistically significant.

Results
Baseline characteristics
A total of 138 newly diagnosed pediatric ITP patients were included. The baseline characteristics are described in Table 1. Median platelet counts at inclusion were 6*10⁹/L [3-10*10⁹/L]. Following randomization 79 (57.2%) patients received a single dose of IVIg (0.8 g/kg bodyweight) within three days after diagnosis, and 59 (42.8%) patients were assigned to the observation arm. A slight uneven numbers of observed (59) and treated patients (79) were noted, resulting from the fact that this study is part of the yet ongoing TIKI study. One week after diagnosis, platelet counts were measured. In the treatment group, 55 (70.5%) of the patients had a platelet count >100*10⁹/L, with a median platelet count of 309*10⁹/L [220-421*10⁹/L], whereas 23 (29.5%) patients failed to respond and showed a median platelet count of 23*10⁹/L [6-56*10⁹/L]. One patient treated with IVIg was lost in the follow up. In the observation group, 13 (22%) patients had a platelet count >100*10⁹/L with a median platelet count of 282*10⁹/L [161-352*10⁹/L] after one week, whereas the remainder 46 (78%) had a median platelet count of 8*10⁹/L [4-29*10⁹/L] one week after diagnosis. Age did not differ significantly between the groups (P=.416), nor did it influence recovery in either group (P=.564 and P=.317 for the IVIg and observation group, respectively).
Table 1 Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>IVIg (n=79)</th>
<th>Observation (n=59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>39(49.4)</td>
<td>33(55.9)</td>
</tr>
<tr>
<td>Female</td>
<td>40(50.6)</td>
<td>26(54.1)</td>
</tr>
<tr>
<td>Age at inclusion (months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-5 years</td>
<td>54(68.4)</td>
<td>30(50.9)</td>
</tr>
<tr>
<td>5-10 years</td>
<td>14(17.7)</td>
<td>12(20.3)</td>
</tr>
<tr>
<td>10-16 years</td>
<td>11(13.9)</td>
<td>17(28.8)</td>
</tr>
<tr>
<td>PLT count at inclusion</td>
<td>6[3-9]</td>
<td>6[3-10]</td>
</tr>
<tr>
<td>Recovery after 1 week (&gt;100PLT)</td>
<td>55(70.5)*</td>
<td>13(22)</td>
</tr>
<tr>
<td>PLT count</td>
<td>309[220-421]</td>
<td>282[160-5-351.5]</td>
</tr>
<tr>
<td>No recovery after 1 week (&lt;100PLT)</td>
<td>23(29.5)</td>
<td>46(78)</td>
</tr>
<tr>
<td>PLT count</td>
<td>23[6-56]</td>
<td>7.5[4-28.6]</td>
</tr>
</tbody>
</table>

Data is represented as numbers (%) or median[IQ-range]

*From 1 patient the platelet count after 1 week is missing.

No differences in FcγR CNV in ITP patients compared to controls

No CNV was found for FcγRIIa. For FcγRIIIa a CNV ranging from one to three was found, whereas a CNV ranging from one to four was found for both FcγRIIIb and FcγRIIc (Table 2). For all these genes the vast majority (>80%) harbored two copies. No individual was identified without a copy, although a single copy was regularly found, in particular for FcγRIIIb and FcγRIIc (~5% of the cases). Importantly, the occurrence of these CNV variations was equally distributed between patients and controls (Table 2).
Table 2 FcyR Copy Number Variation (CNV) in newly diagnosed pediatric ITP

<table>
<thead>
<tr>
<th>CNV</th>
<th>Controls (n=199)</th>
<th>Patients (n=138)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCGR2A</td>
<td>2</td>
<td>199(100)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12(6.0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>169(84.9)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15(7.5)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3(1.5)</td>
</tr>
<tr>
<td>FCGR2B</td>
<td>1</td>
<td>11(5.5)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>162(81.4)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>21(10.6)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5(2.5)</td>
</tr>
<tr>
<td>FCGR2C</td>
<td>1</td>
<td>1(0.5)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>187(93.5)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12(6.0)</td>
</tr>
</tbody>
</table>

Individuals with 1,2,3 or 4 copies of a gene were observed. Data is represented as numbers (%).

Allele frequencies in pediatric ITP

Allele frequencies of six FcyR alleles were determined in ITP patients and controls (Table 3). No differences between ITP patients and controls were observed for the FCGR2A*H/R131, FCGR2C*I/T232, FCGR3B*HNA1a/HNA1b/HNA1c polymorphisms or the FCGR2B promoter haplotype 2B.4. In concordance with the literature, we found an increased frequency for the FCGR3A*V158 allele in ITP patients compared to controls (P=.02). In addition, we found an overrepresentation of the functional FCGR2C*C-ORF but also of the non-functional FCGR2C*NC-ORF in ITP patients (P <.01). Furthermore, the incidence of FCGR2B/2C promoter haplotype -386C-120T (known as 2B.2) was significantly increased in ITP patients (P=.014).

Genotypic variations in pediatric ITP

Next, FcγR genotype variations were assessed between ITP patients and controls (Table 4). Statistical analysis of genotype variations was complicated by the high variability in certain FcγR genotypes by the occurrence of both CNV and allotype variations, of which some combinations were very rare. Therefore, for these FcγR we decided to determine the occurrence of at least one specific allele or haplotype versus its absence. No differences were observed for the presence of the FCGR2A*H131, FCGR2B*T232, FCGR2C*NC-ORF, FCGR3A*V158 or FCGR3B*HNA1a allele. In contrast, the frequency of FCGR2C*C-ORF was higher.
in ITP patients (P<0.01), as was the incidence of FCGR2B/2C promoter haplotype 2B.2 and 2B.4 (P<0.01 and P<0.05 respectively).

Table 3 FcγR SNP allele frequency in newly diagnosed pediatric ITP

<table>
<thead>
<tr>
<th></th>
<th>Genotype</th>
<th>Controls (n=199)</th>
<th>Patients (n=138)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCGR2A</td>
<td>131H</td>
<td>214(53.5)</td>
<td>149(53.8)</td>
</tr>
<tr>
<td></td>
<td>131R</td>
<td>186(46.5)</td>
<td>128(46.2)</td>
</tr>
<tr>
<td>FCGR2B</td>
<td>232I</td>
<td>352(88.4)</td>
<td>243(88.0)</td>
</tr>
<tr>
<td></td>
<td>232T</td>
<td>46(11.6)</td>
<td>33(12.0)</td>
</tr>
<tr>
<td>FCGR2C</td>
<td>STOP</td>
<td>365(86.7)</td>
<td>222(77.1)</td>
</tr>
<tr>
<td></td>
<td>C-ORF</td>
<td>45(10.7)</td>
<td>48(16.7)</td>
</tr>
<tr>
<td></td>
<td>NC-ORF</td>
<td>11(2.6)</td>
<td>18(6.2)*^</td>
</tr>
<tr>
<td>FCGR3A</td>
<td>158V</td>
<td>135(32.8)</td>
<td>117(41.6)</td>
</tr>
<tr>
<td></td>
<td>158F</td>
<td>276(67.2)</td>
<td>164(58.4)*</td>
</tr>
<tr>
<td>FCGR3B</td>
<td>HNA1a</td>
<td>164(39.9)</td>
<td>101(35.9)</td>
</tr>
<tr>
<td></td>
<td>HNA1b</td>
<td>237(57.7)</td>
<td>173(61.6)</td>
</tr>
<tr>
<td></td>
<td>HNA1c</td>
<td>10(2.4)</td>
<td>7(2.5)</td>
</tr>
<tr>
<td>Promotor haplotype (-386G/T,-120A/T)</td>
<td>2B.1</td>
<td>727(88.9)</td>
<td>471(83.5)</td>
</tr>
<tr>
<td></td>
<td>2B.4</td>
<td>42(5.1)</td>
<td>40(7.1)</td>
</tr>
<tr>
<td></td>
<td>2B.2</td>
<td>49(6.0)</td>
<td>53(9.4)*^</td>
</tr>
</tbody>
</table>

Data are numbers (%). Significance levels are indicated by symbols.

*P <.05
^Both, Stop vs C-ORF and STOP vs NC-ORF have a P <.05
GT vs CT have a P <.05

Linkage disequilibrium (LD)

Recently, Nagelkerke et al. determined linkage disequilibria (LD) between all known FcγR SNPs, demonstrating high LDs between FCGR3A*V158 and FCGR2C*C-ORF (0.96), FCGR3A*V158 and promoter haplotype 2B.2 (0.94) in FCGR2C. This promoter haplotype 2B.2 is also strongly associated with FCGR2C*C-ORF (0.98) as well as flanking FCGR2A*H131 allele (0.89). As our findings indicate that FCGR3A*V158, FCGR2C*C-ORF and promoter haplotype 2B.2 are associated with susceptibility to ITP (Tables 2-3), the biologically relevant SNP may be originating from only one of these variants. This association proved too high for FCGR2C*C-ORF and the promoter haplotype 2B.2 polymorphism to be able to determine which SNP was dominant, as logistic regression could not be performed due to a too strong LD, with each variant occurring separately too rarely to be analyzed. Combined however, these two SNP have indeed a higher prevalence in ITP patients (P <.05). However, after logistic regression analysis for FCGR3A*V158 in combination with FCGR2C*C-
ORF the association between acquiring newly diagnosed ITP with FCGR3A*V158 was lost (P=.09), whereas the association with FCGR2C*C-ORF remained (P <.01).

**Table 4** FcγR genotypes and incidence in newly diagnosed pediatric ITP

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (n=199)</th>
<th>Patients (n=138)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FCGR2A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>58(29.1)</td>
<td>38(27.5)</td>
</tr>
<tr>
<td>HR</td>
<td>97(48.7)</td>
<td>72(52.2)</td>
</tr>
<tr>
<td>RR</td>
<td>44(22.1)</td>
<td>27(19.6)</td>
</tr>
<tr>
<td>H</td>
<td>155(77.9)</td>
<td>111(80.4)</td>
</tr>
<tr>
<td>no H</td>
<td>44(22.1)</td>
<td>27(19.6)</td>
</tr>
<tr>
<td><strong>FCGR2B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>160(80.4)</td>
<td>108(78.3)</td>
</tr>
<tr>
<td>IT</td>
<td>32(16.1)</td>
<td>27(19.6)</td>
</tr>
<tr>
<td>TT</td>
<td>7(3.5)</td>
<td>3(2.2)</td>
</tr>
<tr>
<td>T</td>
<td>39(19.6)</td>
<td>30(21.7)</td>
</tr>
<tr>
<td>no T</td>
<td>160(80.4)</td>
<td>108(78.3)</td>
</tr>
<tr>
<td><strong>FCGR2C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STOP</td>
<td>8(4.0)</td>
<td>5(3.6)</td>
</tr>
<tr>
<td>STOP/STOP</td>
<td>127(63.8)</td>
<td>71(51.4)</td>
</tr>
<tr>
<td>STOP/STOP/STOP</td>
<td>13(6.5)</td>
<td>9(6.5)</td>
</tr>
<tr>
<td>STOP/STOP/STOP/STOP</td>
<td>5(2.5)</td>
<td>0(0)</td>
</tr>
<tr>
<td>C-ORF</td>
<td>3(1.5)</td>
<td>1(0.7)</td>
</tr>
<tr>
<td>C-ORF/C-ORF</td>
<td>5(2.5)</td>
<td>1(0.7)</td>
</tr>
<tr>
<td>NC-ORF</td>
<td>0(0)</td>
<td>2(1.4)</td>
</tr>
<tr>
<td>STOP/C-ORF</td>
<td>27(13.6)</td>
<td>37(26.8)</td>
</tr>
<tr>
<td>STOP/STOP/C-ORF</td>
<td>4(2.0)</td>
<td>3(2.2)</td>
</tr>
<tr>
<td>C-ORF/NC-ORF</td>
<td>0(0)</td>
<td>1(0.7)</td>
</tr>
<tr>
<td>C-ORF/NC-ORF/NC-ORF</td>
<td>0(0)</td>
<td>2(1.4)</td>
</tr>
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<td>C-ORF/C-ORF/NC-ORF</td>
<td>0(0)</td>
<td>1(0.7)</td>
</tr>
<tr>
<td>STOP/NC-ORF</td>
<td>3(1.5)</td>
<td>2(1.4)</td>
</tr>
<tr>
<td>STOP/STOP/NC-ORF</td>
<td>4(2.0)</td>
<td>3(2.2)</td>
</tr>
<tr>
<td>C-ORF</td>
<td>39(19.6)</td>
<td>46(33.3)</td>
</tr>
<tr>
<td>no C-ORF</td>
<td>160(80.4)</td>
<td>92(66.7)**</td>
</tr>
<tr>
<td>NC-ORF</td>
<td>7(3.5)</td>
<td>11(8.0)</td>
</tr>
<tr>
<td>no NC-ORF</td>
<td>192(96.5)</td>
<td>127(92.0)</td>
</tr>
<tr>
<td><strong>FCGR3A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1(0.5)</td>
<td>0(0)</td>
</tr>
<tr>
<td>VV</td>
<td>24(12.1)</td>
<td>25(18.1)</td>
</tr>
<tr>
<td>VVV</td>
<td>0(0)</td>
<td>1(0.7)</td>
</tr>
<tr>
<td>F</td>
<td>0(0)</td>
<td>1(0.7)</td>
</tr>
<tr>
<td>FF</td>
<td>85(42.7)</td>
<td>46(33.3)</td>
</tr>
<tr>
<td>FFF</td>
<td>5(2.5)</td>
<td>2(1.4)</td>
</tr>
<tr>
<td>VF</td>
<td>77(38.7)</td>
<td>60(43.5)</td>
</tr>
<tr>
<td>VFF</td>
<td>6(3.0)</td>
<td>2(1.4)</td>
</tr>
<tr>
<td>VVF</td>
<td>1(0.5)</td>
<td>1(0.7)</td>
</tr>
<tr>
<td>Continued…</td>
<td></td>
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</tr>
<tr>
<td>V</td>
<td>109(54.8)</td>
<td>89(64.5)</td>
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</table>
**Homozygous FCGR2B*T232 genotype associates with failure to respond to IVIg therapy**

In addition to determining which FcγR isoforms are related to disease prevalence, we investigated whether certain FcγR genotypes are associated with response to IVIg treatment, or the lack thereof. No differences were found in genotype distribution of FCGR2A*H/R131 alleles, nor in the prevalence of FCGR3A*V158, FCGR2C*C-ORF, FCGR3B*HNA1a, or the promotor haplotypes 2B.2 and 2B.4. In contrast, patients homozygous for FCGR2B*T232 were overrepresented in patients who did not respond to IVIg treatment (P <.05) (Table 5a). We then tested if individuals homozygous for the FCGR2B*I232 variant and carrying the FCGR2B promoter haplotype 2B.4, might respond even better to IVIg. Of the 14 patients who carried the combination of homozygous FCGR2B*I232 and promoter haplotype 2B.4, 13 responded well (platelet counts >100*10^9/L) to IVIg, whereas only 42 of 64 patients without this genetic combination responded well, albeit only borderline significant (P=.054). However, in the observation group receiving no treatment, 8 out of 18 patients with the combination of homozygous FCGR2B*I232 and promotor haplotype...
2B.4 recovered spontaneously, while this was case for only 5 out of 41 without this genetic profile (P < .01) (Table 5b).

Table 5a FcγR SNP and response to IVIg in newly diagnosed pediatric ITP

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients treated with IVIg (n=78)</th>
<th>no complete response (platelets &lt;100x10⁹/L) (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>complete response (platelets ≥100x10⁹/L) (n=55)</td>
<td>n=78</td>
</tr>
<tr>
<td>FCGR2A</td>
<td>HH 22 (40.0)</td>
<td>4 (17.4)</td>
</tr>
<tr>
<td></td>
<td>HR 25 (45.5)</td>
<td>14 (60.9)</td>
</tr>
<tr>
<td></td>
<td>RR 8 (14.5)</td>
<td>5 (21.7)</td>
</tr>
<tr>
<td></td>
<td>no HH 33 (60.0)</td>
<td>19 (82.6)</td>
</tr>
<tr>
<td></td>
<td>HH 22 (40.0)</td>
<td>4 (17.4)</td>
</tr>
<tr>
<td>FCGR2B</td>
<td>II 43 (78.2)</td>
<td>15 (65.2)</td>
</tr>
<tr>
<td></td>
<td>IT 12 (21.8)</td>
<td>5 (21.7)</td>
</tr>
<tr>
<td></td>
<td>TT 0 (0)</td>
<td>3 (13.0)</td>
</tr>
<tr>
<td></td>
<td>no TT 55 (100)</td>
<td>20 (87.0)</td>
</tr>
<tr>
<td></td>
<td>TT 0 (0)</td>
<td>3 (13.0)*</td>
</tr>
<tr>
<td>FCGR2C</td>
<td>C-ORF 21 (38.2)</td>
<td>5 (21.7)</td>
</tr>
<tr>
<td></td>
<td>no C-ORF 34 (61.8)</td>
<td>18 (78.3)</td>
</tr>
<tr>
<td>FCGR3A</td>
<td>minimum VV 9 (16.4)</td>
<td>3 (13.0)</td>
</tr>
<tr>
<td></td>
<td>no minimum VV 46 (83.6)</td>
<td>20 (87.0)</td>
</tr>
<tr>
<td>FCGR3B</td>
<td>HNA1a 40 (72.7)</td>
<td>14 (60.9)</td>
</tr>
<tr>
<td></td>
<td>no HNA1a 15 (27.3)</td>
<td>9 (39.1)</td>
</tr>
<tr>
<td>Promotor haplotype (-386G/T, -120A/T)</td>
<td>2B.4 16 (29.1)</td>
<td>3 (13.0)</td>
</tr>
<tr>
<td></td>
<td>no 2B.4 39 (70.9)</td>
<td>20 (87.0)</td>
</tr>
<tr>
<td></td>
<td>2B.2 22 (40.0)</td>
<td>5 (21.7)</td>
</tr>
<tr>
<td></td>
<td>no 2B.2 33 (60.0)</td>
<td>18 (78.3)</td>
</tr>
</tbody>
</table>

Data is presented as numbers (%). P values denote the results of chi square tests except for cells with small numbers (<5 or <10 if there is only one degree of freedom) where we used Fisher Exact tests. * P < .05
Table 5b Combination of genetic profiles FcγRIIb with enhanced expression and inhibitory potential

<table>
<thead>
<tr>
<th>Patients treated with IVIg (n=78)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>complete response (platelets ≥100x109/L), n=55</td>
<td>no complete response (platelets &lt;100x109/L), n=23</td>
</tr>
<tr>
<td>FCGR2B*II232-2B.4</td>
<td>13 (24)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>no FCGR2B*II232-2B.4</td>
<td>42 (76)</td>
<td>22 (96)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patients observed (n=59)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>complete response (platelets ≥100x109/L), n=13</td>
<td>no complete response (platelets &lt;100x109/L), n=46</td>
</tr>
<tr>
<td>FCGR2B*II232-2B.4</td>
<td>8 (62)</td>
<td>10 (22)</td>
</tr>
<tr>
<td>no FCGR2B*II232-2B.4</td>
<td>5 (38)</td>
<td>36 (78)**</td>
</tr>
</tbody>
</table>

Data is presented as numbers (%). Significance levels are indicated by symbols. *P < .05  **P < .01

Discussion

Specific genetic variations in genes encoding low-affinity FcγRs are known to modify FcγR expression and function, affecting individuals’ capacity to mount humoral immune responses and FcγR-mediated destruction of IgG-opsonized cells, thereby possibly predispose individuals to the development of autoimmune diseases or chronic inflammatory disorders. Indeed, some of these variations are associated with e.g systemic lupus erythematosus (SLE), Guillain-Barre syndrome (GBS), Myasthenia Gravis (MG), Wegeners Granulomatosis (WG) Kawasaki, and ITP.7;9;11;21;26-31;45;46 Here, we investigated the distribution of known FcγR polymorphisms and CNV in newly diagnosed pediatric ITP patients and controls, all of West European background. Furthermore, we explored the relation between FcγR profiles and response to IVIg treatment. All data were corrected for LDs between FcγR-encoding genes and alleles as determined by Nagelkerke et al.23

In concordance with the literature, we observed an increased prevalence of the FCGR3A*V158 allele in ITP patients. Our data however, suggest that the genetic association observed for FCGR3A*V158 in ITP is concomitant to the dominant prevalence of FCGR2C*C-ORF together with promoter haplotype 2B.2. The latter is mostly found in relation to FCGR2C, although it might also be a promoter for FCGR2B.44 FcγRIIc is an activating receptor, which is expressed by neutrophils, B cells, monocytes, DCs and NK cells.13;21;47;48 Expression of an extra activating FcγR alters the balance of activating and inhibitory FcγR signaling in these cells, which may lead to enhanced phagocytic activity or to impaired
downregulation of B cell responses and thereby may predispose to ITP. Moreover, since FcγRIIc is also expressed on NK cells and mDCs, which are capable of strong ADCC activity and antigen presentation respectively, they might be at least partly responsible for the onset of pediatric ITP following a viral infection, by causing accelerated platelet clearance and killing.

Moreover, the recently reported expression of FcγRIIc on B cells in FCGR2C*C-ORF individuals, that otherwise only express the inhibitory FcγRIIib, together with their lower activation threshold and association with autoimmunity, suggest that this receptor may be critical for tipping the balance of the humoral immune system towards autoreactivity.

The incidence of promoter haplotype 2B.2 polymorphisms, found in the genes encoding for both FcγRIIib and FcγRIIc, could not be analyzed separately from FCGR2C*C-ORF, since the ORF variant is almost always inherited together with 2B.2 (LD of .98). Promoter haplotype 2B.4 on the other hand is associated with a 1.5 fold increased expression of FcγRIIib. Somewhat counterintuitive, this promoter haplotype was observed more frequently in ITP patients than in controls. Su et al. had a similar finding in SLE patients. It might point at alternative mechanisms of action, as is described in mice in which DCs increased their antigen production. Another explanation would be that expression levels are not solely dependent on the promoter haplotype.

Furthermore, we observed that the homozygous FCGR2B*T232 genotype is associated with a lack of response to IVIg treatment. It has been demonstrated that the FCGR2B*T232 variant is functionally impaired compared to FCGR2B*I232 due to decreased capacity to be incorporated into lipid rafts and down regulate ITAM signaling of activating FcγR and B cell receptors, thereby providing functional evidence how this allele is predisposing to autoimmunity. In addition, several studies performed in experimental murine ITP models underline the importance of FcγRIIb as the crucial point of application for IVIg therapy. IVIg has been reported to result in a 60% increase of FcγRIIb expression, whereas therapy failed altogether in FcγRIIb KO mice. Leontyev et al. on the contrary concluded that observed differences were due to different mice strains. Although our finding indicates a possible mechanism of action of IVIg via FcγRIIb signaling in humans, it has to be considered with care due to the rarity of the genotype. Our results suggest that any genetic variation of FCGR2B, impairing FcγRIIb function (e.g. the FCGR2B*T232 allele or absence of the promotor haplotype 2B.4) counteracts good recovery and/or good response to IVIg. Conversely, the genotypic
combination of homozygous $FCGR2B^*I232$ and promoter haplotype 2B.4 is beneficial for recovery. Since the positive effect on recovery of a homozygous $FcyR2B^*I232$ genotype combined with promoter haplotype 2B.4 was very strong in the observation group as well, this genotype might be inclined to a quick recovery in general, with only minor influence on response to IVIg.

Taken together, we show that $FCGRIIC^*C-ORF$ is more prevalent in newly diagnosed pediatric ITP compared to controls. In addition, we demonstrated that a patients’ short-term response to IVIg is linked to its $FcyRIIb$ genotype. While homozygous $FCGR2B^*T232$ predicted a lack of response to IVIg, the combination of homozygous $FCGR2B^*I232$ and promoter haplotype 2B.4 was associated with a good response to therapy and spontaneous recovery. Though further research with larger patient cohorts and both newly diagnosed as well as chronic ITP patients is necessary, we believe that $FcyR$ polymorphisms play an important role in ITP pathology, and might be a useful tool to enable personalized future medicine.

**Acknowledgments**

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**Authorship Contributions**

AL, KH, TK, MB, ES, MH and GV designed the study. SN and SC performed the experiments. AL and KH carried out the data analysis. All authors contributed to data interpretation. AL, KH, MH and GV wrote the manuscript. All authors contributed to revising the manuscript for intellectual content and style and all approved the final version.

**Conflict of Interest Disclosures**

The authors declare no competing financial interests.
Reference list


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