

# **Predisposing and modulating genetic factors in idiopathic thrombocytopenic purpura (ITP)**

Johannes Rischewski\*, Karl Heinimann°, Thomas Kühne\*.

\*Pediatric Hematology and Oncology and

°Medical Genetics, University Children's Hospital Basel (UKBB), Switzerland

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### **Synopsis**

#### **Introduction:**

Control of bleeding depends on an adequate number of blood platelets (thrombocytes). Any reduction in the number of platelets (thrombocytopenia) can lead to a bleeding tendency. If the bleeding occurs into the skin, it leads to a deep-red color, called purpura. In idiopathic-thrombocytopenic purpura (ITP) an immune mediated mechanism leads to an elimination of blood platelets from the circulation. Antibodies, that normally help eliminating foreign materials such as potentially dangerous invaders of the body, bind to platelet surface receptors. Subsequently they get destroyed by other cells. Cells of the immune system are normally able to distinguish between self and foreign structures. Cells with potential autoreactivity (i.e. with the potential to destroy self-structures) are usually inactivated. Thus, autoreactivity reflects a dysbalanced immune system, which is termed autoimmune disease. Genes encoding structures involved in the destruction of autoreactive cells ("apoptosis") could be involved in the genesis of ITP. Apoptosis consists of a complex machinery of the body in order to destroy cells which are no longer needed. Within the immune system, the mechanism is needed to destroy potentially autoaggressive effector cells. In ITP, defective apoptosis mechanisms could hence allow for the survival of autoreactive effector cell populations resulting in the destruction of thrombocytes. A preliminary analysis of registry data of ITP patients and of patients in our hospital has detected a surprisingly high number of patients with suspected ITP with affected family members, suggesting genetic risk factors.

#### **Hypothesis:**

Genetic risk factors predispose to and influence the clinical course of ITP. Analysis of familial ITP cases focussing on apoptotic pathways might reveal involved genes.

#### **Methods:**

DNA from a blood sample will be used to analyze target genes by molecular genetic techniques. Results will be compared with healthy controls. Unknown genes involved in diseases can be searched by using known sequence variations, called single nucleotide polymorphism's ("SNP"). If SNP's show a different distribution in affected and healthy individuals, genes near to the SNP's can be analyzed for a possible role in ITP.

#### **Proposal:**

A study on the potential involvement of genes in ITP. Evaluation of apoptosis-genes, followed by a genome wide search for yet unknown genes involved in the pathogenesis of ITP.

#### **Conclusion and vision:**

We aim for a better understanding of ITP in children. ITP can be seen as a model for autoimmune disorders. Understanding underlying causes could allow to develop more targeted therapies with less side effects than the used drugs, and could help to identify patients with an increased risk for bleeding complications.

## **Introduction:**

Idiopathic or immune-thrombocytopenic purpura (ITP) is defined as a bleeding disorder of children and adults with the hallmark of autoimmune mediated thrombocytopenia. ITP is still a diagnosis of exclusion. The term ITP is likely to define a group of etiologically diverse diseases with an heterogeneous and complex genetic background. Etiological and pathophysiological uncertainties about ITP and a significant lack of data concerning the optimal clinical management in 1997 lead to the foundation of the Intercontinental Childhood ITP Study Group (ICIS). ICIS ([www.unibas.ch/itpbasel](http://www.unibas.ch/itpbasel)) provides a worldwide network for clinicians involved in research and clinical management of ITP patients. The Pediatric and Adult Registry on Chronic ITP (PARC-ITP) (<https://www.parc-itp.net>) was started by ICIS in 2004. PARC-ITP collects data on adult and pediatric patients enabling subsequent research projects. Goals are analyses of the heterogeneity in ITP, of the etiopathology of the disease, and of the value of diagnostic methods and criteria. The data collection is coordinated by the ICIS office in Basel.

The study of genetic aspects of ITP as a model for autoimmune disorders is the first substudy of PARC-ITP. The goal is to identify genetic loci which potentially predispose to ITP, or that could influence the course of the disease.

Within the international cooperation and the University Children's Hospital Basel (UKBB) the collection of a case-control cohort for genetic analyses is in progress. The Basel ethics committee has approved the proposed study.

We propose a molecular genetic study on the genetic background of ITP, which allows us to start the analysis of the genetic background in a defined area, i.e. apoptosis.

## **Prevalence of mutations and polymorphism's in the FAS-receptor (TNFRSF6, CD95) gene in patients with ITP and a positive family history**

### **1.1 Hypothesis**

FAS receptor gene mutations are more common in patients with acute or chronic ITP with a positive family history for auto-immune disorders or lymphoproliferative disorders than in the normal population. FAS receptor mutations can be associated with a chronic course of ITP.

### **1.2 Rationale**

ALPS (autoimmune lymphoproliferative syndrome, OMIM 601859) was first described in 1967 by Canale and Smith as a syndrome in childhood, defined by lymphadenopathy, splenomegaly and autoimmune-cytopenia.

According to the underlying genetic defects three groups can be differentiated:

ALPS1A with mutations in the FAS-receptor gene, ALPS1B with mutations in the FAS-ligand gene, ALPS2A and 2B with mutations in the caspase 8 and 10 genes, and ALPS3 with unknown gene defects, possibly representing patients with somatic mosaicism, carrying heterozygous FAS-gene mutations in the double negative T-cell fraction only (Holzelova E et al, 2004)<sup>i</sup>.

Autoimmune cytopenia is common in ALPS patients (Straus SE et al, 1999)<sup>ii</sup>. In a study with nine ALPS patients, 1/9 had an ITP, 2/9 an Evans syndrome (hemolytic anemia and ITP), and 6/9 hemolytic anemia. Eight of nine patients carried FAS-gene mutations, and heterozygous carriers without manifestation of the disease showed in vitro abnormalities

of the FAS-mediated apoptosis (Snellere al, 1997)<sup>iii</sup>. Recently it was shown that 7/12 patients with Evans syndrome had an increased number of double negative T-cells as well as a deficient FAS-mediated apoptosis (Teachey DT et al, 2005)<sup>iv</sup>. The importance of this study lies in the indication, that ALPS is likely to be much more common than expected. Only very few ITP-patients have been studied for FAS-gene mediated apoptotic defects (Dianzani U et al, 1997)<sup>v</sup>. In two patients with ITP a defective FAS mediated apoptosis without lymphoproliferation was found. Both patients had normal double negative T-cell numbers (Shenoy S et al, 2001)<sup>vi</sup>.

Considering the available data, it is possible, that in cases of ITP with positive family history either with or without fulfilling the diagnostic criteria of ALPS or Evans-Syndrome, an increased incidence of FAS-receptor mutations can be present.

### 1.3 Objectives of the study

The study will try to answer the following questions:

- a) Can FAS-receptor gene mutations be detected in patients with ITP and positive family history?
- b) Are double negative T-cells (T-cell receptor alpha-beta+CD3+CD4-CD8-) in these patients detectable, as in ALPS patients?
- c) Can other genetic abnormalities be identified in addition to FAS receptor mutations?

### 1.4 Aims of the study

The definition of underlying genetic disorders may help to diagnose ITP, and could allow for targeted therapies.

For ALPS patients, new substances are under phase 1 clinical tests (pyrimethamine and sulfadoxin), which have the potential of altering the clinical course of the disease (van der Werff Ten Bosch J et al, 2002)<sup>vii</sup>. If FAS-receptor gene mutations are involved in the genesis of ITP, these new immune-modulating strategies could also be evaluated for ITP.

If additional genes of interest can be identified, further molecular genetic studies are warranted.

### 1.5 Material and methods

- 1) Collection of EDTA blood samples from ITP patients and all available family members, if a further member of the family suffers an autoimmune or lymphoproliferative disease.
- 2) Mutation analysis of the FAS receptor gene (9 exon's, 1007bp coding sequence) by direct DNA sequencing of genomic DNA. Restriction digest analysis of the FAS promotor polymorphism c.IVS1-670G>A and of the polymorphism c.IVS7-154C>T.
- 3) Flow cytometric analysis of CD3+CD4-CD8- cells of all affected family members.
- 4) SNP based genome wide analysis of the collected families. Estimation of LOD scores within the families, estimation of H-LOD scores after analysis of several families, which show evidence of the same mode of genetic transmission. Search of putative genes of interest within the defined genomic regions using the HUGO genebank. Direct sequencing of genes of interest, if identified.

## 1.5 Interim report and proposed modifications of the initial project:

### a) Sample acquisition:

A total 30 pediatric ITP samples could be collected in the Argentinian cohort.

A total of 8 pediatric and 25 adult ITP samples could be collected in the Korean cohort.

A total of 83 pediatric samples could be collected in the Basel cohort, of those 32 are ITP index cases, of those 6 are ITP cases with a positive family history. The remaining samples are family members (affected and unaffected), and index patients, that have to be excluded, because a different than ITP cause was identified as underlying disease. One familial case is included in the analysis, which is not part of the Basel cohort.

**Summary a):** The aim to collect samples and to extract DNA sufficient for target gene analysis has been reached.

### b) Basel cohort sample studies:

The cohort from Basel is studied extensively concerning their clinical and laboratory chemical features. Two result papers have been submitted, one is accepted and in press, one has been submitted, and is under review. Both papers are attached in submitted form, and have to be kept confidential until publication. The first paper excludes celiacs disease as a major risk factor for childhood ITP. The paper is important, as numerous case reports have been published, which made a relation of the autoimmune disorder celiacs disease and ITP likely. Our work shows, that the autoimmune mechanisms leading and predisposing to celiacs disease are unlikely to cause or influence the course of ITP. The second paper shows, that there is strong evidence both in the literature and in our data, that disturbed apoptotic mechanisms causing ALPS are possible to be involved in certain, familial subtypes of ITP. This finding is important, as it has direct impact on the initial diagnostic steps taken in patients presenting with ITP, as the diagnosis of ALPS defines a patient subset with a different spectrum of at risk diseases in the clinical cause, as lymphoproliferation, and possibly an increased risk to develop lymphomas. Most important- this finding supports the chosen strategy of target gene analysis in our collected cohort samples.

**Summary b):** The studied cohorts reveal important and new findings concerning risk constellations and possible underlying genetic causes. Two papers have been submitted on these results.

### c) Molecular genetic results:

FAS and FAS-ligand PCR has been established. Sequencing for these two target genes is established. A possible disease related mutation in the FAS gene as been detected in one familial case by direct sequencing of all exons. Due to this finding, a high throughput screening strategy to allow for a cost effective screening of FAS and FAS-ligand has been established, called denaturing high-pressure liquid chromatography, allowing for mutation detection directly on the PCR product without sequencing. This screening strategy allows to focus on aberrant PCR samples in

DHPLC screening only. All ITP samples are now amplified by PCR, and mutation screening of the samples is currently done.

Due to the findings so far, it does not seem to be cost effective to follow the proposed path of analysing the caspase 8 and 10 genes as well. Due to the clinical characteristics of the cohort, a new target gene has been selected, being involved in the differentiation of certain T-cell subsets. Please understand full confidentiality for this target to be kept, until results are available. The same screening approach as for FAS and FAS-ligand will be taken. We hope you agree with our change of target strategy, as clinical data on the cohort studied make an analysis of the described differentiation gene very attractive.

**Summary c):** A potential causative FAS gene mutation could be identified, and a technique to screen the whole cohort for FAS mutations has been established. A further target gene has been identified, and is under mutation screening.

**d) Genome wide SNP analysis:**

Due to the pedigree details of the collected familial cases, and due to now published data on genome wide SNP analysis in other autoimmune disorders (rheumatoid arthritis), we had to change the initially described approach. As our familial case do not share a common pattern of heritability, a genome wide SNP analysis of sporadic cases of 100 cases seems unlikely to reveal target genes<sup>viii</sup>. It seems more likely, that several groups of ITP patients may be identifiable, that do have an underlying genetic cause, or a combination of several genetic influencers, but the groups of ITP patients are unlikely to have the identical underlying genetic alteration. We therefore propose to be allowed to follow our already chosen approach to speculate on target genes taking the clinical details of our investigated familial cases into account, and to perform target gene analyses of the possibly underlying genes. Within the familial cohort, we will try to collect sufficient family members, to perform a segregation analysis. However, this approach can only be taken, if further family members can be acquired.

**Summary d):** The genome wide SNP analysis is unlikely to reveal the aimed results. We propose to be allowed for further target gene analyses as mentioned under c), and aim for a segregation analysis of the familial cases.

**e) Supplementary funding:**

The ITP foundations trust and funding of our group allowed us to establish a matching fund for genetic analyses in ITP in our hospital, and industry sponsors could be convinced to further support the project.

**Summary e):** Further financial support to the project could be allocated.

**f) Scientists involved in the project:**

A biologist was full time involved in the laboratory initiation of the project. Two medical students doing their thesis work perform continuously laboratory work and collect clinical data. Two specialist laboratories, one in Switzerland and one in Germany, support the project by flowcytometric analyses and apoptosis tests. A

large clinical and research laboratory in Switzerland supports the project by allowing the use of their laboratory equipment, especially for DHPLC analyses.

**Summary f):** The analysis network could be established. Personal could be recruited due their scientific interest.

**g) Publications and presentations directly resulting out of this project, mentioning the ITP foundation:**

Rischewski JR, Paulussen M, Kühne T. Celiacs disease is not a major risk factor for the development of childhood idiopathic thrombocytopenic purpura. JPHO 2007, in press

Rischewski JR, Ehl S, Imbach P, Kühne T. Evans syndrome and cooccurrence of idiopathic thrombocytopenic purpura in families: consider autoimmune lymphoproliferative disease. PBC 2007, submitted

Heritability of Idiopathic Thrombocytopenic Purpura: Fact or Artifact? Johannes R. Rischewski, Juliette Fong, Karl Heinemann, Andreas Huber, Heung S. Kim, Regina Kohan, Silvie Stocker, Paul Imbach, Thomas Kühne. Presentation: Schweizer Forschungstag Pädiatrie, Bern 2007

Genetische Analysen bei Patienten mit ITP. Silvie Stocker, Johannes R. Rischewski. Presentation: Laborlunch Kantonsspital Aarau 15.11.2007

**Summary a-g):** The funding of the ITP foundation allowed us to establish a research network involving different laboratories and scientists, to study possible genetic causes of ITP in the cohorts of patients from Basel, Argentina and Korea. It seems promising to proceed the chosen path of evaluations: thorough clinical investigation of the ITP patients from Basel and the collected familial cases, and subsequent target analysis of suspected involved genes. The current setup allows for a high throughput screening of samples, and following the FAS and FAS-ligand analysis a further target gene will be included, whose involvement in T-cell subset differentiation in ITP seems possible. We will proceed to collect familial samples, and will try to complete the sample collection within the pedigrees. Supplementary funding could successfully be allocated.

We thank the ITP Foundation for their trust and support of our group.

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<sup>i</sup> Holzelova E et al: Autoimmune lymphoproliferative syndrome with somatic FAS mutations. *N Engl J Med* 2004;351:1409-18

<sup>ii</sup> Straus SE et al: An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Ann Intern Med* 1999;130:591-601

<sup>iii</sup> Snellere et al: Clinical, immunological, and genetic features of an autoimmune lymphoproliferative syndrome associated with abnormal lymphocyte apoptosis. *Blood* 1997;89:1341-1348

<sup>iv</sup> Teachey DT et al: Unmasking Evans Syndrome: T cell phenotype and apoptotic response reveal autoimmune lymphoproliferative syndrome (ALPS). *Blood* 2005;105:2443-8

<sup>v</sup> Dianzani U: deficiency of the Fas apoptosis pathway without Fas gene mutations in pediatric patients with autoimmunity/ lymphoproliferation. *Blood* 1997;89:2871-2879

<sup>vi</sup> Shenoy S et al: Defective apoptosis in lymphocytes and the role of IL-2 in autoimmune hematological cytopenia. *Clin Immunol* 2001;99:266-275

<sup>vii</sup> van der Werff Ten Bosch J et al: Reversion of autoimmune lymphoproliferative Syndrome with an animalarial drug: preliminary results of a clinical cohort study and molecular observations. *Br J Hematol* 2002;117:176-188

<sup>viii</sup> Plenge RM et al: Two independent alleles at 6q23 associated with risk of rheumatoid arthritis. *Nat Genet.* 2007 Nov 4; (Epub ahead of print)