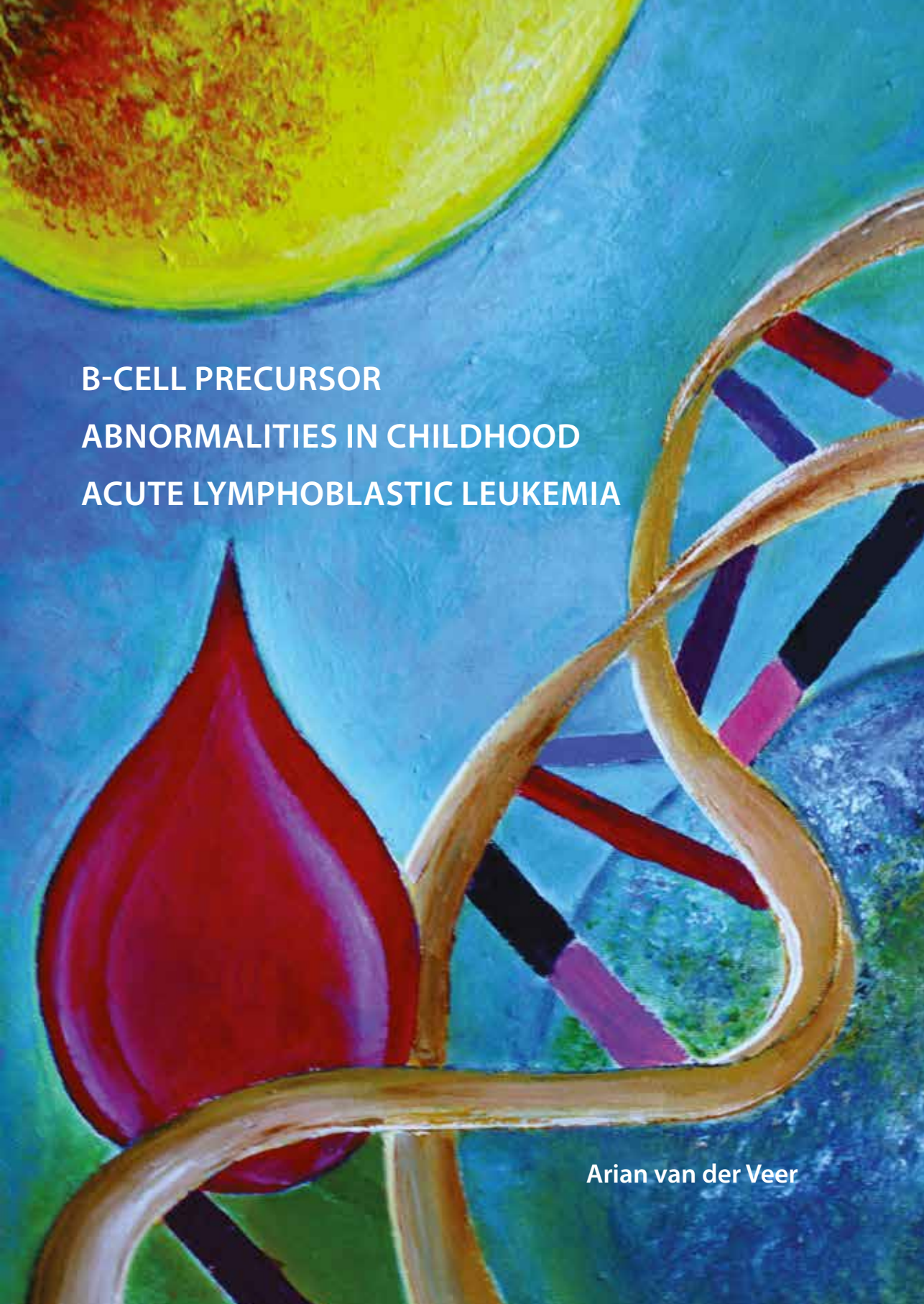

B-CELL PRECURSOR ABNORMALITIES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

Arian van der Veer

B-Cell Precursor Abnormalities in Childhood Acute Lymphoblastic Leukemia

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Explanation: As described in Greek mythology, Ikaros attempted to escape from Crete by means of wings made of feathers and wax. His father warned him not to fly too high to the sun, because the high temperature of the sun would melt his wings. Ikaros ignored this warning, his wings melted and he drowned in the sea.

Since daily sunlight induces radicals and thereby causing DNA damage, Ikaros is represented as a DNA molecule on the front cover. The DNA (Ikaros) is damaged, resulting in abnormal blood (drop of blood) and leading to leukemia (fall into the sea).

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CONTENTS

CHAPTER 1

General introduction

HEMATOPOEISIS

The hematopoiesis is the process of the formation of blood cells.[1] Three types of blood cells are formed: 1) thrombocytes, which are responsible for primary blood clothing, 2) erythrocytes, which transport oxygen to the organs, and 3) leukocytes, which are essential for the immune response against pathogens. All these types of blood cells originate from a pool of self-renewing hematopoietic stem cells (HSC).[1] The hematopoiesis starts during the first weeks of embryogenesis in the yolk sac and HSCs migrate via the dorsal aorta to the liver and spleen.[1-3] At the end of the fetal period, the bone marrow is the most important site of hematopoiesis. During infancy the total bone marrow can produce hematopoietic cells. In time, the hematopoiesis becomes more and more restricted to the central skeleton and proximal ends of femurs and humeri.[3] Other bone marrow sites, the spleen and liver retain their hematopoietic capacity which becomes evident in the development of hematopoietic malignancies.

LEUKOCYTES

Leukocytes are white blood cells that either originate from myeloid lineage or the lymphoid lineage. The myeloid progenitor cells develop into granulocytes, monocytes and mast cells (**Figure 1**).[4] These cells belong to the innate immune system and are not capable of adaptation to a specific antigen.[4,5] The lymphoid precursors give rise to T-lymphocytes, B-lymphocytes and natural-killer (NK)-cells (**Figure 1**). The B-lymphocytes, subject of this thesis, and T-lymphocytes are directed to specific antigens.[4,5]

B-CELL DEVELOPMENT

B-cells develop in the bone marrow and mature to immunoglobulin producing plasma cells in the peripheral lymphoid tissues (i.e. lymph nodes, tonsils, spleen). This development is regulated by differentiation factors such as *IKZF1*,[6] *EBF1*,[7] *TCF3*,[8] *PAX5* (**Figure 2**).[9] It is characterized by VDJ recombination of the *Immunoglobulin heavy chain* (IGH) first (**Figure 3**). The translated protein Immunoglobulin heavy chain together with VPREB1 and LAMBDA 14.1 constitute the pre-B-cell receptor (pre-BCR) in large pre-BII cells.[4,5] Activation of this receptor initiates clonal expansion of the B-cell precursor and further differentiation and gene rearrangement of *Immunoglobulin light* chains (*IGK* and eventually the *IGL* locus) and therefore is important in the B-cell development.[10] If the light chain is rearranged in frame, the immunoglobulin heavy chain together with light chain forms the B-cell receptor and is expressed on the cell membrane of the immature B-cell. Activation of this receptor results into differentiation to mature B-cells.

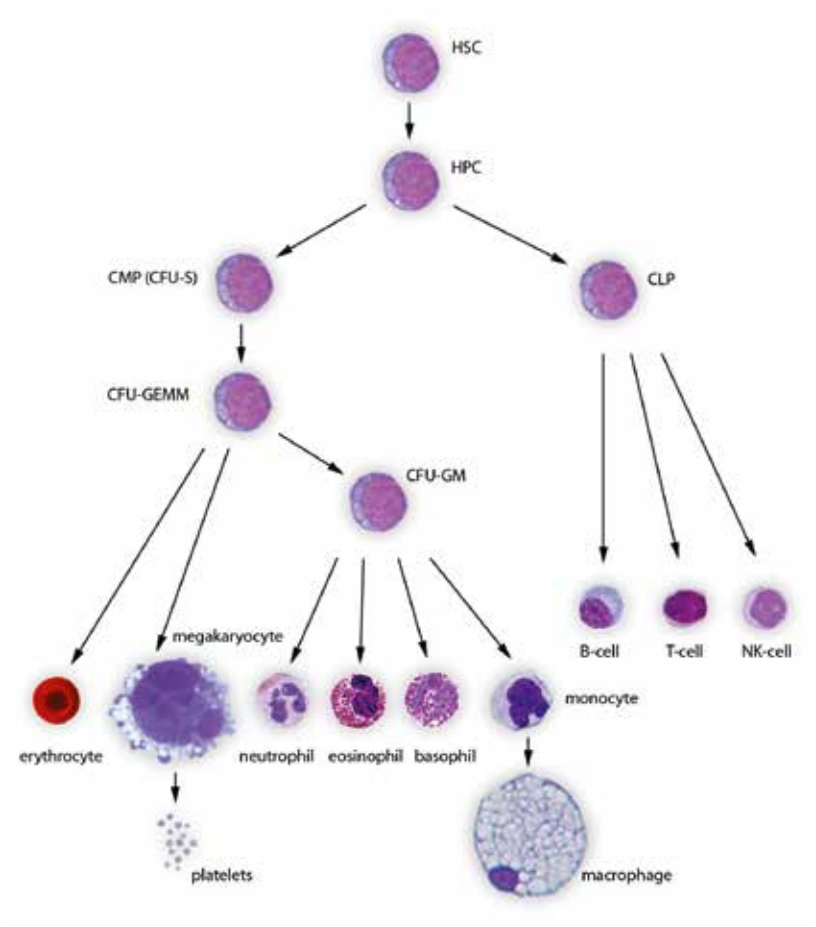

Figure 1 l Hematopoiesis. (daley.med.harvard.edu/assets/Willy/hematopoiesis.jpg)

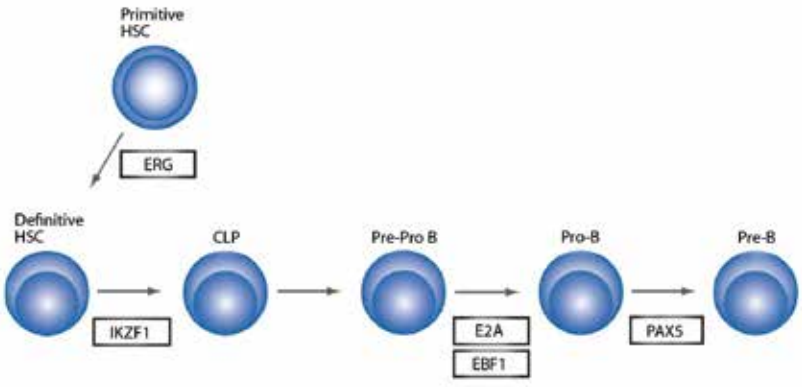

Figure 2 | Transcription factors in B-cell development. Transcription factors are denoted in boxes.
HSC, hematopoietic stem cell; CLP, common lymphoid progenitor.[4]

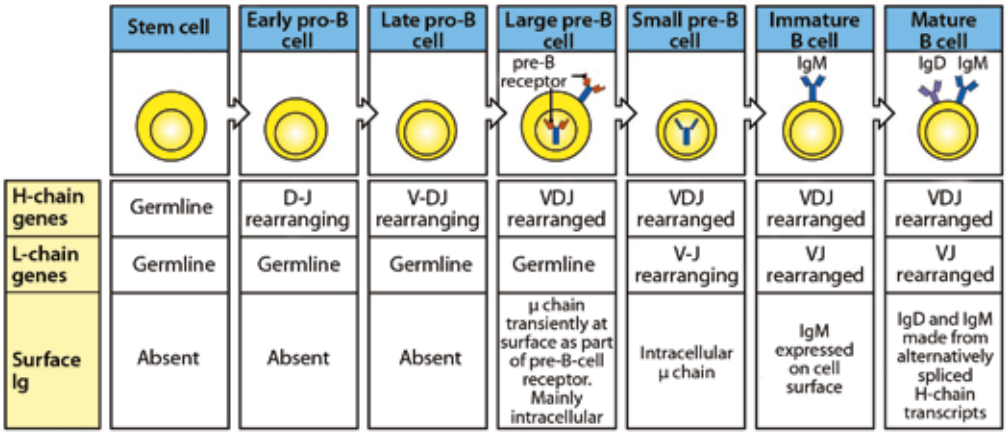

Figure 3 | Immunoglobulin heavy(H) and light(L) chain rearrangements during B-cell maturation.[4]

LEUKEMIA

A differentiation block and uncontrolled proliferation of hematopoietic precursor cells characterize leukemia. The most common form of this disease in childhood is B-cell precursor acute lymphoblastic leukemia (BCP-ALL).[1,11] The malignant behavior of these precursor B-cells is caused by acquired genetic defects influenced by coincidence, environmental (e.g. ionization radiation)[12] and constitutional factors (e.g. Down syndrome).[13]

THE PROGNOSIS OF BCP-ALL

In the sixties approximately 20% of children with BCP-ALL survived. Current treatment protocols result in an overall survival of 80%.[14] This improvement is based on rationally designed treatment regimes that contain an induction, consolidation intensification and maintenance phase. This therapy is adjusted by the risk of relapse, leading to a more intensive treatment for high-risk BCP-ALL. In contrast, low-risk patients receive less intense treatment with the intention to reduce side effect on short term, but also on long term.[15-18] The relapse risk prediction is based on the early response to therapy measured by quantification of the minimal residual disease (MRD)[19] and on cytogenetic findings.[20]

PROGNOSTIC GENETIC ABNORMALITIES IN CHILDHOOD BCP-ALL

In childhood BCP-ALL certain recurrent genetic alterations are predominant.[21,22] About half of the children with BCP-ALL cases are characterized by a translocation affecting the *ETV6*-gene and the *RUNX1*-gene (*ETV6-RUNX1*) or by an increased copy number of chromosomes (>50) called hyperdiploidy.[22] These two genetic alterations are correlated to a good clinical outcome in children. The less frequent *MLL*-rearrangement is predominantly found in infant BCP-ALL.[23] The *BCR-ABL1*-translocation is also rare in childhood (<5%) and is mainly detected in children older than 10 years.[24,25] These two genetic alterations are correlated to an unfavorable outcome.[20] The *TCF3*-rearrangement occurs in approximately 5% of childhood BCP-ALL cases and is correlated to an intermediate outcome on contemporary treatment protocols (**Figure 4)**.[22,26]

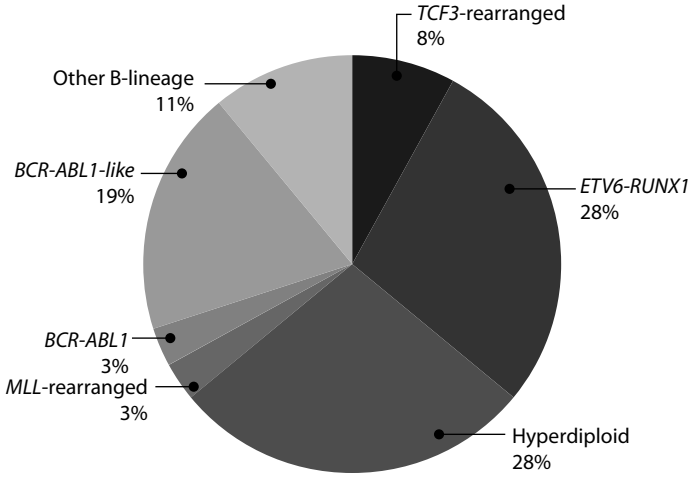

Figure 4 | Genetic groups in BCP-ALL. Before the discovery of the *BCR-ABL1-like* group, cases in this group were classified as other B-lineage comprising together 30% of BCP-ALL in children.

IDENTIFICATION OF NEW MOLECULAR STRATIFICATION MARKERS

Despite the implementation of risk stratification, more than half of the relapsed cases have not been recognized as high risk at initial diagnosis of the disease (**Figure 5**).[27] Furthermore, 30% of all BCP-ALL cases does not belong to one of the genetic groups and therefore remains unclassified as B-other (**Figure 4**). A significant part of relapses occurs in this B-other group. Both observations warrant the identification of new prognostic markers in children with BCP-ALL.

Different techniques can be used to further identify new genetic abnormalities in children with BCP-ALL. Conventional karyotyping can be used to identify DNA translocations, amplifications and

deletions. Although the resolution of this technique is very low, chromosome 7p deletions were discovered and correlated to an unfavorable prognosis in *BCR-ABL1*-positive BCP-ALL.[28] Fluorescent in-situ hybridization (FISH) resulted in the discovery of the intrachromosomal amplification of chromosome 21 (iAMP 21) which is correlated to an unfavorable outcome.[29-32] Furthermore this technique demonstrated the occurrence of *CRLF2*-rearrangements in 10% of the BCP-ALL patients.[33,34] The prognostic value of this rearrangement is controversial.[33,35-39] Although FISH resulted in the discovery of various recurrent aberrations, it is not a genomic wide screening method, but can only be used to detect specific genomic deletions, amplifications and translocations. In contrast single-nucleotide polymorphisms (SNP) array and array comparative genomic hybridization (aCGH) are genome wide techniques to detect deletions, amplifications and unbalanced translocations. Interestingly recurrent deletions were found in the B-cell development genes *IKZF1*,[40-42] *EBF1*,[40,41] *TCF3*,[40,41] *PAX5*,[40,41] *VPREB1*,[40] and *LEF1*.[41] *IKZF1* deletions were correlated to an unfavorable prognosis and occur in 10-15% of the BCP-ALL cases.[35,42-46] Although the resolution of SNP array and aCGH is higher than conventional karyotyping, intragenic deletions and amplifications are hard to detect. Multiplex ligation-dependent probe amplification (MLPA) has a higher resolution and is found to be a robust technique for a reliable detection of deletions and amplifications in a candidate gene approach.[47] This technique revealed that different variants of *IKZF1* deletions occur in BCP-ALL.[43,44,46] DNA sequencing provides information about single nucleotide mutations and small insertions and deletions (InDels). However, the DNA-fragment that can be analyzed is relatively short (<1000 basepairs). Inactivating mutations in the *IKZF1*-gene have, been identified using DNA sequencing.[44-46]

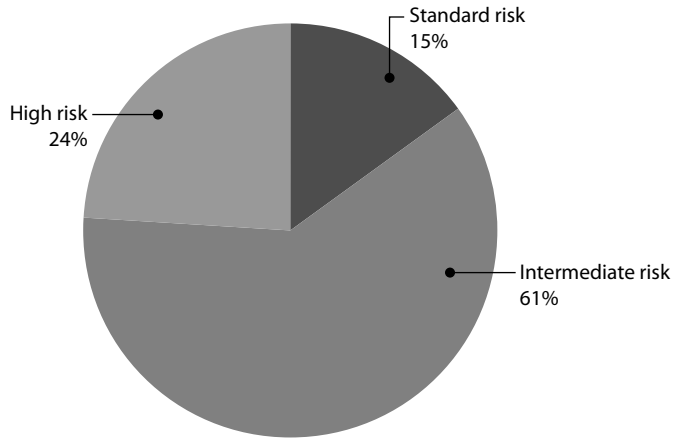

Figure 5 | Initial stratification of relapsed cases. Cases were treated according to DCOG ALL-10 (MRD-based) treatment protocol. Data were kindly provided by DCOG, the Hague, the Netherlands (unpublished)

Genetic classification can also be done by unique gene expression signatures.[40,48,49] Furthermore, this gene expression profiling technique identified a group that was originally identified as unclassified

B-other. The gene expression and poor prognosis of this group was similar to the *BCR-ABL1*-positive group and therefore was called the *BCR-ABL1-like* group.[26,40,45,50,51] Approximately, 15%-20% of BCP-ALL cases has such a gene expression signature (**Figure 4**).

Overall these techniques identified various new prognostic genetic abnormalities in children with BCP-ALL. Some of these new genomic lesions may simultaneously be found in one patient, and hence the independent prognostic value needs to be established. Examples are the presence of a *BCR-ABL-like* gene expression signature together with an *IKZF1* deletion and high expression of *CRLF2*.[40,45,50,51] The interaction and/or independence of these new prognostic markers was unknown at the start of this thesis project.

AIMS OF THIS THESIS

The general aim of this thesis was to provide new tools for the diagnosis and risk stratification of children with BCP-ALL. More specifically this study aimed to:

1. Identify new diagnostic and prognostic markers for BCP-ALL
2. To find drugable targets by studying the pathobiology of subtypes of BCP-ALL

OUTLINE OF THIS THESIS

Chapter 2 aimed to determine the occurrence and independent prognostic relevance of *BCR-ABL1-like* gene expression signature, deletions in *IKZF1* and high expression of *CRLF2* in children with newly diagnosed BCP- ALL.

The incidence of all *IKZF1* deletions together is approximately 10-15% in *BCR-ABL1*-negative BCP-ALL. These deletions are correlated to a poor prognosis in this group. However, various studies demonstrated that the incidence of deletions is even much higher (approximately 70%) in poor risk *BCR-ABL1*-positive BCP-ALL. But yet, the prognostic value of these deletions in *BCR-ABL1*-positive childhood BCP-ALL is unknown. In **chapter 3** we aimed to clarify the prognostic relevance of *IKZF1* deletions in children with *BCR-ABL1*-positive BCP-ALL treated with or without imatinib.

Studies addressing the prognostic value of *IKZF1* deletions in children with BCP-ALL have mainly focused on the prognostic value of all types of *IKZF1* deletions taken yet together. However, different genomic variants exist which may differ in clinical outcome. In **chapter 4** we aimed to clarify which genomic deletion variants of *IKZF1* have the strongest prognostic value in children with BCP-ALL.

TCF3-rearranged BCP-ALL is characterized by the expression of cytoplasmic immunoglobulin heavy chain. This protein is important in the pre-BCR pathway. The function of the pre-BCR pathway in *TCF3*-rearranged BCP-ALL had not been studied yet. In **chapter 5** we aimed to clarify the biological and therapeutic role of this pathway in *TCF3*-rearranged BCP-ALL.

Finally **chapter 6** comprises the general discussion of this thesis and provides future perspectives for research.

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CHAPTER 2

Independent prognostic value of *BCR-ABL1-like* signature and *IKZF1* deletion, but not high *CRLF2* expression, in children with B-cell precursor ALL

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ABSTRACT

Most relapses in childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) are not predicted using current prognostic features. Here, we determined the co-occurrence and independent prognostic relevance of three recently identified prognostic features: *BCR-ABL1-like* gene signature, deletions in *IKZF1* and high *CRLF2* mRNA expression (*CRLF2*-high). These features were determined in four trials representing 1128 children with ALL: DCOG ALL-8, ALL-9, ALL-10 and COALL-97/03. *BCR-ABL1-like, IKZF1*-deleted and *CRLF2*-high cases constitute 33.7% of *BCR-ABL1*-negative, *MLL* wild-type BCP-ALL cases, of which *BCR-ABL1-like* and *IKZF1* deletion (co)occurred most frequently. Higher cumulative incidence of relapse was found for *BCR-ABL1-like* and *IKZF1*-deleted – but not *CRLF2*-high – cases relative to remaining BCP-ALL cases, reflecting the observations in each of the cohorts analyzed separately. No relapses occurred among cases with *CRLF2*-high as single feature, whereas 62.9% of all relapses in *BCR-ABL1*-negative, *MLL* wild-type BCP-ALL occurred in cases with *BCR-ABL1-like* signature and/or *IKZF1* deletion. Both the *BCR-ABL1-like* signature and *IKZF1* deletions were prognostic features independent of conventional prognostic markers in a multivariate model, and both remained prognostic among cases with intermediate minimal residual disease. The *BCR-ABL1-like* signature and an *IKZF1* deletion, but not *CRLF2*-high, are prognostic factors and are clinically of importance to identify high-risk patients who require more intensive and/or alternative therapies.

INTRODUCTION

The 5-year event-free survival rate of childhood acute lymphoblastic leukemia (ALL) currently exceeds 80%.[1] This is attributed to risk-adjusted treatment which implements risk factors such as an early treatment response and cytogenetic abnormalities in leukemic cells as well as by the implementation of rationally designed phases in the treatment backbone of ALL including remission induction, central nervous system prophylaxis, intensification and maintenance phases of treatment. More than half of the children in whom contemporary therapies failed, were initially classified as non-high risk,[2] highlighting the need for improved prognostic markers.

The IKAROS transcription factor, encoded by *IKZF1*, was recently associated with an unfavorable prognosis in childhood B-cell precursor (BCP)-ALL.[3-6] A monoallelic (often partial) deletion in *IKZF1* results in a loss of its tumor suppressor function.[7,8] *EBF1*, *MSH2* and *MCL1* were demonstrated as target genes for IKAROS.[9] These genes contribute to the development of B-cells (*EBF1*), DNA repair (*MSH2*) and survival/anti-apoptosis (*MCL1*). Deletions in *IKZF1* aborted the activation of these genes which may facilitate B-cell leukemogenesis.[9] Ten to fifteen per cent of BCP-ALL cases have an *IKZF1* deletion, which therefore represents the most frequently observed genetic marker for unfavorable outcome identified in children.[3-6]

The *BCR-ABL1-like* gene expression signature is a second recently identified unfavorable prognostic marker in childhood BCP-ALL. *BCR-ABL1-like* ALL was identified based on a gene expression signature of its leukemic cells, which is similar to that of *BCR-ABL1*-positive ALL, although these leukemic cells do not harbor the *BCR-ABL1*-translocation.[5,10-12] Approximately 15% of BCP-ALL cases have *BCR-ABL1-like* ALL, which is associated with a 5-year event-free survival of <60%, similar to that observed in *BCR-ABL1*-positive BCP-ALL.[5,10-12] More than 80% of *BCR-ABL1-like* ALL cases have abnormalities in genes involved in B-cell development, a.o. *IKZF1* deletions in ~40%.[5,10-12]

A third recently identified adverse marker is linked to increased expression of *cytokine receptor-like factor 2* (*CRLF2*).[13-16] CRLF2 and interleukin-7 receptor alpha (IL7RA) form the thymic stromal-derived lymphopoietin receptor, which promotes B cell progenitor proliferation by activating the Jak2-Stat5 pathway.[16] The high expression of *CRLF2* in BCP-ALL arises either via a cryptic deletion which juxtaposes *CRLF2* to the *P2RY8* promoter (*P2RY8-CRLF2*), or from the positioning of *CRLF2* under control of the *immunoglobulin heavy chain* locus (*IGH@-CRLF2*) enhancer element.[16,17] In addition, *CRLF2* activity can be increased by gain-of-function mutations either in *CRLF2* itself[18] or in its partner gene, *IL7RA*.[19] High *CRLF2* mRNA expression (*CRLF2*-high) occurs in 5-14% of childhood BCP-ALL cases, and its incidence is remarkably high in Down-syndrome ALL (50-55%).[14,17,20]

The presence of the aforementioned features was determined in several studies that used different inclusion and outcome criteria and a variety of techniques. Therefore, the relationship between these features -as well as their prognostic independence within a given patient population- remains unclear. Here, we studied these three molecular features in 1128 cases from four independent cohorts of children with newly diagnosed ALL. This study shows that both the *BCR-ABL1-like* signature and *IKZF1* deletions are strong and independent prognostic features, whereas *CRLF2*-high is not uniformly predictive for an unfavorable outcome.

METHODS

Detailed methods are described in the **Supplementary Appendix**.

Patients and leukemic cell samples

This study comprised 1128 children with newly diagnosed ALL in three consecutive Dutch Childhood Oncology Group trials (DCOG ALL-8, ALL-9 and ALL-10)[21] and two German Cooperative ALL trials (COALL 06-97 and 07-03)[22] that were combined for analysis and are referred to hereafter as COALL-97/03. Patients were stratified in low, intermediate and high risk according to stratification markers in the treatment protocol (DCOG ALL-8[21], ALL-9[21], ALL10 (**Supplementary Table S1)** COALL 06-97[22] and 07-03[22]). Written informed consent was obtained from parents or guardians and institutional review boards approved the use of excess of diagnostic material for research purposes. Minimal residual disease (MRD) of DCOG ALL-10 patients was diagnosed by Sanquin (Amsterdam) and Erasmus MC (Rotterdam).[23] Cases were screened for hyperdiploidy (>50 chromosomes or DNA-index ≥1.16), *ETV6-RUNX1* and *BCR-ABL1* fusion products and rearrangements of *TCF3* and *MLL* by routine diagnostic procedures and complementary data were generated by the Erasmus MC laboratory. Cases negative for these cytogenetic markers were assigned to the B-other group **(Supplementary Figure S1).**

BCR-ABL1-like signature

New *BCR-ABL1-like* cases were identified using gene-expression profiling of leukemic cells with Affymetrix U133 plus 2.0 microarrays. The same set of 110 gene probes and clustering procedure was used to identify novel *BCR-ABL1-like* cases in a newly arrayed cohort of 572 BCP-ALL cases using the previous study cohort of 107 DCOG cases as reference (**Supplementary Figure S2, Supplementary Table S2**).[10] Newly arrayed cases that hierarchically clustered together with previously identified *BCR-ABL1-like* and *BCR-ABL1*-positive cases were identified as new *BCR-ABL1-like* cases if proven negative for the *BCR-ABL1*-translocation (**Supplementary Figure S2**).[10]

IKZF1 deletions and mutations

IKZF1 deletions identified using the SALSA P335 ALL-IKZF1 Multiplex Ligation-dependent Probe Amplification (MLPA) assay (MRC-Holland) were confirmed using the P202 MLPA assay, a previously described in-house *IKZF1* MLPA probe set,[4] or comparative genomic hybridization analysis (SurePrint G3 180K array, Agilent).[10] The presence of inactivating mutations in *IKZF1* exons 4, 5, 6 and 8 was measured using Sanger sequencing in *BCR-ABL1-like* cases.[4,5]

Abnormalities in *CRLF2* by genetic lesions and gene expression

P2RY8-CRLF2 and *IGH@-CRLF2* (detected by interphase FISH)[16] are linked to high *CRLF2* mRNA expression levels as determined by Affymetrix gene expression microarrays (**Supplementary Figure S3**).[24] Those cases with a signal intensity of the Affymetrix probe set 208303_s_at above the 90th percentile of the total BCP-ALL group were classified as *CRLF2*-high.

Statistics

Categorical and continuous variables were analyzed using non-parametric statistical tests. The cumulative incidence of relapse (CIR) with death as a competing event was calculated as described by Fine and Gray[25] considering relapse and non-response to induction chemotherapy as events. Event-free survival (EFS) rates were determined using Cox's univariate and multivariate proportional hazard analyses with relapse, non-response and death by leukemia considered as events. Differences with a p-value <0.05 were considered significant.

RESULTS

A total of 1128 newly diagnosed children with BCP (n=971) and T-lineage (n=157) ALL were analyzed for the *BCR-ABL1-like* signature, *IKZF1* deletions and/or high *CRLF2* expression.

The DCOG ALL-10 study subset was highly representative of the entire cohort of eligible cases, whereas the other three subsets had higher WBC counts than the cases in each of the trial cohorts (**Supplementary Table S3**). To prevent bias from known high-risk factors in the outcome analyses, CIR and Cox's EFS analyses were analyzed in BCP-ALL patients after excluding *BCR-ABL1*-positive and *MLL*-rearranged cases (**Supplementary Figure S1**).

Frequency and cumulative relapse incidences of BCP-ALL with *BCR-ABL1-like* expression signature

Ninety-four (16%) new *BCR-ABL1-like* cases were identified among the total of 572 BCP-ALL with gene expression data (**Table 1, Supplementary Figure S2**). *BCR-ABL1-like* cases were identified in fifty-two per cent (30/58) of DCOG ALL-10 B-other cases, i.e. negative for hyperdiploidy, *ETV6-RUNX1* fusion, *TCF3*-rearrangement, *MLL*-rearrangement or *BCR-ABL1*-translocation. Similar frequencies were observed in the other cohorts (50% ALL-8 (16/32), 64% ALL-9 (25/39), 49% COALL-97/03 (23/47). None of the T-ALL patients were found to carry the *BCR-ABL1-like* signature. *BCR-ABL1-like* cases were more often assigned to intermediate and high-risk treatment than other BCP-ALL cases as a consequence of higher WBC count or age at diagnosis (**Supplementary Tables S6 and S7**).

The 94 *BCR-ABL1-like* ALL had a 5-year CIR of 32% (95% CI: 27-37%) compared to 11% (95% CI: 9-12%) for the 442 *BCR-ABL1*-negative/*MLL* wild-type BCP-ALL cases (p<0.001; **Figure 1A, Supplementary Table S8**). Similar significant differences were measured within each of the four study cohorts (**Supplementary Table S9, Supplementary Figure S5**). *BCR-ABL1-like* patients who were treated in the standard-risk (p=0.05) and medium-risk (p=0.06) arms of the most recent DCOG ALL-10 study exhibited a trend towards an unfavorable outcome (**Supplementary Figure S9**).

Frequency and cumulative relapse incidences of *IKZF1*-deleted BCP-ALL

IKZF1 deletions were identified in 16% (136/857) of *BCR-ABL1*-negative and *MLL* wild-type BCP-ALL cases (**Table 1**). Whole-gene deletions encompassing *IKZF1* exons 1-8 accounted for 40.4% of these deleted cases. In the remaining cases, partial deletions of exons 4-7 (26.5% of cases), exons 2-7 (9.6%), exons 4-8 (7.3%), exons 2-3 (8.1%), exons 2-8 (4.4%) and other exons (3.7%; **Supplementary**

Figure S4) were detected. Whole-gene or partial deletions in the protein-coding exons 2-8 were considered to comprise one biological group, as reported previously.[4-6]

Table 1 | Frequency of new prognostic features in subtypes of pediatric ALL

Subgroup	*BCR-ABL1-like*		Subtype	*IKZF1*-deleted		*CRLF2*-high	
	n	%		n	%	n	%
Hyperdiploid	0/130	0%		32/208	15%	25/130	19%
ETV6-RUNX1-positive	0/162	0%		7/225	3%	0/162	0%
TCF3-rearranged	0/19	0%		1/19	5%	0/19	0%
MLL-rearranged	0/12	0%		2/27	7%	1/12	8%
B-other*	94/176	53%	*BCR-ABL1-like***	37/92	40%	15/94	16%
			remaining B-other	16/80	20%	10/82	12%
BCR-ABL1-positive	0/24	0%		16/23	70%	0/24	0%
unknown BCP-ALL***	0/49	0%		43/233	18%	5/49	10%
Total BCP-ALL	**94/572**	**16%**		**154/907**	**17%**	**56/572**	**10%**
T-ALL	**0/80**	**0%**		**5/157**	**3%**	**1/80**	**1%**

* B-other are negative for hyperdiploidy, *ETV6-RUNX1*, *TCF3*-rearrangement, *MLL*-rearrangement, *BCR-ABL1*-translocation. ** *BCR-ABL1-like* cases are negatively tested for *BCR-ABL1*-translocation, *BCR-ABL1*-positive cases are allocated to the *BCR-ABL1*-positive group ***Unknown BCP-ALL cases are negative for *BCR-ABL1*-translocation, *MLL*-rearrangement and *BCR-ABL1-like* signature but other cytogenetic lesions were not determined

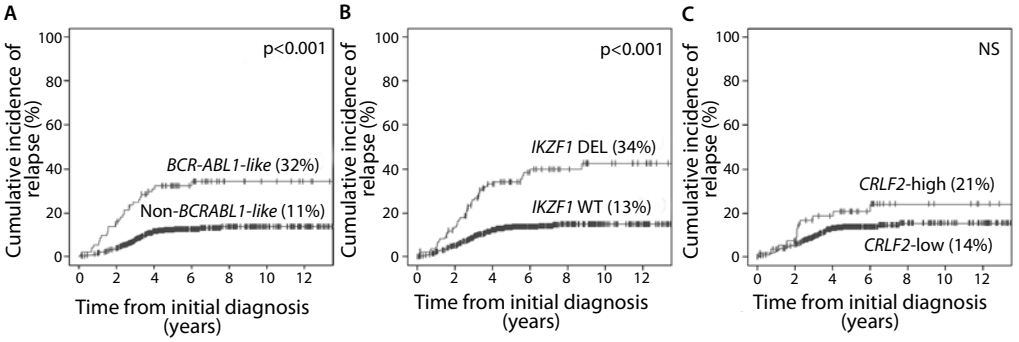


Figure 1 | Cumulative incidence of relapse for the *BCR-ABL1-like* expression signature, *IKZF1*-deleted and high *CRLF2* mRNA expression in newly diagnosed children with *BCR-ABL1*-negative, *MLL* wild-type BCP-ALL. Cumulative incidence of relapse (CIR) with death as a competing event was calculated using the method of Fine and Gray[25] in a pooled analysis of all four study cohorts and plotted against the time from initial diagnosis. For each feature, CIR at the 5-year follow-up is given in parentheses. **A)** CIR of 94 *BCR-ABL1-like* and 442 non-*BCR-ABL1-like* BCP-ALL cases. **B)** CIR of 136 *IKZF1*-deleted (DEL) and 721 *IKZF1* wild-type (WT) cases. **C)** Comparison between 55 cases with high *CRLF2* expression and 481 cases with low *CRLF2* expression.

The highest incidence of *IKZF1* deletions occurred in *BCR-ABL1*-positive (70%; 16/23) and *BCR-ABL1-like* (40%; 37/92) cases (**Table 1**). *IKZF1* deletions were also identified in other BCP-ALL types, including hyperdiploid (15% of cases), *TCF3*-rearranged (5%), *ETV6-RUNX1*-positive (3%), *MLL*-rearranged (7%) and remaining B-other ALL cases (20%). Three percent of T-ALL cases (5/157) contained an *IKZF1* deletion (**Table 1**).

The 136 *IKZF1*-deleted BCP-ALL cases had a 5-year CIR of 34% (95% CI: 30%-38%) compared to 13% (95% CI 12%-15%) in the 721 non-deleted *BCR-ABL1*-negative/*MLL* wild-type cases (p<0.001; **Figure 1B**, **Supplementary Table S8**). Each treatment protocol exhibited similar prognostic differences, except for DCOG ALL-8 treated cases (**Supplementary Table S9** and **Supplementary Figure S6**). A deletion in *IKZF1* was primarily predictive of poor outcome in patients treated in the medium-risk arm (p=0.008) of the DCOG ALL-10 protocol (**Supplementary Figure S9**). The most discriminating prognostic values for *IKZF1* deletions were observed in hyperdiploid (p=0.04) and non-*BCR-ABL1-like* B-other (p=0.02) cases, whereas no prognostic difference was observed for *IKZF1* deletions in the *BCR-ABL1-like* and *ETV6-RUNX1*-positive genetic subtypes in the DCOG ALL-10 study (**Figure 2**). Inactivating mutations in exons 4-6 and 8 were not identified in *BCR-ABL1-like*, *IKZF1* wild-type cases.

Frequency and cumulative relapse incidences of BCP-ALL with high *CRLF2* mRNA expression levels

High *CRLF2* mRNA expression, irrespective of *CRLF2*-rearrangement status, was found in hyperdiploid (19% of cases), *BCR-ABL1-like* (16%), *MLL*-rearranged (8%) and B-other (12%) cases, whereas no *CRLF2*-high cases were observed in *ETV6-RUNX1*-positive, *BCR-ABL1*-positive or *TCF3*-rearranged ALL (**Table 1**). High *CRLF2* mRNA expression was observed in 1% (1/80) of T-ALL cases.

No statistically significant difference in CIR was found for *CRLF2* mRNA expression status using the 90th percentile cutoff value in a pooled analysis of all patients or in the individual treatment cohorts, except for COALL-97/03 (p=0.007; **Figure 1C**, **Supplementary Figure S7** and **Tables S8** and **S9**). The CIR analyses did not reveal a significant difference if the 92.5th and the 95th percentile were used as cutoff value for high *CRLF2* mRNA expression nor did the CIR differ between cases with and without genetic *CRLF2* aberration (*IGH@-CRLF2* and *P2RY8-CRLF2*; **Supplementary Figure S8**). In addition, *CRLF2*-high (90th percentile cutoff) had no prognostic value within the subset of *BCR-ABL1-like* cases (**Supplementary Figure S12**).

Univariate and multivariate event-free survival analyses of BCP-ALL with *BCR-ABL1-like* signature, *IKZF1* deletion and/or *CRLF2*-high expression

The results of the CIR analyses of *BCR-ABL1-like*, *IKZF1*-deleted and *CRLF2*-high cases among the cohort of 507 *BCR-ABL1*-negative/*MLL* wild-type BCP-ALL patients for which all three features were determined (**Supplementary Table S4**, **Supplementary Figure S10**), were similar to those obtained by separate analyses of larger sample sets of *BCR-ABL1-like* signature (n=536), *IKZF1* status (n=857) and *CRLF2* expression (n=536; **Figure 1**). The three molecular features together comprised 33.7% of BCP-ALL cases (**Figure 3A**) of which the *BCR-ABL1-like* signature and *IKZF1* deletions were the most prominent both as single lesions and in combination (**Figure 3B, Supplementary Table S5A**). No relapses were predicted by the 14.6% of cases with high *CRLF2* expression alone. In contrast, 62.9%

(44/70) of all relapses in BCP-ALL (negative for *BCR-ABL1* and *MLL* wild-type) occurred in cases with the *BCR-ABL1-like* signature and/or an *IKZF1* deletion. Of these 44 relapsed cases, 30 had a *BCR-ABL1-like* signature, 25 an *IKZF1* deletion and thus 11 cases had both a *BCR-ABL1-like* signature and an *IKZF1* deletion (**Figure 3C**, **Supplementary Table S5B**).

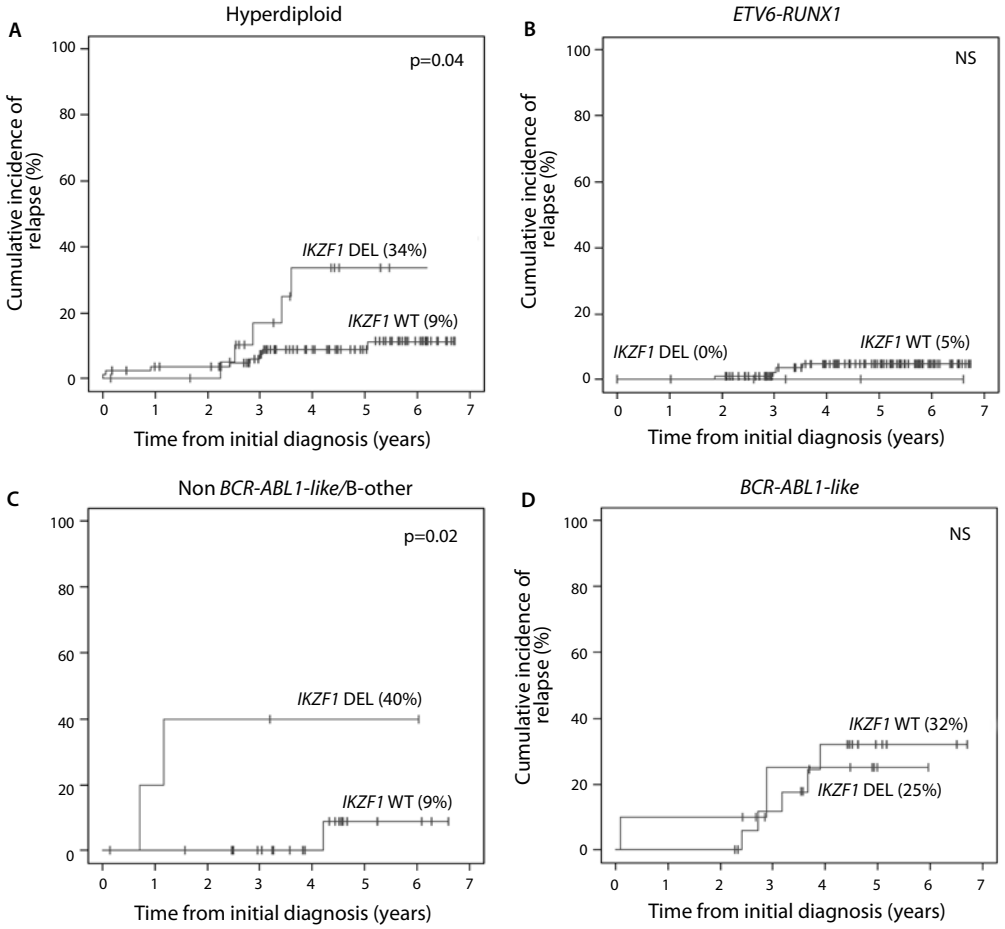


Figure 2 | Cumulative incidence of relapse for *IKZF1* status among subtypes of ALL. Cumulative incidence of relapse with death as a competing event was calculated for the DCOG ALL-10 study cases with **A**) high hyperdiploid ALL (>50 chromosomes) including 21 *IKZF1*-deleted and 87 wild-type cases; **B**) *ETV6-RUNX1*-positive ALL including 4 *IKZF1*-deleted and 100 wild-type cases; **C**) non-*BCR-ABL1-like* B-other ALL including 5 *IKZF1*-deleted and 22 wild-type cases; and **D**) *BCR-ABL1-like* ALL including 10 *IKZF1*-deleted and 19 wild-type cases. For each feature the cumulative incidence of relapse (CIR) at 5 years of follow-up is indicated in parentheses. DEL: deleted; WT: wild-type.

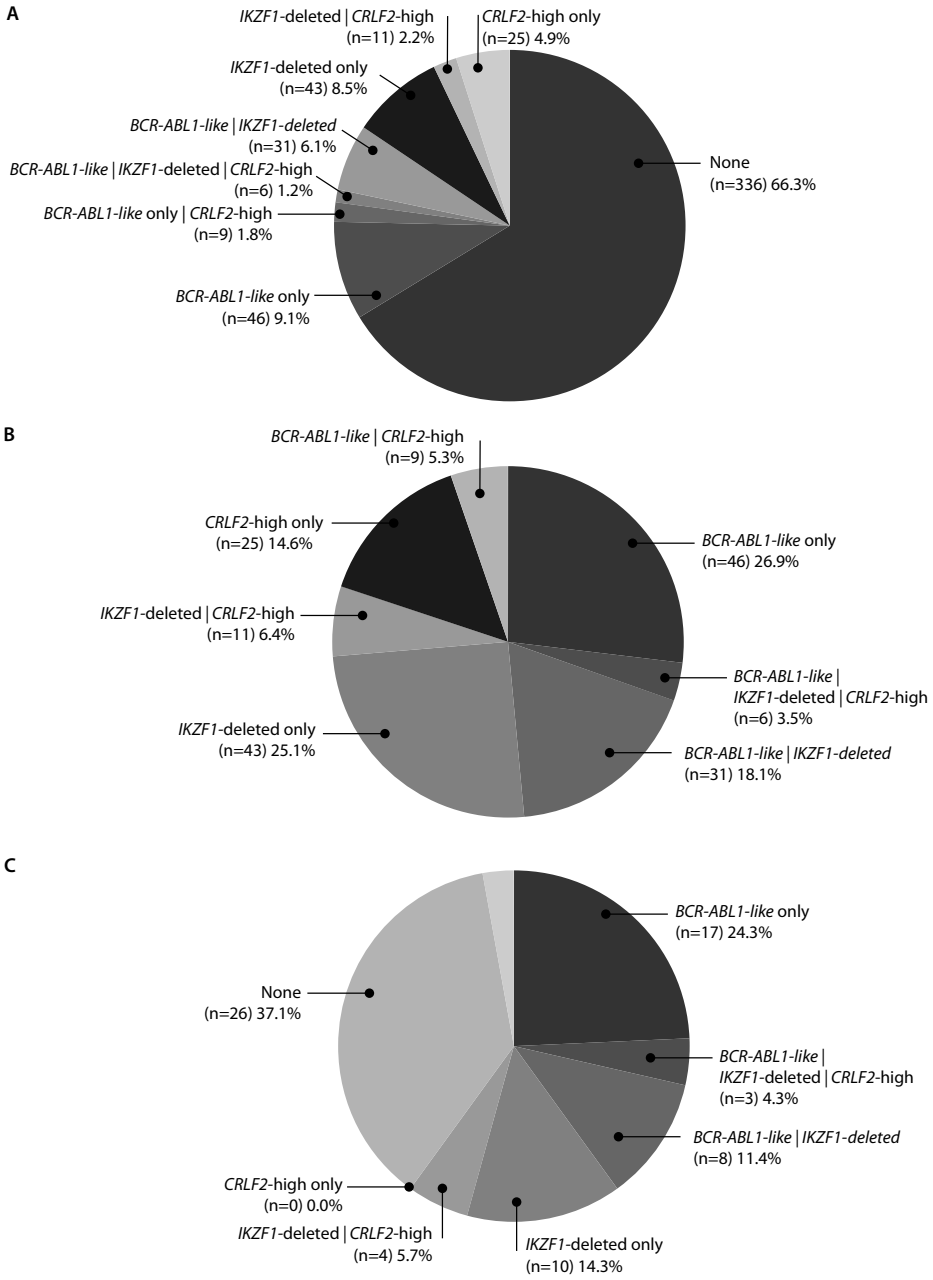


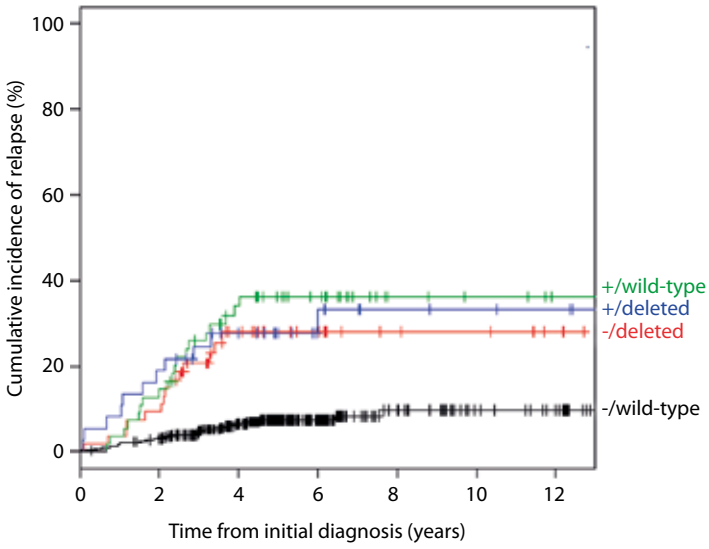
Figure 3 | Frequency of *BCR-ABL1-like, IKZF1*-deleted and *CRLF2*-high mRNA expression features among children with *BCR-ABL1*-negative, *MLL* wild-type BCP-ALL. A) Distribution of cases with *BCR-ABL1-like*, *IKZF1*-deleted and/or *CRLF2*-high mRNA expression in 507 children with *BCR-ABL1*-negative, *MLL* wild-type BCP-ALL. See also **Supplementary Table S5A. B)** Pie chart depicting the distribution of patients carrying one or more of the aforementioned features. Collectively, these three features constitute 33.7% of *BCR-ABL1*-negative, *MLL* wild-type BCP-ALL cases as shown in panel A. See also **Supplementary Table S5A. C)** Pie chart depicting the percentages of relapsed patients carrying one, two or three features. In total, 62.9% of all relapsed cases were associated with *BCR-ABL1-like* and/or *IKZF1*-deleted features. See also **Supplementary Table S5B**.

Univariate analysis of event-free survival of the 507 patients stratified according to treatment revealed that the *BCR-ABL1-like* signature (HR 3.5, 95% CI 2.2−5.7, p<0.001), *IKZF1* deletions (HR 2.8, 95% CI 1.7−4.6, p<0.001), WBC count of ≥50 cells/nl (HR 2.3, 95% CI 1.4−3.8, p=0.001) were associated with an unfavorable prognosis, whereas, high *CRLF2* expression, age and gender were not (**Supplementary Table S10A**). Multivariate analysis stratified according to treatment protocol suggested that the *BCR-ABL1-like* signature, a high WBC count and male gender had independent prognostic value (HR 3.1, 95% CI 1.8−5.2, p<0.001; HR 1.8, 95% CI 1.1−3.1, p=0.02; HR 1.8, 95% CI 1.1v3.0, p=0.02, respectively). In a similar model applied to *IKZF1* status, deletions in *IKZF1* (HR 2.5, 95% CI 1.5−4.2, p=0.001), high WBC count (HR 2.0, 95% CI 1.4−3.4, p=0.007) and male gender (HR 1.7, 95% CI 1.1−2.9, p=0.03) were significant. High *CRLF2* expression did not have prognostic value in this multivariate approach (**Supplementary Table S10B**).

Interestingly, among the *BCR-ABL1-like* cases, CIR was not affected by the *IKZF1* deletion status. Conversely, CIR among the *IKZF1*-deleted cases was not affected by the *BCR-ABL1-like* signature (**Figure 4**). A multivariate interaction model including both *BCR-ABL1-like* signature and *IKZF1* status showed that the hazard ratios for *BCR-ABL1-like* ALL alone (HR 5.3, 95% CI 2.9−9.7), *IKZF1*-deleted alone (HR 4.4, 95% CI 2.3−8.4) or combined *BCR-ABL1-like* signature and *IKZF1*-deleted cases (HR 3.7, 95% CI 2.0−8.4) were similar adverse for each of these categories compared to cases without the *BCR-ABL1-like* signature and non-deleted *IKZF1* (p<0.001, treatment-stratified analysis; **Supplementary Table S10C**).

Relationship between MRD status and *BCR-ABL1-like* ALL, *IKZF1-deleted* and *CRLF2*-high in DCOG ALL-10 study

MRD levels were prospectively measured for eligible DCOG ALL-10 cases and used to stratify patient risk (see Supplementary Appendix). All three MRD categories contained *BCR-ABL1-like*, *IKZF1*-deleted and/or *CRLF2*-high cases, but the distribution was skewed towards the MRD-intermediate and MRD-high categories for both *BCR-ABL1-like* (p=0.001) and *IKZF1*-deleted (p=0.03) cases relative to control BCP-ALL cases (**Supplementary Table S6**). More precisely, 17.9% (5/28), 6.7% (2/30) and 5.6% (1/18) of the *BCR-ABL1-like*, *IKZF1*-deleted and *CRLF2*-high BCP-ALL cases, respectively, were assigned to the MRD-high category (**Supplementary Table S11**), collectively, representing five distinct BCP-ALL cases. Remarkably, *BCR-ABL1-like* was the common denominator of these five MRD-high cases, although this finding is preliminary (**Supplementary Table S11**). Both the *BCR-ABL1-like* signature (HR 3.7, 95% CI 1.2−11.5, p=0.026) and *IKZF1*-deleted (HR 2.7, 95% CI 1.0-7.0, p=0.043) – but not *CRLF2*-high- remained predictive of poor clinical outcome among the MRD-intermediate BCP-ALL cases (**Supplementary Table S12**).



BCR-ABL1-like	*IKZF1*	n	5-year CIR	*p-value*
+	wild-type	55	36%	<0.001
+	deleted	37	27%	<0.001
-	deleted	54	28%	<0.001
-	wild-type	361	7%	reference

Figure 4 | Interaction between *BCR-ABL1-like* and *IKZF1* status for predicting relapse. Cumulative incidence of relapse (CIR) with death as a competing event for cases with or without a deletion in *IKZF1* among *BCR-ABL1-like* and non-*BCR-ABL1-like* ALL cases. The box below the graphs indicates the colour legends, 5-year CIR and p-values compared to the reference group of non-*BCR-ABL1-like* and *IKZF1* wild-type cases (**black line**). *BCR-ABL1*-positive and *MLL*-rearranged cases were excluded from this analysis.

DISCUSSION

Recently, new molecular features were discovered that can predict unfavorable outcome in childhood BCP-ALL and include a *BCR-ABL1-like* gene-expression signature,[10,12] a (partial) deletion in *IKZF1*[3-6] and high expression of *CRLF2*.[13-16] These factors are predictive among cases that do not exhibit known poor-prognostic features and therefore have potential clinical value. However, to date, these leukemia cell characteristics have been assessed separately in discrete patient cohorts with different inclusion criteria, limiting an analysis of their independent prognostic relevance. Moreover, variable technical approaches and cut-off values for positivity have hampered comparisons between published reports.

This is the first study to analyze the prognostic value and interaction between these three recently identified molecular features using standardized conditions and uniform inclusion criteria for newly diagnosed childhood ALL patients enrolled in four different treatment protocols. We show that 16%,

17% and 10% of BCP-ALL cases have the *BCR-ABL1-like* signature, *IKZF1* deletions and high *CRLF2* mRNA expression, respectively. These frequencies are consistent with previous observations in single marker studies in BCP-ALL.[4-6,10,12,15]

A relative high frequency of *IKZF1* deletions was found in *BCR-ABL1-like* cases identified in the present study (40%) and those described by the St. Jude Children's Research Hospital and Children's Oncology Group (80−90%), although the technique for the identification of *BCR-ABL1-like* cases was different.[11,12] The St. Jude and Children's Oncology Group identified the *BCR-ABL1-like* (or Ph-like) cases by using gene expression profiling and gene set enrichment analysis,[5] a recognition of outliers by sampling ends (ROSE) clustering approach (so-called R8 cluster),[11,12] or a prediction analysis for microarrays (PAM) classifier model.[11,26] These approaches used a dynamic, non-fixed classifier which differed per study. In the present study, we used a fixed model using our previously identified 110 gene probes and the same identical hierarchical clustering method as in our discovery study.[10] A second difference is that the St. Jude and Children's Oncology Group studies analyzed patients enrolled in high-risk protocols (e.g. P9906 and AALL0232) whereas in the present study the *BCR-ABL1-like* cases were identified in both standard, medium and high risk arms. In the present more population-based study, the *BCR-ABL1-like* signature, *IKZF1* deletions and *CRLF2*-high cases together constitute 33.7% of *BCR-ABL1*-negative, *MLL* wild-type BCP-ALL cases, of which concomitant *BCR-ABL1-like* signature and *IKZF1* deletion was the most frequent (**Figure 3**).

The *BCR-ABL1-like* and *IKZF1*-deleted features were predictive of unfavorable prognosis in most studied treatment protocols, including the MRD-based DCOG ALL-10 study. This indicates that *BCR-ABL1-like* and *IKZF1*-deleted cases are not yet recognized by other means and that no sufficient risk-adapted treatment is provided in contemporary regimens. Correspondingly, multivariate analysis revealed that the adverse prognoses of *BCR-ABL1-like* signature and *IKZF1*-deleted cases are independent of the conventional risk factors and remained predictive for an unfavorable outcome in the group of patients with intermediate MRD levels. This highlights the value of both features as strong independent risk factors. Importantly, both *BCR-ABL1-like* and *IKZF1*-deleted cases were identified across all stratification arms and were either exclusively (*BCR-ABL1-like*) or primarily (*IKZF1* deletion) present in the B-other category, for which new prognostic markers are urgently needed (**Table 1**).

The biology underlying *BCR-ABL1-like* ALL is largely unknown, but there is a relative high frequency of deletions in B-cell development genes (e.g. *IKZF1*, *EBF1*, *PAX5, VPREB1*) and in cell cycle genes (*CDKN2A/2B*), whereas mutations and translocations are mainly observed in genes associated with cytokine signaling (e.g. *CRLF2*, *IL7R, EPOR*) and kinase pathways (e.g. *ABL1* and *JAK2*).[5,10-12,26,27] The genomic deletions are most often recurrent (e.g. *IKZF1* deletion) whereas the mutations and translocations most often affect single genes and different fusion partners. Exceptions are recurrent lesions found for *CRLF2* and *JAK*-family genes and to a lesser extend for the *EBF1-PDGFRB* translocation in an independent validation cohort.[11,27] In the present cohort of BCP-ALL cases, we infrequently found mutations and translocations affecting *JAK2* (<2%). These lesions were mainly found in non-*BCR-ABL1-like* cases with high *CRLF2* expression and represent only a minority of our *CRLF2*-high cases (J. Marchante, M.L. den Boer et al., preliminary observation). This low frequency of *JAK2* abnormalities therefore cannot explain the poor prognosis ascribed to the *BCR-ABL1-like*

signature or an *IKZF1* deletion in the present study. The *ABL1*-translocated cells were shown to be sensitive to the ABL1 kinase domain inhibitors imatinib and dasatinib whereas *JAK2*-affected leukemic cells were sensitive to the JAK-inhibitor Ruxolitinib.[11,28] Although there is no common genomic denominator (yet) identified for *BCR-ABL1-like* ALL, screening of patients for presence of *ABL1* and *JAK*-family lesions may identify *BCR-ABL1-like* patients who may benefit of implementing targeted tyrosine kinase inhibitors in their treatment.

IKZF1 deletions were observed in every ALL subtype – including at low frequency in T-ALL – and primarily showed unfavorable prognostic value in hyperdiploid and non-*BCR-ABL1-like* B-other cases (**Figure 2**; **Supplementary Figure S11**). Together, this indicates that a broad range of ALL types are affected by aberrant IKAROS function. *IKZF1* deletions contribute to uncontrolled proliferation in *BCR-ABL1*-positive ALL,[7,8] most likely by interfering with c-Myb and Bmi1-regulated transcription of B-cell differentiation genes.[29,30] Interestingly, *IKZF1* deletions had no additive adverse effect on the prognosis of *BCR-ABL1-like* cases (**Figure 4**). Moreover, the unfavorable prognosis of *BCR-ABL1-like* cases without *IKZF1* deletion could not be explained by presence of inactivating mutations in *IKZF1*. The role of aberrant IKAROS in *BCR-ABL1-like* and other ALL subtypes is yet unclear, and the current findings underscore the importance of elucidating these mechanisms. One intriguing observation is that expression of the immunoglobulin joining chain (*IGJ*) gene is deregulated in both *IKZF1*-deleted and *BCR-ABL1-like* ALL.[10,12] IGJ is involved in immunoglobulin maturation,[31-33] whereas IKAROS controls the processing of immature B-cell receptors into immunoglobulins.[34,35] Whether *IKZF1* deletions functionally affect this maturation process clearly warrants further investigation.

In this study, high *CRLF2* mRNA expression was the weakest prognostic feature, being predictive of an unfavorable outcome in COALL-97/03 but not in the three DCOG-trials. The group with high *CRLF2* mRNA expression included all cases with genetic *CRLF2* aberrations (**Supplementary Figure S3**), which is in correspondence with previous observations.[15] We cannot rule out that high *CRLF2* expression will show some prognostic relevance when larger sample sizes are used. We therefore do not want to state that deregulated *CRLF2* completely lacks prognostic relevance in pediatric BCP-ALL but we want to emphasize that the discriminative prognostic value of high *CRLF2* mRNA expression is lower than that of the *BCR-ABL1-like* signature and *IKZF1* deletion. The prognostic and clinical value of *CRLF2* expression levels in childhood BCP-ALL is controversial, and appears to be dependent on the treatment protocol: predictive for adverse outcome in US, German (e.g. COALL97/03 in the present study),[15,24,36,37] but not predictive in Dutch (DCOG ALL-8, ALL-9 and ALL-10) and British treatment protocols.[14,37] Other possible explanations of this discrepancy include differences in methodology (e.g. mRNA expression versus genomic rearrangement) and inclusion criteria (e.g. inclusion of cases with Down syndrome or cases of Hispanic/Latino ethnicity)[17,24] and the possibility that the treatment and/or stratification criteria could mitigate any adverse effects of high *CRLF2* expression. Moreover, we observed that *CRLF2*-high cases that lacked *IKZF1* deletions and/or the *BCR-ABL1-like* signature did not relapse, suggesting that the prognostic value attributed to this factor may arise from the co-occurrence of *IKZF1* deletions and/or the *BCR-ABL1-like* signature.[13-16]

We conclude that the *BCR-ABL1-like* signature and *IKZF1* deletions in BCP-ALL cells are strong independent prognostic features that can identify patients at high risk for treatment failure, even among cases with intermediate MRD levels. In contrast, high *CRLF2* mRNA expression lacks such

discriminative prognostic power in our studies. These findings highlight the important clinical value of these two molecular features for identifying patients at high risk for treatment failure who might otherwise remain unrecognized. The implementation of *BCR-ABL1-like* signature as new risk factor in DCOG trials is being considered and to this aim a feasibility study for gene expression profiling in clinical practice has been initiated. Since the *IKZF1* deletion can be detected using DNA, which is less vulnerable to degradation than mRNA, this genomic marker can be more easily implemented in clinical practice. In the recently initiated DCOG ALL-11 protocol it was therefore decided to first implement the *IKZF1* status as new risk factor. In the DCOG ALL-11 trial, medium risk patients with a deletion in *IKZF1* will receive an additional year of maintenance therapy.

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CHAPTER 3

IKZF1 status as a prognostic feature in *BCR-ABL1*-positive childhood ALL

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ABSTRACT

Childhood *BCR-ABL1*-positive B-cell precursor acute lymphoblastic leukemia (BCP-ALL) has an unfavorable outcome and is characterized by a high frequency of *IKZF1* deletions. The prognostic value of *IKZF1* deletions was evaluated in two cohorts of children with *BCR-ABL1*-positive BCP-ALL, before (pre-TKI) and after introduction of imatinib (EsPhALL). *IKZF1* deletions were found in 126/191 (66%) of the patients. In the pre-TKI cohort, *IKZF1*-deleted patients had an unfavorable outcome compared to wild-type patients (4-yr DFS 30.0±6.8% versus 57.5±9.4%, p=0.01). In the EsPhALL-cohort, the *IKZF1* deletions were associated with an unfavorable prognosis in patients who were stratified by early clinical response in the good-risk arm (4-yr DFS 51.9±8.8% for *IKZF1*-deleted versus 78.6±13.9% for *IKZF1* wild-type; p=0.03), even when treated with imatinib (4-yr DFS 55.5±9.5% for *IKZF1*-deleted versus 75.0±21.7% for *IKZF1* wild-type; p=0.05). In conclusion, *IKZF1* deletions are predictive for a highly unfavorable outcome in children with *BCR-ABL1*-positive BCP-ALL irrespective the introduction of imatinib. These results underscore the urgent need for alternative therapy for *IKZF1*-deleted *BCR-ABL1*-positive patients. In contrast, good-risk patients with *IKZF1* wild-type responded remarkably well to imatinib-containing regimens, thus providing a rationale to potentially avoid the use of hematopoietic stem cell transplantation in this subset of *BCR-ABL1*-positive children.

INTRODUCTION

The introduction of risk-adjusted treatment protocols has significantly improved the overall prognosis of childhood acute lymphoblastic leukemia (ALL).[1] A highly unfavorable abnormality is the translocation of BCR at chromosome 22q11 to *ABL1* at chromosome 9q34 (t(9;22)(q34;q11) or *BCR-ABL1*-translocation), resulting in the so-called Philadelphia-chromosome. This translocation occurs in 2-3% of children and 25% of adults with B-cell precursor ALL (BCP-ALL).[2] Both adults and children are treated with intensive chemotherapy frequently followed by hematopoietic stem cell transplantation (HSCT). However, despite this intensive therapy, the prognosis of *BCR-ABL1*-positive BCP-ALL remains poor with a 5-year event free survival of less than 50% in both children and adults.[3-6] The introduction of tyrosine kinase inhibitors (TKIs), such as imatinib and dasatinib, targeting the *BCR-ABL1*-fusion protein has significantly improved treatment outcome.[7-10] However, *BCR-ABL1*-positive ALL is a biologically and clinically heterogeneous disease with large individual differences in response to chemotherapy.[11-13] Therefore studies that elucidate the biological and prognostic variation in *BCR-ABL1*-positive ALL may help to rationally assign patients to more appropriate treatment modalities.

Recent studies correlate deletions in the gene encoding IKAROS (*IKZF1*) with an unfavorable outcome in *BCR-ABL1*-negative BCP-ALL.[14-19] *IKZF1* is involved in B-cell proliferation and differentiation processes, most likely by modulating the rearrangement of the immunoglobulin heavy chain locus (*IGH*) and signaling of the immature B-cell receptor (pre-BCR).[20] Deletions of the whole gene as well as partial deletions that affect at least the starting codon located in exon 2 result in haplo-insufficiency. Deletions that affect the DNA-binding domain in exons 4−7 (known as isoform 6) exert a dominant negative effect over the unaffected allele resulting into a loss of the tumor suppressor function attributed to wild-type *IKZF1*.[21,22] Together, deletion variants have been considered as indicators of poor prognosis in previous studies.[14-19,23] In contrast to the relatively low frequency of deletions (15%) in *BCR-ABL1*-negative BCP-ALL, the frequency of deletions is high (70%) in *BCR-ABL1*-positive BCP-ALL,[19,23-26] suggesting that the unfavorable prognosis of *BCR-ABL1*-positive ALL is correlated with *IKZF1* deletions. All *IKZF1* deletion variants taken together were shown to be highly predictive of an adverse clinical outcome in adults with *BCR-ABL1*-positive ALL.[23] However, the prognostic value in children with *BCR-ABL1*-positive ALL is unknown.

In this international multicenter study, we investigated the prognostic role of *IKZF1* deletions in 191 *BCR-ABL1*-positive childhood BCP-ALL patients before (pre-TKI) and after the introduction of imatinib. Our data showed that deletions of *IKZF1* are predictive of a poor outcome in both the pre-TKI era and in imatinib-treated patients. Remarkably, good risk-stratified patients with wild-type *IKZF1* who received imatinib had a favorable outcome, which is comparable to the outcome results for *BCR-ABL1*-negative BCP-ALL patients treated with contemporary protocols. Recently, it was demonstrated that HSCT does not improve the prognosis of *BCR-ABL1*-positive patients who are treated with chemotherapy and TKI's.[8] Together with the present study, these findings provide a strong rationale to avoid HSCT in *IKZF1* wild-type *BCR-ABL1*-positive patients who are stratified as good-risk patients based on a good early clinical response to a therapeutic window with prednisone or induction therapy at day 21.

METHODS

Patients

Leukemic cells of 191 *BCR-ABL1*-positive BCP-ALL patients who achieved complete remission were analyzed for the presence of *IKZF1* deletions. These cases were collected from the "Ponte di Legno" (pre-TKI) and the European Study for Philadelphia-positive ALL (EsPhALL) cohorts, and were representative for the total cohort with respect to outcome *(***Supplementary Figure S1**) and clinical variables (**Supplementary Table S1**). Additional data on 11 pre-TKI patients who did not achieve complete remission are not included in the analysis: seven had an *IKZF1* deletion and four wild-type (no induction failures were observed in the EsPhALL cohort).

Bone marrow samples from these patients were collected from the Associazione Italiana di Ematologia Pediatrica (AIEOP, Italy), the German Berlin-Frankfurter-Munster study group (BFM, Germany), Childhood Leukemia Investigation Prague (CLIP, Czech Republic), the Dutch Childhood Oncology Group (DCOG, the Netherlands), the Children Leukemia Group of the European Organization for Research and Treatment of Cancer (EORTC-CLG), French Acute Lymphoblastic Leukemia Study Group (FRALLE, France) and Children's Cancer and Leukemia Group (CCLG, United Kingdom). All experimental tests were performed in the national reference laboratories. The study was approved by each institutional review board and written informed consent was obtained.

Pre-TKI cohort; 351 patients were diagnosed between 1995 and 2005, entered the national study protocols and achieved complete remission upon induction therapy in the seven participating countries participating in the so-called Ponte di Legno study group. DNA for analysis of the *IKZF1* status analysis was available for 84 patients, of which 18 cases received imatinib due to compassionate use at the discretion of the treating physicians, but which was not standardized in terms of frequency, duration and dosage.

E**sPhALL cohort**; DNA of patients enrolled for this study was available for 107 out of 213 patients registered between 2005 and 2010 in the seven participating countries. These patients were assigned to the good-risk (n=63) or the poor-risk group (n=44) according to their early clinical response. A good early clinical response was defined as blast cell count of less than 1000 cells per µl in peripheral blood after 7 days of treatment with prednisone and a single intrathecal dose of methotrexate, or ≤5% leukemic blast cells in the bone marrow at day 21, depending on national induction protocols. Good-risk patients were initially randomized for imatinib treatment (**EudraCT: 2004-001647-30, ClinicalTrials.gov: NCT00287105**). Following amendment of the protocol in December 2009, all patients received imatinib. All patients stratified as poor-risk received imatinib. This study was monitored by the EsPhALL International Trial data Centre (University of Milano-Bicocca, Monza, Italy).

Sample preparation

BCR-ABL1-positive BCP-ALL bone marrow samples were collected prior to initial treatment. Mononuclear fraction of cells was isolated by Ficoll gradient separation and DNA isolated according to local laboratory procedures.

IKZF1-status analysis

The presence of *IKZF1* deletions was investigated by the Multiplex Ligation-dependent Probe Amplification (MLPA) assay SALSA p335 kit (MRC-Holland, Amsterdam, the Netherlands) using 125ng of genomic DNA. The assays were performed according to manufacturers' protocol. Electrophoresis and quantification of fluorescein amidite (FAM)-labeled amp icons were performed on an ABI-3130 genetic analyzer (Applied Bio systems, Carlsbad, CA, USA). The resulting peak intensities were normalized to manufacturers' control probes and to normal DNA as a reference. An intensity ratio between 0.75 and 1.3 was considered to represent a normal copy number; a ratio between 0.25 and 0.75 a mono-allelic deletion and a ratio below 0.25 a bi-allelic deletion. Deletions were validated by either an independent MLPA SALSA p335, SALSA MLPA p202, array comparative genomic hybridization (aCGH, Sureprint G3 Human CG 180K arrays, Agilent technologies, Santa Clara, CA, USA) as described previously[24] or by single nucleotide polymorphism array analysis (GeneChip Human Mapping, 100K Array set, Affymetrix, Santa Clara, CA, USA) as performed previously.[27]

Deletions were classified by assumed effect on protein function in three different groups: the 'dominant negative group' including all cases with at least one exons 4-7 deleted allele;[22] the 'haplo-insufficiency group', including whole gene deletions and deletions affecting exon 2;[13,22] the 'miscellaneous group' representing all remaining variants.

Genomic aberrations in other B-cell differentiation genes

Aberrations in other B-cell genes (*PAX5*, *ETV6*, *RB1*, *BTG1*, *EBF1*, *CDKN2A, CDKN2B*, *P2RY8-CRLF2* were investigated by the same MLPA SALSA p335 kit as used for the detection of *IKZF1* deletions. Data were interpreted as defined for *IKZF1* deletions.

Statistical analysis

Disease free survival (DFS) was calculated from date of first remission to the date of event which included relapse, death in complete remission, or second malignancy, whichever occurred first. Overall survival (OS) was calculated from the date of first remission to the date of death from any cause. Observations of patients were censored at the date of last contact when no events were observed. Follow-up was on December 31th 2008 for the pre-TKI cohort, and on December 31th 2010 for the EsPhALL cohort, with a median (interquartile range) follow up of 5.0 years (2.8−6.0) and 2.8 years (1.5−3.7), respectively. DFS was chosen as primary endpoint for both cohorts, as EsPhALL treated patients were registered at the end of national induction treatment. The Kaplan-Meier method was used to estimate the probabilities of DFS and OS, with standard errors (SE) calculated according to Greenwood. Curves were compared using the log-rank test. Cumulative incidence of relapse (CIR) were estimated adjusting for competing risks of death and were statistically analyzed by the Gray test.[28] The Cox model was used to investigate the prognostic role of *IKZF1* status. The $\chi 2$ test was used to assess the association between *IKZF1* status and clinical features. All tests were two-sided. Analyses were performed using SAS 9.2 (SAS institute, Cary, NC, USA) at the EsPhALL Trial Center.

RESULTS

The characteristics of *IKZF1*-deleted versus wild-type patients are shown in **Table 1**. *IKZF1*-deletions were identified in 126/191 (66%) *BCR-ABL1*-positive BCP-ALL samples, confirming the frequency observed in previous studies.[19,23-26] The frequency of *IKZF1* deletion did not differ between the pre-TKI (65%) and EsPhALL trials (66%). All *IKZF1*-deleted cases together had a higher white blood cell count than wild-type patients (p=0.006, **Table 1)** The 'haplo-insufficient' group accounted for 36.5%, the 'dominant negative' for 52.4% and the 'miscellaneous' for 11.1% of all *IKZF1*-deleted cases **(Table 1)**. This distribution was different from that was found in other studies in *BCR-ABL1*-negative BCP-ALL (p<0.001)[18,19,29] with a 2-fold higher frequency of the 'dominant negative' variant and a 1.7-fold lower frequency of 'haplo-insufficient' variants in a representative cohort of Dutch *BCR-ABL1*-positive BCP-ALL cases **(Supplementary Figure S2)**.

Table 1 | Patients' characteristics

		***IKZF1* wild-type**		***IKZF1*-deleted**		**p-value**
		n	**%**	**n**	**%**	
Gender						
	Male	44	67.7%	85	67.5%	0.97
	Female	21	32.3%	41	32.5%	
Age (years)						
	<10	43	66.2%	71	56.3%	0.19
	≥10	22	33.8%	55	43.7%	
White blood cell count (cells *10e9/L)[§]						
	<50	42	65.6%	58	46.0%	
	50-100	10	15.6%	15	11.9%	0.006
	≥100	12	18.8%	53	42.1%	
Early clinical response*						
	yes	38	63.3%	81	66.4%	0.68
	no	22	36.7%	41	33.6%	
***IKZF1* status**						
	wild-type	65	100.0%	-	-	-
	haplo-insufficient	-	-	46	36.5%	
	dominant negative	-	-	66	52.4%	
	miscellaneous	-	-	14	11.1%	

[§]White blood cell count was unknown in one patient (EsPhALL cohort). *Early clinical response was defined as: <1000 cells/µl in peripheral blood after 7 days of treatment with prednisone and a single intrathecal dose of methotrexate, or ≤5% leukemic blast cells in the bone marrow at day 21 (depending on national induction protocols). Early clinical response was unknown for 5 wild-type and 4 *IKZF1*-deleted patients (all from the pre-TKI cohort).

Prognostic value of all types of *IKZF1* deletions together

In the pre-TKI cohort, *IKZF1*-deleted patients had a lower DFS compared to wild-type patients (4-year DFS 30.0% (SE 6.8) vs. 57.5% (SE 9.4), p=0.013) (**Figure 1A**). This was mainly due to relapses as indicated by the 4-year cumulative incidence of relapse (CIR) of 57.2% (SE 7.4) for *IKZF1*-deleted patients and 20.9% (SE 7.8) for *IKZF1* wild-type patients (p=0.026, **Figure 1B**, **Table 2**). This resulted in a trend for superior overall survival (OS) for *IKZF1* wild-type patients with a 4-year OS of 75.1% (SE 8.2) versus 48.7% (SE 7.3) for *IKZF1*-deleted patients (p=0.075) (**Figure 1C***)*. 19/29 (65.5%) of the *IKZF1* wild-type patients received a HSCT which did not significantly differ from the 28/55 (50.9%) of *IKZF1*-deleted patients who received HSCT (**Supplementary Table S2**).

In the EsPhALL study, *IKZF1*-deleted patients had a trend for inferior DFS and OS compared to wild-type patients, but not an inferior CIR (**Figure 1D-F**, **Table 2**). Within the good-risk stratum of the EsPhALL protocol (n=63), the 20 *IKZF1* wild-type patients had a highly favorable prognosis (4-year DFS 78.6%, SE 13.9) compared to that of the 43 *IKZF1*-deleted patients (4-year DFS 51.9%, SE 8.8; p=0.027) (**Figure 2A**), although the difference in CIR did not reach statistical significance (**Figure 2B**). Interestingly, only two out of 20 *IKZF1* wild-type patients relapsed at 1.5 and 2.5 years from achieving complete remission compared to 11 out of 43 *IKZF1*-deleted patients who relapsed at a median time of 1.4 years (interquartile range: 1.2−1.9) (**Table 2**). All deaths, whether in CCR or after relapse, occurred in the *IKZF1*-deleted group (4-year OS 56.7% (SE 10.8), p=0.012, **Figure 2C**). HSCT was given in 12/20 (60.0%) of the wild-type patients and in 29/43 (67.4%) of the *IKZF1*-deleted patients (**Supplementary Table S2**, p=NS).

51 good-risk EsPhALL patients were treated with imatinib. The *IKZF1* wild-type good-risk patients had a 4-year DFS of 75.0% (SE 21.7) compared to 55.5% (SE 9.5) for *IKZF1*-deleted patients (p=0.051) (**Figure 2D**). Although only one out of 15 *IKZF1* wild-type good-risk patients relapsed in contrast to eight of 36 *IKZF1*-deleted good-risk patients, this difference in CIR did not reach statistical significance (**Figure 2E**). *IKZF1*-deleted good-risk patients had an OS of 65.8% (SE 9.0) compared to 100.0% for wild-type good-risk patients (p=0.039) (**Figure 2F**).[25] of the 36 (69.4%) *IKZF1*-deleted patients received HSCT versus 9/15 (60.0%) of the wild-type patients (**Supplementary Table S2**, p=NS). Multivariate analysis including age, white blood cell count, and treatment with imatinib indicated that *IKZF1* deletions were of independent unfavorable prognostic value in good-risk EsPhALL patients (hazard ratio=4.30, 95% CI 0.98-19.0, p=0.05) (**Table 3**).

There was no significant difference in DFS, CIR, or in OS between *IKZF1*-deleted and wild-type patients observed in the EsPhALL poor-risk group treated with imatinib (**Supplementary Figure S3**).

Additional genetic lesions in B-cell differentiation genes (*P2RY8-CRLF2, CDKN2A, CDKN2B, PAX5, ETV6, BTG1*, *RB1* and/or *EBF*1) were found in 73% (92/126) of *IKZF1*-deleted samples compared to 48% (28/65) of the wild-type samples (p<0.001). Furthermore, among imatinib treated good-risk patients, 47% (7/15) of *IKZF1* wild-type patients had additional genetic lesions versus 86% (31/36) of *IKZF1*-deleted (p=0.0058). Although these are significant differences, they did not impact on outcome. Indeed, none of the imatinib treated good-risk and *IKZF1* wild-type patients suffered from a relapse despite having additional genetic lesions in one or more of the above mentioned genes. Therefore, although the number of patients is limited, the good prognosis of imatinib treated *IKZF1* wild-type cases cannot be explained by absence of lesions in other B-cell maturation related genes.

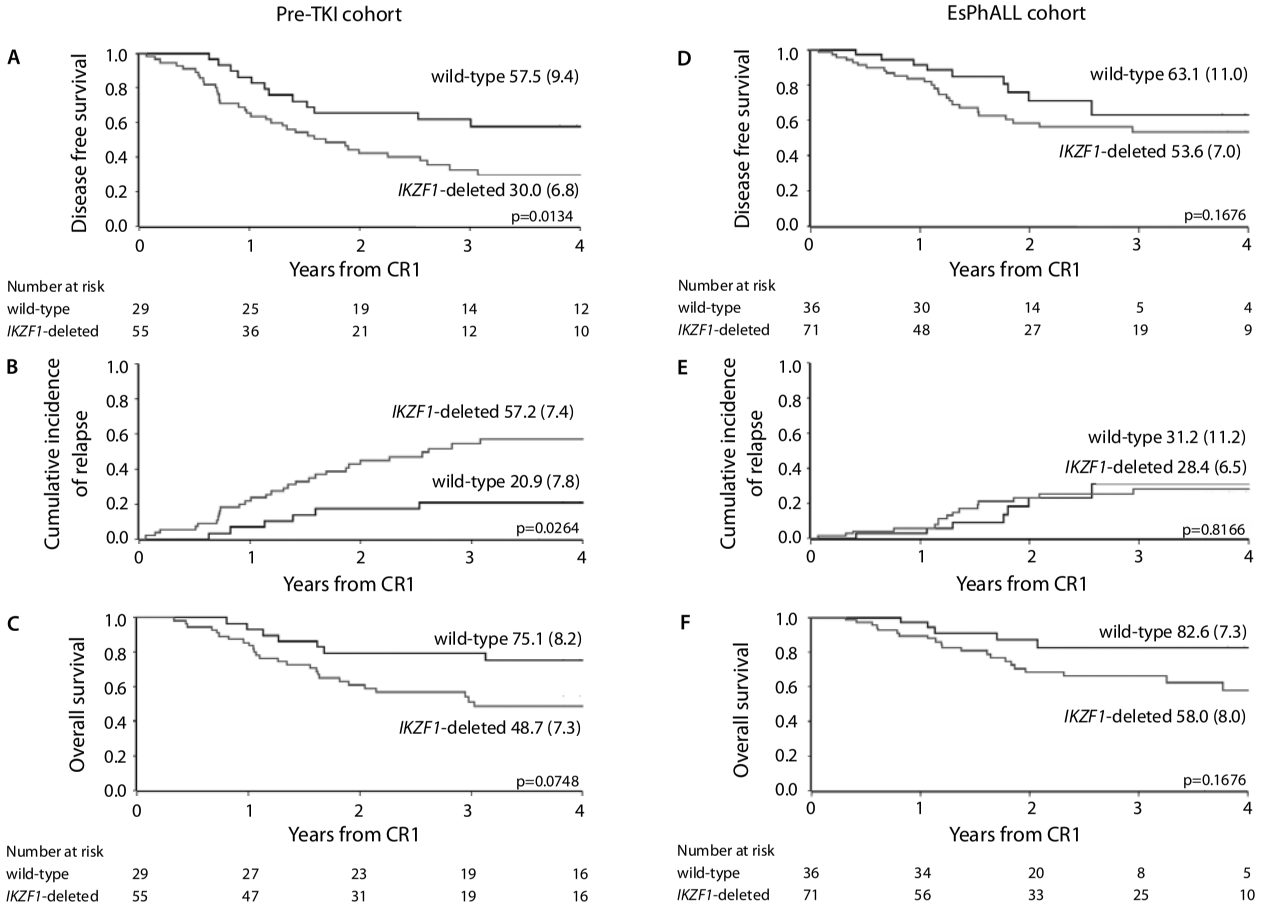


Figure 1| Outcome by cohort and *IKZF1* status. Disease free survival, cumulative Incidence of relapse and overall survival curves by *IKZF1* status in the pre-TKI cohort (Panel **A**, **B**, and **C**, respectively) and in the EsPhALL cohort (Panel **D**, **E**, and **F**, respectively), with 4-year estimates (standard error).

CR1: First complete remission. Three additional events occurred after more than 4 years from CR1 (2 relapses in *IKZF1*-deleted patients and 1 relapse in wild-type) and are therefore not depicted in the plots of Panel A and B. Two deaths that occurred after more than 4 years from CR1 in wild-type, relapsed pre-TKI patients are not depicted in Panel C.

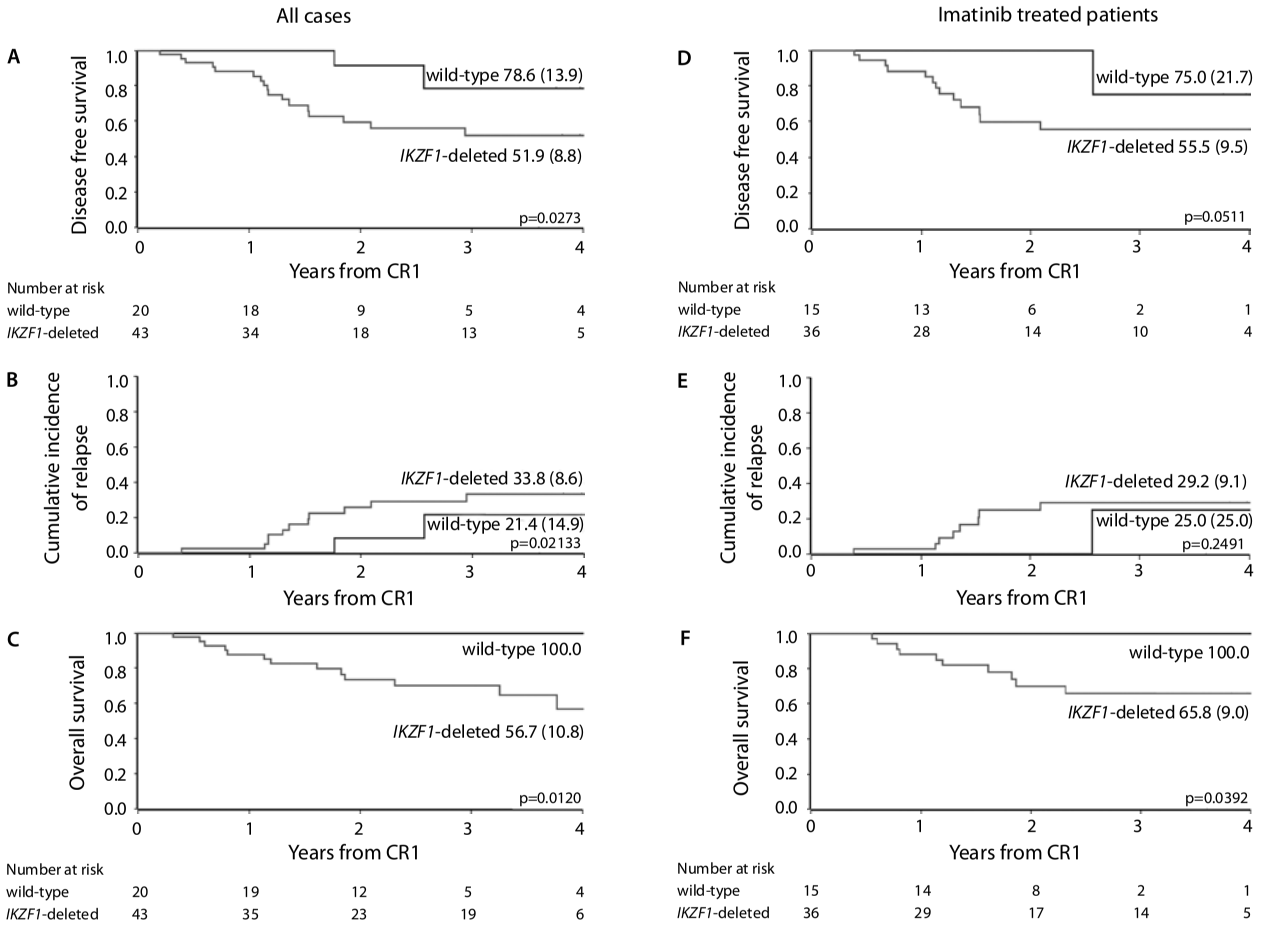


Figure 2 | Outcome by *IKZF1* status in EsPhALL good-risk patients. Disease free survival, cumulative incidence of relapse and overall survival curves by *IKZF1* status of EsPhALL good-risk patients (Panel **A**, **B**, and **C**, respectively) and cumulative incidence of relapse of EsPhALL Good-risk patients treated with imatinib (Panel D), with 4-year estimates (standard error).

CR1: First complete remission.

Table 2 | Clinical events by cohort and *IKZF1* status

	IKZF1 wild-type		*IKZF1*-deleted	
	n	**%**	**n**	**%**
Pre-TKI				
N. of patients	29	100.0	55	100.0
Relapses (Death after relapse)	7 (3)	24.1	31 (19)	56.4
BM involvement	7		25	
Isolated extra-medullary	0		6	
Deaths in CCR	6	20.7	7	12.7
death after HSCT	5		4	
EsPhALL overall				
N. of patients	36	100.0	71	100.01
Relapses (Death after relapse)	7 (4)	19.4	15 (10)	21.1
BM involvement	6		12	
Isolated extra-medullary	1		3	
Deaths in CCR	2	5.6	11	15.5
death after HSCT	2		8	
EsPhALL good-risk				
N. of patients	20	100.0	43	100.0
Relapses (Death after relapse)	2 (0)	10.0	11 (7)	25.6
BM involvement	1		8	
Isolated extra-medullary	1		3	
Deaths in CCR	0	0.0	6	14.0
death after HSCT	0		4	
EsPhALL poor-risk				
N. of patients	16	100.0	28	100.0
Relapses (Death after relapse)	5 (3)	31.3	4 (3)	14.3
BM involvement	5		4	
Isolated extra-medullary	0		0	
Deaths in CCR	2	12.5	5	17.9
death after HSCT	2		4	

BM, bone marrow; CCR, continuous complete remission. No second malignant neoplasm was observed in the 2 cohorts. EsPhALL cases were stratified in good-risk or poor-risk based on their early clinical response. Fifteen out of 20 *IKZF1* wild-type EsPhALL good-risk patients received imatinib: 1 relapsed in BM and testis and none died in CCR. Thirty-six out of 43 *IKZF1*-deleted EsPhALL good-risk patients received imatinib: 8 suffered from a relapse (5 in BM and 3 isolated extramedullary) and 5 died in CCR (4 after HSCT in CR1).

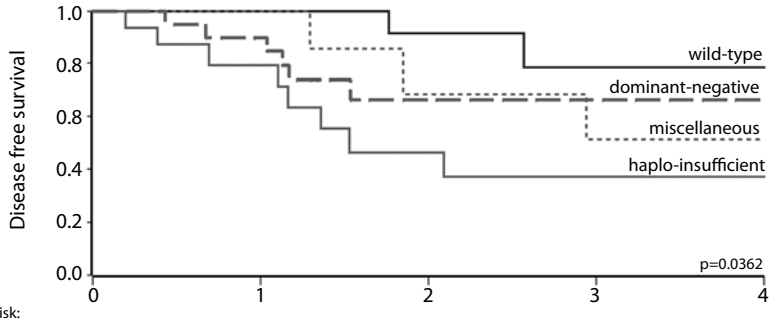
Table 3 | Multivariate analysis of DFS including potential risk factors and *IKZF1* status in *BCR-ABL1*-positive ALL.

	HR	95% CI	p-value
EsPhALL good-risk (n=62)			
Age at diagnosis, years			
<10	1		
≥10	1.69	0.61 - 4.68	0.31
WBC at diagnosis, ***10e9/L**			
<50	1		
≥50	1.53	0.62 - 3.78	0.36
imatinib exposure			
Yes	1		
No	1.52	0.51 - 4.51	0.45
***IKZF1* status**			
wild-type	1		
deleted	4.30	0.98 - 19.0	0.05

For 62 of EsPhALL good-risk patients all covariates were known. HR: hazard ratio; 95% CI 95% confidence interval. EsPhALL treated patients with a good early clinical response were stratified into the good-risk arm.

Prognosis according to the functional type of *IKZF1* deletion variant

In the EsPhALL good-risk group, outcome depended on the type of deletion (p=0.036, **Figure 3)**. The outcome of patients with deletions in *IKZF1* resulting in the dominant negative variant and miscellaneous variants did not statistically differ from wild-type patients. Interestingly, *IKZF1* haplo-insufficient patients showed an unfavorable outcome (4-year DFS: 37.1% (SE 14.2)) compared to wild-type patients versus (4-year DFS 78.6% (SE 13.9)) (p=0.0042) (**Figure 3**). Taking both poor- and good-risk EsPhALL patients together, haplo-insufficient patients showed an unfavorable outcome compared to wild-type patients (p=0.010) (**Supplementary Figure S4A)**. The poor outcome of haplo-insufficient patients was sustained by the outcome of patients with whole gene or exon 2−7 deletions, which were the most frequent variants in the haplo-insufficient group, compared to those with the dominant-negative isoform 6 (**Supplementary Figure S4B**).



Number at risk:					
wild-type	20	18	9	5	4
haplo-insufficient	16	10	5	3	2
dominant negative	20	17	9	7	3
miscellaneous	7	7	4	3	0

	No. patients	No. events	No. relapses	Disease free surivial (SE) 2-year	4-year
wild-type	20	2	2	91.7 (8.0)	78.6 (13.9)
haplo-insufficient	16	8	5	46.4 (14.2)	37.1 (14.2)
dominant negative	20	6	3	66.4 (11.5)	66.4 (11.5)
miscellaneous	7	3	3	68.6 (18.6)	

Figure 3 | DFS by *IKZF1* deletion variant in EsPhALL good-risk patients. Disease free survival curves of EsPhALL good-risk patients by *IKZF1* wild-type and deletion variant, with 2- and 4-year estimates (standard error).

CR1, First complete remission; dominant negative: samples with exons 4–7 deletions involving at least one allele; haplo-insufficient: samples with total deletions plus exons 2–7 deletions plus samples with exon 2 involved one sole allele; miscellaneous: samples not classified in previous groups.

DISCUSSION

The overall prognosis of childhood BCP-ALL has improved enormously throughout the recent decades, reaching 80 to 85% EFS rates at 5 years from diagnosis.[1] However, the prognosis of *BCR-ABL1*-positive BCP-ALL remained highly unfavorable in the pre-tyrosine kinase inhibitor (TKI) era with 5-year EFS below 50%.[3-5] Generally, until mid 2000 these patients were treated according to the high-risk protocols of individual national study groups. The introduction of TKI's, such as imatinib and dasatinib, has markedly improved the prognosis of *BCR-ABL1*-positive BCP-ALL.[7-10]

Recent studies have shown that *BCR-ABL1*-positive BCP-ALL is characterized by a very high frequency (70%) of *IKZF1* deletions.[19,23-26] In *BCR-ABL1*-negative childhood BCP-ALL these deletions of *IKZF1* were 4-fold less frequent but predictive for an unfavorable outcome in children, and recent studies showed that this predictive value is independent of other known risk-factors.[14-19] As the frequency of *BCR-ABL1*-positive BCP-ALL in children is low (<5% of newly diagnosed patients), the predictive value of *IKZF1* deletions remained unaddressed. Moreover, the recent introduction of TKI's may modify the predictive value of *IKZF1* deletions in *BCR-ABL1*-positive ALL. Therefore this international collaborative study was undertaken to address the prognostic value of *IKZF1* deletions

in children with *BCR-ABL1*-positive ALL both in patients treated in the pre-TKI era and in patients treated with imatinib.

We here show that *IKZF1* deletions are related to a poor outcome in *BCR-ABL1*-positive BCP-ALL. With the introduction of imatinib the 4-year DFS of *IKZF1*-deleted patients improved to ~50% compared to ~30% for *IKZF1*-deleted patients in the pre-TKI cohort. Of interest, *IKZF1* deletions remain associated with an unfavorable clinical outcome even in imatinib-containing therapies. Importantly, this difference in poor outcome could not be explained by a difference in the frequencies of HSCT between *IKZF1*-deleted and wild-type patients. In the pre-TKI cohort a lower frequency of HSCT was observed in the *IKZF1*-deleted group, because many *IKZF1*-deleted patients suffered from an early relapse excluding the possibility for a first-line HSCT.

As the *BCR-ABL1*-translocation itself is a strong adverse risk factor, it is even more remarkable that the *IKZF1* deletions can further dissect out patients at high-risk of relapse. This implies that *IKZF1*-deleted patients should receive more intensive and/or an alternative therapy. *IKZF1* deletions were shown to be a second hit in *BCR-ABL1*-positive BCP-ALL thereby inducing a more aggressive type of leukemia.[11,30] Deletions in *IKZF1* may lead to activated JAK-STAT pathway[13] and a pre-B-cell receptor signaling via BTK activation.[31] If confirmed, JAK and/or BTK inhibitors may offer an alternative strategy for treatment in *BCR-ABL1*-positive ALL with *IKZF1* deletions.

The frequency of deletions in exons 4 to 7 of *IKZF1*- resulting in the dominant negative isoform 6 – is higher in *BCR-ABL1*-positive than in *BCR-ABL1*-negative BCP-ALL. *BCR-ABL1*-positive BCP-ALL cells were demonstrated to have an increased activity of RAG1/RAG2 recombination genes.[25,26,32] Heptamer recombination signal sequences (RSS) have been localized to the break points of exons 4 and 7 of *IKZF1*.[23,25] The hyperactivity of RAG enzymes may therefore be related the increased frequency of exons 4 to 7 deletions in *BCR-ABL1*-positive BCP-ALL. Intriguingly, we observed that the less-frequent haplo-insufficient patients had a poorer prognosis than the more frequent dominant negative patients. Interestingly, loss of chromosome 7, which carries the *IKZF1*-gene, was shown to predict for an unfavorable prognosis in *BCR-ABL1*-positive BCP-ALL.[33] A recent study also indicated that the leukemic cells of *BCR-ABL1*-positive with monosomy 7, have a different gene expression profile compared to leukemic cells from patients with the dominant negative isoform 6 and those with *IKZF1* wild-type.[13] Whether this aberrant gene expression profile also yields insight into the underlying biology that may explain the difference in clinical outcome needs further study.

The most striking observation in our study is the highly favorable clinical outcome for EsPhALL good-risk patients with *IKZF1* wild-type. The majority (>60%) of the patients received HSCT. A recent study showed that *BCR-ABL1*-positive patients treated with TKI and chemotherapy have a comparable outcome to patients treated with TKI, chemotherapy and HSCT.[8] Taken together with our data, this suggests that *IKZF1* wild-type patients with a good early clinical response should be spared from HSCT, thereby reducing treatment-related morbidity. HSCT may then be used as salvage therapy for those *IKZF1* wild-type patients who suffer from a relapse.

In conclusion, as in children with *BCR-ABL1*-negative BCP-ALL, *IKZF1* deletions are a strong adverse prognostic factor in *BCR-ABL*1-positive patients, even if treated with imatinib. Studies on the pathobiological role of *IKZF1* deletions in *BCR-ABL1*-positive ALL should yield new clues for alternative treatment. Imatinib treated good-risk patients with wild-type *IKZF1* may be spared from HSCT, since their prognosis is as good as for *BCR-ABL1*-negative BCP-ALL cases. All together, we

propose to implement the *IKZF1* status in the risk stratification of childhood *BCR-ABL1*-positive BCP-ALL in future treatment protocols.

CONTRIBUTORS

AvdV, MZ, FM, MSc, AB, RP, MSt, MLdB and GC designed this study and the experimental set-up. AvdV, MZ, FM, GtK, CH, JT, MLdB and VS performed and/or analyzed the MLPA's. Statistics were done by AvdV, PdL and MGV. Manuscript was written and approved by all authors.
All authors declare to have no conflict of interest

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CHAPTER 4

Prognostic value of rare *IKZF1* variants in childhood B-cell precursor acute lymphoblastic leukemia: an international collaborative study

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Manuscript in preparation

ABSTRACT

Background: Deletions in *IKZF1* are found in ~15% of children with B-cell precursor acute lymphoblastic leukemia (BCP-ALL). There is strong evidence for the poor prognosis of *IKZF1* deletions affecting exons 4-7 (DEL 4-7) and exons 1-8 (DEL 1-8). Evidence for the remaining 33% of cases harboring other variants of *IKZF1* deletions is lacking. In an international multi-center study we analyzed the prognostic value of each of these rare variants.

Methods: Each *IKZF1*-deleted case was matched to three wild-type controls based upon cytogenetic subtype, treatment protocol, stratification arm, white blood cell count and age at diagnosis. We compared the cumulative incidence of relapse (CIR) between each rare deletion group and their controls using the Gray's test. Matched pair regression was used for event-free survival analysis (EFS).

Findings: The 5-year CIR for all 134 cases with rare deletions was higher compared to 402 matched wild-type controls (40% vs. 22%, $p<0.001$). Separate analyses showed higher 5-year CIR for 32 cases with DEL 2-7 (38% vs. 18%, $p=0.05$), for 15 cases with DEL 2-8 (60% vs. 31%, $p=0.02$), for 34 residual DEL-Other cases (45% vs. 24%, $p=0.04$), and a trend for 26 cases with DEL 2-3 (28% vs. 17%, $p=0.06$). Matched pair EFS revealed a poor prognostic hazard ratio (HR) for all rare variants together (HR 1.8, $p<0.001$), DEL 2-7 (HR 2.0, $p=0.03$), DEL 2-8 (HR 2.2, $p=0.002$), and DEL-Other (HR 2.2, $p<0.001$). The HR of DEL 2-3 (HR 1.8, $p=0.1$) and DEL 4-8 (HR 1.0, $p>0.1$) did not statistically differ from wild-type matched controls. The prognosis of each of the rare variants, including DEL 2-3 and DEL 4-8, was equal or even worse compared with the poor prognosis of DEL 4-7 and DEL 1-8 cases. We therefore recommend using all variants of *IKZF1* deletions as high-risk marker in the stratification of children with BCP-ALL.

INTRODUCTION

The prognosis of childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) has improved enormously since the past decades due to the introduction of risk-adapted therapies and better supportive care.[1] However, most relapses still occur in patients who were initially not considered to be at high risk for treatment failure.[2] Additional predictive markers are therefore essential to improve the clinical outcome in childhood BCP-ALL. Deletions in the B-cell transcription factor IKAROS have been linked to an unfavorable clinical outcome of BCP-ALL in different treatment protocols.[3-8] These studies also indicated that deletions in IKAROS were adverse prognostic in both high risk and non-high risk groups of patients, illustrating the power of this new genetic marker as adverse risk factor.[3,4,6]

The *IKZF1* gene located on chromosome 7p12.2 encodes IKAROS. The gene consists of 8 exons. Exons 2 to 8 contain the protein-coding sequence of IKAROS (**Figure 1**).[9] Transcription and translation of *IKZF1* is essential in the differentiation of B-cell progenitor cells by regulating pre-B-cell receptor signaling and cell cycle progression.[10,11] An intact IKAROS thereby induces differentiation of the cycling pre-B cell to a resting pre-B-cell.[12] The N-terminal DNA-binding domain of IKAROS consists of four zinc fingers, which are encoded by exons 4 to 6. This DNA-binding domain is essential for the tumor suppressive function attributed to wild-type IKAROS.[13] Exon 8 encodes two zinc fingers that mediate the dimerization of IKAROS as homodimer or as heterodimer of IKAROS with other transcription factors, including HELIOS, AIOLOS and GATA3.[13-15] Genomic deletions in *IKZF1* are found in approximately 15% of childhood and ~40% of adult BCP-ALL cases.[3-7,16] The frequency is remarkably high in *BCR-ABL1*-positive (~70%) and *BCR-ABL1-like* (~40%) BCP-ALL.[4,17] However, the number of exons that are deleted varies between patients. The most frequent *IKZF1* deletions in BCP-ALL affect the whole gene or exons 4 to 7.[3-7] The latter deletion includes the DNA-binding region and results in a dominant-negative isoform (isoform 6). The dominant-negative isoform scavenges the wild-type protein and thereby stimulates cell proliferation and impairs cell differentiation in CD34+ lymphoid progenitor cells.[13,18,19] Silencing of this dominant-negative variant partially restored B-cell commitment of *BCR-ABL1*-translocated BCP-ALL cells.[20] In addition, deletions affecting exons 2 to 3, exons 2 to 7 and exons 2 to 8 have also been observed, although the frequency of these variants is much lower than deletions of the whole gene and isoform 6 variants.[3-6,8,21] Since exon 2 comprises the translational ATG starting site,[9] deletions affecting exon 2 are in general considered to result in haplo-insufficiency for IKAROS.

Recently published reports combined the different deletions into one group to study the prognostic value of *IKZF1* deletions in BCP-ALL.[3-7] As the prognostic value of the individual rare *IKZF1* deletion variants is yet unknown, the present international collaborative study was initiated. *IKZF1*-deleted cases were matched to controls from the same treatment protocol and with similar cytogenetic and clinical features. This study comprises the data of 10 international study groups and provides statistical evidence for the adverse clinical outcome of these rare variants. The results obtained in this study pave the way to implement, besides the well-known whole gene (DEL 1-8) and isoform 6 (DEL 4-7) deletion variants, all types of *IKZF1* deletions as genetic marker to identify patients at high risk of treatment failure.

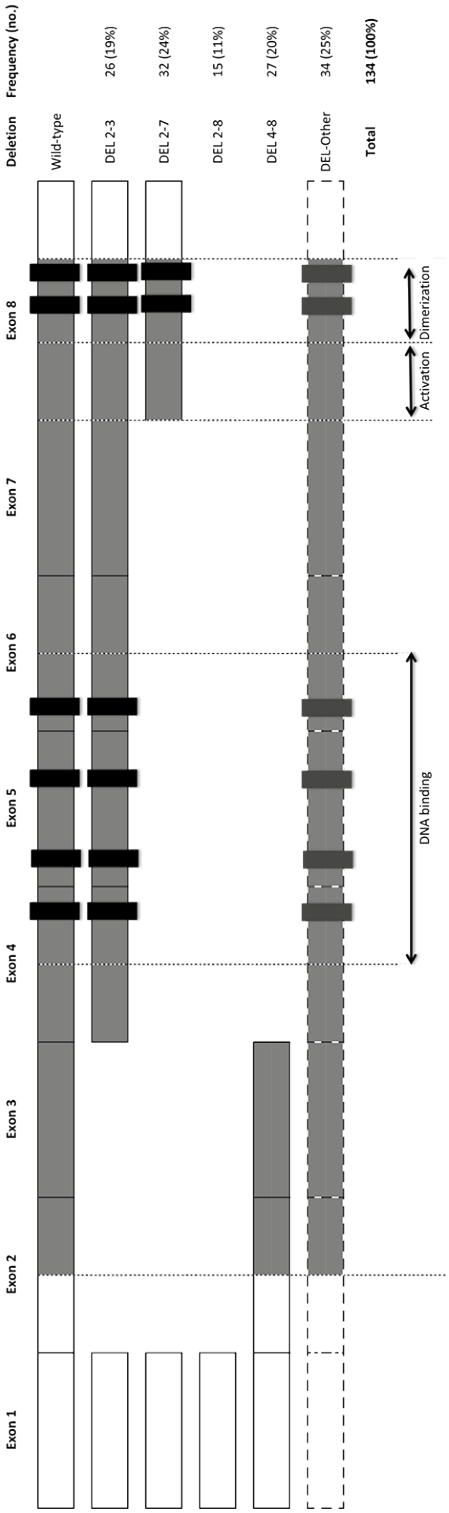

Figure 1 | Distribution and composition of IKZF1 deletion variants within this study. Transparant boxes represent non-coding parts of mature transcript. Gray boxes represent coding parts. Zinc fingers are depicted in black bars.

METHODS

Inclusion criteria

All types of deletions in *IKZF1,* with the exception of whole gene deletions (DEL 1-8) and isoform 6 (DEL 4-7), were called rare deletions in the present study since each of these variants comprised less than 10% of *IKZF1* deletions in children with BCP-ALL.[4] BCP-ALL cases with rare *IKZF1* deletions were selected from Dutch Childhood Oncology (DCOG), German (Berlin-Frankfurt-Münster-Germany [BFM-G] and COALL), UK Children's Cancer and Leukemia Group (CCLG), Australian and New Zealand Children Oncology Group (ANZCHOG), Austrian (Berlin-Frankfurt-Münster-Austria [BFM-A]), Italian (Associazione Italiana Ematologia Oncologia Pediatrica [AIEOP]), Polish Pediatric Leukemia Lymphoma Study Group (PPLLSG), Czech Pediatric Hematology Group (CPH), and Brazilian Pediatric Hematology-Oncology Program study groups. In accordance with the Declaration of Helsinki, written informed consent was obtained from parents or guardians, and institutional review boards approved the use of excess diagnostic material for research purposes. Selection of cases was based on availability of multiplex ligation-dependent probe amplification (MLPA) data, collected by the national study groups. Genomic DNA of patients' bone marrow aspirates was isolated according to local DNA isolation protocols. The MLPA assays (SALSA MLPA P335 ALL-IKZF1 and/or SALSA MLPA P202 IKZF1; MRC-Holland, Amsterdam, the Netherlands; **Supplemental Figure S1**) were locally performed according to the manufacturer's protocol and analyzed as previously described.[4] For 31 cases the locally produced data were confirmed by the research laboratory of Pediatric Oncology, Erasmus MC, the Netherlands.

Case-control selection

For each case with a rare *IKZF1* deletion, three control samples with *IKZF1* wild-type were selected (1:3 match). The matching was based on treatment protocol, stratification arm (standard/intermediate/high risk), cytogenetic group, white blood cell (WBC) count, and age (**Table 1**). The matched controls were selected by choosing samples with a date of diagnosis as close as possible to its corresponding case with a maximum difference of 2 years (**Table 1**). All cases were tested for *MLL*-rearrangements and *BCR-ABL1*-translocations. *BCR-ABL1*/*MLL*-rearrangement negative BCP-ALL cases that were not tested for *ETV6-RUNX1*, hyperdiploidy ([>50 chromosomes] and/or DNA index ≥1.16), and *TCF3*-rearrangements were allocated to the unclassified group. Cases that were negatively tested for *ETV6-RUNX1*, hyperdiploidy, *TCF3*-rearrangements, *BCR-ABL1*, and *MLL*-rearrangements were allocated to the cytogenetic B-other group. Cases with DEL 1-8 or DEL 4-7 were selected from the same study groups and only served as reference for the unfavorable outcome across all study groups.

Association with clinical outcome

A competing risk model − with death as a competing event − was used to estimate the cumulative incidence of relapse for all rare deletion variants and their controls. Gray's test was employed to assess the statistical difference between the estimated CIR corresponding to each group.[22] The 5-year CIR with the corresponding standard error was reported. A Cox 's regression model was used in which the standard errors have been calculated using the jack-knife sandwich estimator to account for

the matching nature of the data.[23] Events were defined as relapse or death. The Hazard Ratio (HR) and event-free survival (EFS) of cases was compared between cases with rare *IKZF1* deletions and matched wild-type controls and corresponding 95% confidence interval (CI) have been given. To avoid bias from known high-risk factors in the outcome analyses, infants (<1 year old), *BCR-ABL1*-positive, and *MLL*-rearranged cases were excluded.

Table 1 | Criteria that were used to match *IKZF1*-deleted cases to wild-type controls

Matching criteria
treatment protocol
stratification arm
cytogenetic group
match diagsnosed with date range of 2 years
age (<10 years or ≥10 years)
white blood cell count (<50 cells/nl or ≥50 cells/nl)

The stratification arm contained standard, intermediate and high-risk. Cytogenetic groups: *ETV6-RUNX1*, hyperdiploidy, *TCF3*-rearranged, unclassified B-other and undertermined BCP-ALL. *BCR-ABL1* and *MLL-r* cases were excluded for this study. A maximum of two years difference in date of diagnosis was used between deleted and wild-type cases. Age and white blood cell count were devided in two categories

Statistical packages

Statistics were centrally performed in Erasmus MC – Sophia Children's hospital. Baseline characteristics were analyzed by SPSS version 20.0 (IBM, New York, USA). Differences in absolute values were tested by the Mann-Whitney U tests. Categorical tests were performed by X^2-test. CIR and EFS were estimated by using R version 3.0.1.24 For CIR analyses the packages cmprsk version 2.2−6,25 and mstate version 0.2.6,[26] were used. EFS values were calculated by using the survival library 2.37−4. All tests were performed two-tailed and p-values ≤0.05 were considered as significant.

RESULTS

134 BCP-ALL cases with a rare *IKZF1* deletion were included. Of these cases, 26 (19%) had a deletion in exon 2 to 3 (DEL 2-3), 32 (24%) in exon 2 to 7 (DEL 2-7), 15 (11%) in exon 2 to 8 (DEL 2-8), 27 (20%) in exon 4 to 8 (DEL 4-8), and 34 (25%) belonged to the remaining group (DEL-Other) (**Figure 2**). An *ETV6-RUNX1* translocation was found in 12 (9%) of these rare cases, 21 (16%) had a hyperdiploid karyotype, 88 (66%) belonged to the B-other group, the remaining 13 (10%) was allocated to the unclassified group (**Table S1**). The cases with a rare *IKZF1* deletion equally represented the different risk categories: 56 (42%) of deleted cases were treated according to standard risk arms of national treatment protocols, 33 (25%) in intermediate risk arms and 45 (34%) in high risk arms (**Table S1**).

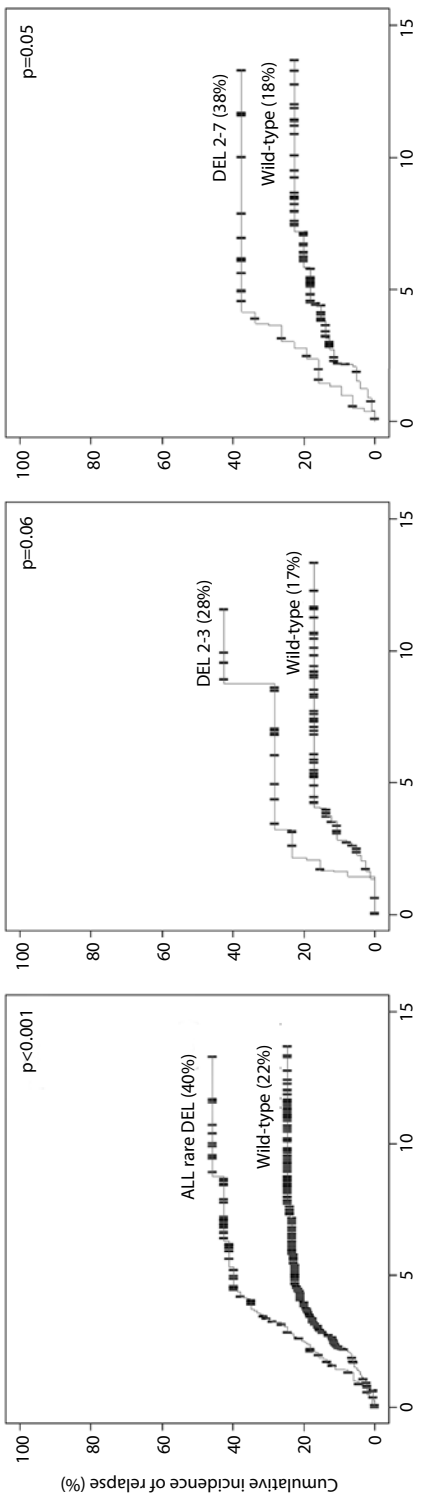

Figure 2 | Cumulative incidence of relapse (CIR) of *IKZF1*-deleted variants cases compared with their matched wild-type controls. Data were summarized in first panel and split up in the other panels. Death was used as a competing event. The 5-years CIR percentages are displayed between brackets. P-values were calculated using the method introduced by Fine and Gray.

Prognosis of all cases with rare variant deletions of *IKZF1*

CIR analysis revealed that all cases with a rare deletion in *IKZF1* had a higher 5-year CIR (40%) compared with wild-type controls (22%) (p<0.001; **Figure 2**). Matched pair EFS analysis revealed a HR 1.8 (95% CI: 1.4−2.3) for cases with rare *IKZF1* deletions compared with wild-type controls (p<0.001; (**Table 2, Supplemental Figure S2B and S3**). **Supplemental Figure S2** showed that the 5-year CIR and the EFS of all cases with rare deletions together is not different from those in the well-known poor prognostic deletion variants *IKZF1* DEL 4-7 and DEL 1-8. Deaths due to toxicity were equally distributed between all cases with rare *IKZF1* deletions and wild-type controls, but also between each of the distinct variant types and their wild-type controls (**Supplemental Table S2**). Next, we studied the outcome of patients with rare *IKZF1* deletions per type of genomic lesion.

Deletion exon 2−3 (DEL 2-3)

Cases with DEL 2-3 were found in all cytogenetic subtypes; 23% of DEL 2-3 cases had a hyperdiploidy (6/26), 27% an *ETV6-RUNX1* translocation (7/26), 38% were B-other (10/26), and 12% were cytogenetically unclassified (3/26) (**Supplemental Table S2**). The 5-year CIRs were 28% and 17% respectively for DEL 2-3 and wild-type controls (p=0.06) (**Figure 2**). The CIR and EFS curves for DEL 2-3 were not different from those for the common DEL 4-7 and DEL 1-8 reference group (**Figure 3, Supplemental Table S3**). Matched pair EFS analysis did not differ significantly (HR 1.8, 95% CI 0.9−3.5, p=0.1) between the DEL 2-3 cases and wild-type controls (T**able 2, Supplemental Figure S3**).

Deletion exon 2−7 (DEL 2-7)

Hyperdiploidy was found in 16% (5/32) of the DEL 2-7 cases, B-other in 72% (23/32), and 13% of these cases was cytogenetically unclassified (4/32); no *ETV6-RUNX1* translocated samples were identified harboring this rare deletion variant (**Supplemental Table S2**). The DEL 2-7 cases had a higher 5-year CIR (38%) compared with wild-type controls (18%) (p=0.05) (**Figure 2**). The CIR and EFS curves did not differ between DEL 2-7 cases and the reference of DEL 4-7 and DEL 1-8 cases (**Figure 3, Supplemental Table S3**). The matched pair analysis showed lower EFS in DEL 2-7 cases (HR for an event 2.0, 95% CI: 1.1−3.7, p=0.03) compared with wild-type controls (**Table 2, Supplemental Figure S3**).

Deletion exon 2-8 (DEL 2-8)

For DEL 2-8 cases, 7% had a hyperdiploidy (1/15), 13% (2/15) had an *ETV6-RUNX1* translocation, 73% was B-other (11/15), and 7% (1/15) had an unclassified genetic alteration (**Supplemental Table S2**). The DEL 2-8 cases had a highly unfavorable 5-year CIR of 60% compared with the 31% for their wild-type controls (p=0.02) (**Figure 2**). The CIR curves for DEL 2-8 cases were more unfavorable than those for DEL 4-7 and DEL 1-8 references (p=0.04 and p=0.01, respectively) and had a more unfavorable EFS than DEL 1-8 cases (p=0.04) (**Figure 3, Supplemental Table S3**). The matched pair EFS was lower in DEL 2-8 cases compared with wild-type controls (HR for an event 2.2, 95% CI 1.3−3.6, p=0.002) (**Table 2, Supplemental Figure S3**).

Table 2 | Hazard ratio's (HR) calculated by mathed pair EFS

EFS paired analysis	HR	95% conficence interval	*p*-value
All rare DEL	1.8	1.4–2.3	<0.001
DEL 2-3	1.8	0.9–3.5	0.1
DEL 2-7	2.0	1.1–3.7	0.03
DEL 2-8	2.2	1.3–3.6	0.002
DEL 4-8	1.0	0.5–1.9	NS
DEL-Other	2.2	1.4–3.5	<0.001

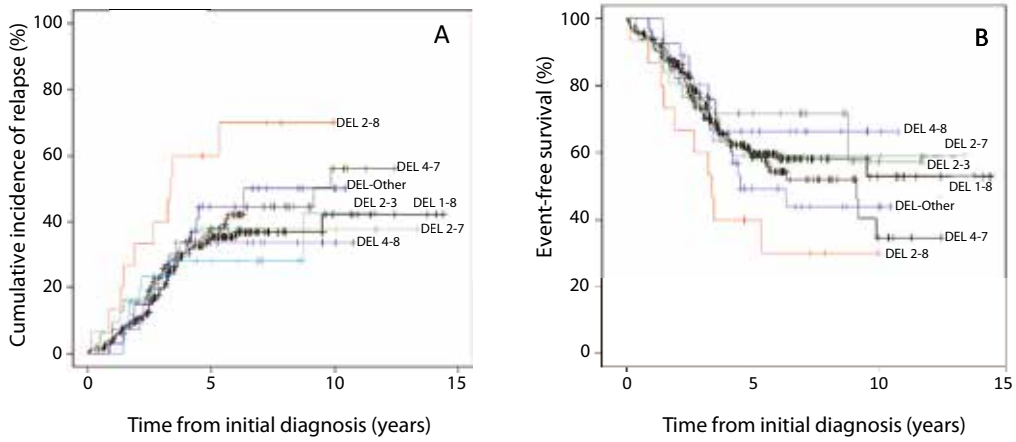

Figure 3 | **Outcome of rare variants compared with DEL 1-8 and DEL 4-7 reference group.** CIR (**A**) and EFS (**B**). DEL 4-7 and DEL 1-8 cases were extracted from the same treatment protocols as rare deleted cases and controls. For statistical tests see: **Supplemental Table S3**.

Deletion 4-8 (DEL 4-8)

A hyperdiploid karyotype was found in 15% (4/27) of the DEL 4-8 cases; 7% (2/27) had an *ETV6-RUNX1* translocation, 70% (19/27) was B-other, and 7% (2/27) was genetically unclassified (**Table 2**). The 5-year CIR did not significantly differ between DEL 4-8 cases and wild-type controls (**Figure 2**) but the CIR cure of DEL 4-8 was neither different from those of the known unfavorable prognostic DEL 4-7 and DEL 1-8 reference groups (**Figure 3, Supplemental Table S3**). No statistical difference in EFS was found for DEL 4-8 cases compared with wild-type controls (**Table 2, Supplemental Figure S3**).

Remaining deletions (DEL-Other)

Cases with DEL-Other had a hyperdiploidy in 15% (5/34), an *ETV6-RUNX1* translocation in 3% (1/34), B-other in 74% (25/34), and 9% of them were cytogenetically unclassified (3/34) (**Supplemental Table S2**). DEL-other cases had an unfavorable 5-year CIR (45%) compared with that of wild-type controls (24%) (p=0.04) (**Figure 2**). The CIR curves of DEL-Other cases did not differ from those of the known unfavorable prognostic DEL 4-7 and DEL 1-8 references (**Figure 3, Supplemental Table S3**). The matched pair EFS was lower for DEL-Other cases (HR 2.2, 95% CI 1.4−3.5, p<0.001) compared with wild-type controls (**Table 2, Supplemental Figure S3**). DEL-Other cases in which at least exon 2 was deleted (which contains the ATG-start codon) were considered as haplo-insufficient (n=15) whereas the remaining cases were allocated to the miscellaneous group (n=19) (**Supplemental Figure S1; Supplemental Table S4**). Haplo-insufficiency of DEL-Other cases was not statistically significantly predictive for an unfavorable outcome in CIR analyses (5-year CIR: 35%) compared with wild-type controls (5-year CIR: 24%; p=0.3). However, the matched pair EFS was lower in haplo-insufficient cases (HR for an event 2.4, 95% CI 1.1−5.5, p=0.04). The miscellaneous DEL-Other cases had an unfavorable prognosis based on both CIR and EFS compared with wild-type cases (5-year CIR: 54% vs. 24%, p=0.05; HR 2.1, 95% CI 1.4−3.2, p<0.001) (**Supplemental Figure S4**).

DISCUSSION

Evidence for the poor prognosis of *IKZF1* deletions in pediatric BCP-ALL is based on studies that use all types of *IKZF1* deletions together without considering that some of these variants might have an altered function, not necessarily a loss-of-function.[3-8] The difference in biology is exemplified by the difference in gene expression signatures of cases with DEL 4-7 compared with that of cases with a DEL 1-8.[27] Besides the known unfavorable prognosis of DEL 4-7 and DEL 1-8 cases, the biological and clinical consequences of the remaining one-third of *IKZF1* deletions (called rare *IKZF1* deletions in the present paper) in *BCR-ABL1*-negative BCP-ALL remains unclear. For stratification based on *IKZF1* deletions it is important to clarify the prognostic role of each type of *IKZF1* deletion. Because of the low frequency, the prognostic value of rare variants can only be analyzed by jointed forces of different study groups, each contributing with a few rare cases. To meet this aim, we initiated a large collaborative study within the international BFM network in which participating study groups provided data of rare variant cases matched to wild-type controls using stringent matching criteria. Each case was matched for treatment protocol, treatment arm, date of diagnosis (±2 years), cytogenetic subgroup, WBC, and age at diagnosis since these features might influence the prognostic value of a putative marker if analyzed in an unbalanced way.[4,28-32] Our study revealed that all types of rare *IKZF1* deletions with the exception of DEL 4-8 cases, were statistically significantly associated with an increased risk of relapse and poorer EFS. Although DEL 4-8 cases had no poorer prognosis than their matched wild-type controls, the prognosis of DEL 4-8 cases was as poor as those of the other rare variants and that of the known high risk variants DEL 4-7 and DEL 1-8. Our experimental design of matched cases and wild-type controls rules out that conventional risk markers (age, WBC, genetic subtype) contribute to the observed unfavorable clinical outcome.

The DEL 4-7 deletion variant of *IKZF1* was shown to result into an elevated expression of the dominant negative isoform 6 and this variant was found to contribute to the development of leukemia.[17] This suggests that a disturbance in the quantity and/or ratio of IKAROS isoforms result in abnormal B-cell development and leukemia.[33] Interestingly, the deletions that we found most discriminative for an adverse prognosis result in haplo-insufficiency by lacking the translational start site in exon 2 (**Figure 1**), i.e. DEL 2-3, DEL 2-7, DEL 2-8. In addition, a deletion of exon 4 to 8 might also affect IKAROS function by lacking the C-terminal dimerization domain. Because IKAROS regulates the differentiation from proliferating cycling pre-B-cell to resting pre-B-cell by interfering with the pre-B-cell receptor,[12] it may be hypothesized that *IKZF1* deletions prevent this important differentiation step and thereby result in uncontrolled proliferation. Alternatively, the poor prognosis of *IKZF1*-deleted cases may reflect genomic instability rather than malfunction of IKAROS, as recently shown for *ETV6-RUNX1* translocated BCP-ALL.[34,35]

At present, genomic deletions in *IKZF1* can only be used as new diagnostic marker to identify patients at high risk of treatment failure. No specific agents are yet available that counteract the biological effect of an *IKZF1* deletion in leukemic cells. Of interest is the finding that *BCR-ABL1*-positive BCP-ALL cells with DEL 4-7 have a higher level of the stem cell marker THY1, which is normally suppressed by wild-type IKAROS.[36,37] In a preliminary study (abstract) retinoid acid was shown to induce the expression of wild-type IKAROS simultaneously with a decrease in THY1 expression in *BCR-ABL1*-positive BCP-ALL, and as an result cells became more sensitive to dasatinib.36 These promising results need further confirmation but suggest that retinoid acid might provide a therapeutic option for *IKZF1*-deleted cases.

In summary, our study shows that all types of *IKZF1* variants are prognostically relevant and are predictive for an unfavorable outcome in pediatric BCP-ALL. Therefore, we advocate to include, besides DEL 4-7 and DEL 1-8, all types of rare variants of *IKZF1* deletions in the risk stratification of pediatric ALL.

CONTRIBUTORS

AvdV, JMB, MZ, AVM, RP, designed this study. Patients' samples and follow up data were supplied by: HAdGK, PH, JT, MH, MdSPdO, WM, AA, RS, AE, MSc, GC, MZ and AVM. Multiplex Ligation-dependent Probe Amplification assays were performed by: AvdV, ES, RPK, MZ, EF, ME, TS, KN, AA, NV, CJS, AV, MSt and GC. AvdV, JMB, DR, MF, HAdGK, MZ, AVM and MldB performed the statistical analysis of data. The manuscript was written and approved by all authors. All authors declare to have no disclosures.

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CHAPTER 5

Interference with pre-B-cell receptor signaling offers a therapeutic option for *TCF3*-rearranged childhood acute lymphoblastic leukemia

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ABSTRACT

TCF3-rearranged B-cell precursor (BCP)-ALL is characterized by constitutive expression of the cytoplasmic immunoglobulin heavy chain (CyIgμ). CyIgμ and the surrogate light chain together constitute the pre-B cell receptor (pre-BCR). Here the pre-BCR pathway was investigated as therapeutic target in *TCF3*-rearranged pediatric BCP-ALL. Immunoglobulin rearrangement patterns were analyzed by multiplex PCR in *TCF3*-rearranged and non-*TCF3*-rearranged cells. Levels pre-BCR related proteins were quantified by reverse phase protein arrays. The sensitivity of leukemic cells to ibrutinib (BTK-inhibitor) was determined by methyl-thiazol-tetrazolium assays. CyIgμ positivity was detected in 84.6% of *TCF3*-rearranged and 20.9% of non-*TCF3*-rearranged B cases ($p<0.001$). *TCF3*-rearranged cases more often had a rearranged immunoglobulin heavy chain without processing of light chain compared to non-*TCF3*-rearranged cases (86.4% versus 17.9%; $p<0.001$). The pre-BCR related proteins were all higher expressed in *TCF3*-rearranged cases compared to non-*TCF3*-rearranged cases ($p<0.05$). All (6/6) *TCF3*-rearranged patients' samples were sensitive to ibrutinib, but none (0/6) of the non-*TCF3*-rearranged samples. Ibrutinib reduced the phosphorylation of ERK1/2 in the *TCF3*-rearranged cell line, but not in the non-*TCF3*-rearranged cell lines. *TCF3*-rearranged BCP-ALL is characterized by a constitutively activated pre-BCR pathway. Targeted interference with pre-BCR-mediated signaling effectively and specifically killed *TCF3*-rearranged cells with high BTK expression and therefore may offer a new therapeutic option for *TCF3*-rearranged BCP-ALL.

INTRODUCTION

Rearrangements of *TCF3* (E2A) occur in <5% of childhood B-cell precursor ALL (BCP-ALL) cases.[1-3] In 90-95% of these rearranged cases *TCF3* (chromosome 19p13) is fused to *PBX1* (chromosome 1q23).[4] Initially, the *TCF3-PBX1* fusion (formerly known as *E2A-PBX1*) was associated with an inferior clinical outcome in pediatric ALL,[5-7] but on contemporary protocols the fusion gene has lost this adverse prognostic value.[2,8]

The *TCF3*-rearranged subtype is characterized by expression of cytoplasmic immunoglobulin heavy chain (CyIgμ) in more than 80% of pediatric patients.[6,9] This CyIgμ positivity is a consequence of an in-frame VDJ rearrangement of the immunoglobulin heavy chain locus (*IGH*).[10-12] Igμ and the surrogate light chain (SLC) together constitute the pre-B cell receptor (pre-BCR).[12,13] Activation of this pre-BCR triggers the clonal expansion of pre-B cells in the bone marrow after which these cells further maturate by initiating the rearrangement of the light chain locus genes *IGK* and *IGL*.[10,11] The transcription factor *TCF3* is essential in the differentiation process of common lymphoid progenitors into B-lineage cells and is a key regulator of further B-cell development. *TCF3*-deficient cells are impaired in rearranging both the immunoglobulin heavy and light chain genes, and *TCF3* regulates the expression of other genes important in B-cell differentiation such as *EBF1* and *PAX5*.[14,15] These findings imply that *TCF3*-rearranged cases might be affected in pre-BCR mediated signaling.

Bruton's tyrosine kinase (BTK) is an important protein in the pre-BCR signaling cascade.[16] BTK stimulation results in downstream activation of PLCγ2 and ERK1/2, resulting in cell survival and proliferation.[17,18] Deficiencies in *BTK* result in an immature B-cell arrest leading to agammaglobulinemia.[19] Interestingly, BTK can be therapeutically targeted by the well tolerated drug ibrutinib (PCI-32765) in chronic lymphoblastic leukemia,[20-22] B-cell non-Hodgkin lymphomas,[21] and multiple myeloma.[23]

The prognosis of children with *TCF3*-rearranged leukemia improved during consecutive trials by optimizing the regimens with traditional chemotherapeutic drugs.[2,8] Implementation of more specific, i.e. targeted, drugs may further improve prognosis and/or reduce the side effects of conventional chemotherapeutics by allowing a dosage reduction. We here investigated components of the pre-BCR pathway in *TCF3*-rearranged BCP-ALL. Our study revealed that *TCF3*-rearranged BCP-ALL cells are arrested at a pre-B-II-large stage, showing IGH rearrangements but no light chain rearrangements, and display a constitutively activated pre-BCR pathway. *TCF3*-rearranged BCP-ALL cells were further shown to be highly sensitive to inhibition of the BTK component of this pre-BCR pathway. Together these data suggests that interference with the pre-BCR pathway offers a therapeutic option for *TCF3*-rearranged BCP-ALL.

METHODS

Samples

Bone marrow aspirates of newly diagnosed BCP-ALL patients (1−18 years) were collected after the patients, parents or guardians had given their written consent. Institutional review boards approved the use of excess of diagnostic material for research purpose.

Cytogenetic abnormalities were identified in BCP-ALL cells as follows: *ETV6-RUNX1*-translocations were identified using real-time quantitative RT-PCR (RQ-PCR) and interphase Fluorescent In-situ Hybridization (FISH), high hyperdiploidy using conventional karyotyping (>50 chromosomes) or DNA-index (≥1.16), *TCF3*-rearrangements by RQ-PCR or split-signal interphase FISH, *BCR-ABL1*-translocations using RQ-PCR, conventional karyotyping or interphase FISH. Immunophenotyping, including CyIgμ expression, was performed by reference laboratories. Samples that contained >30% leukemic cells expressing CyIgμ were marked as CyIgμ positive. The cell lines MHH-CALL3 (*TCF3*-rearranged BCP-ALL), MHH-CALL4 (non-*TCF3*-rearranged BCP-ALL) and Nalm6 (non-*TCF3*-rearranged BCP-ALL) were obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

The mononuclear fraction of bone marrow aspirates was isolated using Lymphoprep sucrose centrifugation (1.077 g/ml, Nycomed Pharma, Oslo, Norway). The leukemic blast percentage was determined after May-Grünwald-Giemsa staining. The blast percentage was increased >90% by depleting normal cells by anti-CD-antibody coated magnetic beads (Dynal, Oslo, Norway) as described previously.[24] DNA and RNA were isolated using TRIzol reagents (Invitrogen, Breda, the Netherlands) according to manufacturer's protocols and dissolved in Tris-EDTA. Cell pellets containing 5 million cells were stored in -80°C upon protein isolation.

Immunoglobulin rearrangement profile

Samples were screened for the presence of IGH (V_H-J_H and D_H-J_H) and IGK-Kde (V_κ-Kde and intron RSS-Kde) rearrangements using genomic PCR heteroduplex analysis.[25] PCR analysis of V_κ-J_κ and V_λ-J_λ rearrangements was performed using the BIOMED-2 multiplex primer-sets (IVS Technologies, San Diego, CA).[26] Based on the type and order of immunoglobulin rearrangements in normal B-cell differentiation, samples were classified as IGH (IGH rearrangements only), IGK (V_κ-J_κ without *IGK*-Kde rearrangement), *IGK*-Kde (IG_K-Kde without V_λ-J_λ rearrangement), or IGL (V_λ-J_λ rearrangements).

Reverse phase protein array

The revere phase protein assay (RPPA) was performed as described previously.[27] Cell pellets were lysed in Tissue Protein Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) supplemented with 300 mM NaCl, 1 mM sodium orthovanadate (Sigma-Aldrich, Zwijndrecht, the Netherlands), 2 mM pefabloc (Roche, Almere, the Netherlands), 5 ug/ml aprotinin (Roche), 5 ug/ml leustatin (Roche) and 5 ug/ml pepstatin (Roche). After incubation on ice, tubes were centrifuged and the supernatant was transferred to new vials. The cell lysates were spotted at 0.5 ug/ul, twice in triplicate on each slide on a glass-backed nitrocellulose-coated array slide (FAST-slide, Whatman Plc, Kent, UK) by an Aushon Biosystems 2470 arrayer (Aushon Biosystems, Billerica, MA, USA). The total amount of loaded protein was determined on the first of every fifteen slides by Sypro Ruby Protein Blot Stain

(Invitrogen, Bleiswijk, the Netherlands), quantified on a NovaRay CCD fluorescent scanner (Alpha Innotech, San Leandro, CA, USA). Slides were stained with antibodies against BTK (#3532), SLP65 (#3587) and IRF4 (#4948) obtained from Cell Signaling Technology (Danver, MA, USA), EBF1 (#1879) from ABnova (Walnut, CA, USA), ZAP70 (#05-253) from Millipore (Billerica, MA, USA) and PI3K-p110δ from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For the incubation with the secondary antibody a DAKO cytomation (Dako, Heverlee, Belgium) autostainer was used. Slides were scanned on a NovaRay-scanner and analyzed with MicroVigene version 2.8.1.0 software (VigeneTech, Carlisle, MA, USA). Protein levels detected by the specific antibodies were normalized for total protein levels in each sample and were corrected for background staining of the secondary antibody.

In vitro BTK-inhibition assay

Sensitivity to the BTK-inhibitor ibrutinib (PCI-32765, Selleckchem, Houston, TX, USA) was analyzed in six *TCF3*-rearranged BCP-ALL cases, two hyperdiploid cases, two *ETV6-RUNX1*-translocated cases, one *BCR-ABL1*-translocated case, one *BCR-ABL1-like* case and the leukemic cell lines MHH-CALL3 (*TCF3*-rearranged), MHH-CALL4 and Nalm6 (both non-*TCF3*-rearranged). The inhibitor was dissolved in dimethyl-sulfoxide (DMSO). The final concentration of DMSO was 0.3%, which did not affect the viability of leukemic cells. Cells were resuspended in RPMI (Invitrogen, Life Sciences, Bleiswijk, the Netherlands) with 20% fetal calf serum at a concentration of 2.5 million cells/ml. All tested primary patients' samples contained ≥90% leukemic cells. The sensitivity to ibrutinib was determined by methyl-thiazol-tetrazolium (MTT) assays. BCP-ALL primary patients' cells and cell lines were incubated with a serial dilution of ibrutinib ranging between 0.6 μM and 50.0 μM, in duplicate in a 96-wells plate for three days at 37°C, 5% CO_2. Cells were incubated with the equivalent DMSO-concentrations as control. Subsequently, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide was added, as previously described.[24] After six hours, acidified propanol was used to dissolve formazan crystals and the amount of formed formazan was quantified at a wavelength of 562 nm by a VersaMax (Molecular Devices, Sunnyval, CA, USA). Dose-response curves were generated for samples with an optimal density read of at least 100 units and more than 70% leukemic cells in the control wells left after 3 days of culture at 37°C. The percentage of viable cells at each ibrutinib concentration was calculated by the ratio in optical density of ibrutinib-exposed and DMSO-exposed control wells.

Detection of downstream effect of ibrutinib by western blot

MHH-CALL3 cells, as a model for *TCF3*-rearranged BCP-ALL and MHH-CALL4 and Nalm6, as a models for non-*TCF3*-rearranged BCP-ALL, were incubated with 0 μM, 50 μM and 150 μM of ibrutinib for 4 hours. Cells were cultured in RPMI with 20% fetal calf serum at 37°C, 5% CO_2. Cells were washed twice with physiologically buffered salt solution (PBS, Invitrogen, Life Sciences, Bleiswijk, the Netherlands), and resuspended in 100 μl lysis buffer. Lysis buffer contained 25 mM Tris (tris[hydroxymethyl]aminomethane) buffer, 150 mM NaCl, 5 mM EDTA (ethylenediaminetetraacetic acid), 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM glycerolphosphate, 1 mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 1% aprotinin (Sigma-Aldrich), 10mM sodium fluoride, and 20 μL of freshly prepared sodium pervanadate. Cells were lysed on ice for 30 minutes. Protein concentrations were measured by the bicinchoninic acid protein assay (Pierce Biotechnology, Etten-Leur, the Netherlands) with a concentration series

of bovine serum albumin (BSA) as standard. Twenty ng of protein per case was separated on a precast gel (Mini-PROTEAN TGX Gels any KD, Biorad, Veenendaal, the Netherlands) and blotted on a nitrocellulose trans-blot (Biorad) using the conditions recommended by the manufacturer. Antibodies against total ERK1/2 (p44/42 MAPK, #4695, Cell Signaling) and phosphorylated ERK1/2 (p44/42 MAPK (T202/Y204), #4370S, Cell Signaling) were incubated overnight at 4°C in TBS-Tween 5% BSA. Antibody against β-actin was incubated for one hour at room temperature. After washing with TBS-Tween 1% BSA, the secondary antibody was incubated for one hour at room temperature. The blots were scanned on an Odyssey Infrared Scanner 9190 (Li-Cor, Lincoln, USA). Western blots were analyzed by Odyssey Software 2.1.

RESULTS

Pre-BCR expression

CyIgμ positivity was found in 11/13 (84.6%) of *TCF3*-rearranged cases and in 68/325 (20.9%) of non-*TCF3*-rearranged BCP-ALL cases (p<0.001; **Figure 1**, **Supplementary Table S1**). These data confirm previous findings by others.[6,9]

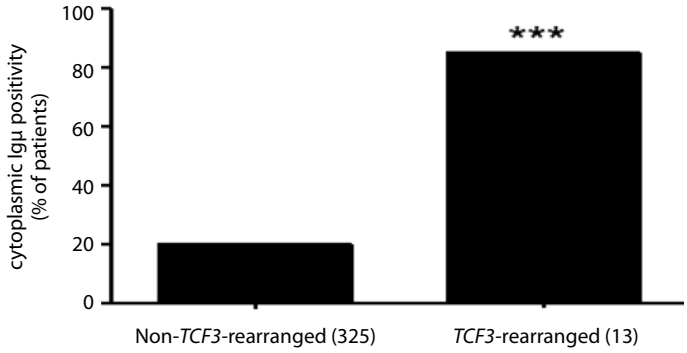

Figure 1 | Cytoplasmic Igμ expression in 13 *TCF3* and 325 non-*TCF3*-rearranged B-cell precursor ALL. Patients were considered CyIgμ positive if >30% of leukemic cells were stained positively for this marker.
***: p<0.001, chi-square test.

The *IGH*, *IGK* and *IGL* rearrangement pattern was analyzed in 212 BCP-ALL samples and has been summarized in **Supplementary Table S2A**. We defined four stages at which the rearrangement had been arrested: samples with only rearrangements of *IGH* (IGH), samples with V_{κ}-J_{κ} rearrangements but no IGK-Kde or V_{λ}-J_{λ} rearrangements (IGK), samples with IGK-Kde and no V_{λ}-J_{λ} rearrangements (IGK-Kde), and samples with V_{λ}-J_{λ} rearrangements (IGL). The *TCF3*-rearranged patients were arrested at the *IGH* stage in 86.4% (19/22) of the cases. Only one case was assigned to the IGK group and two cases to the IGL group. In contrast, only 17.9% (34/190) of the non-*TCF3*-rearranged cases were

arrested at the IGH stage and the frequency of cases with light chain rearrangements was significantly increased to 18.4% (35/190) for samples arrested at IGK, 38.4% (73/190) for IGK-Kde and 24.7% (47/190) for IGL (p<0.001). In one sample, i.e. the *BCR-ABL1-like* sample, neither rearrangements of the heavy chain (IGH) nor rearrangements in the light chain genes (IGK, IGL) were detected (**Figure 2**, **Supplementary Table S2B**).

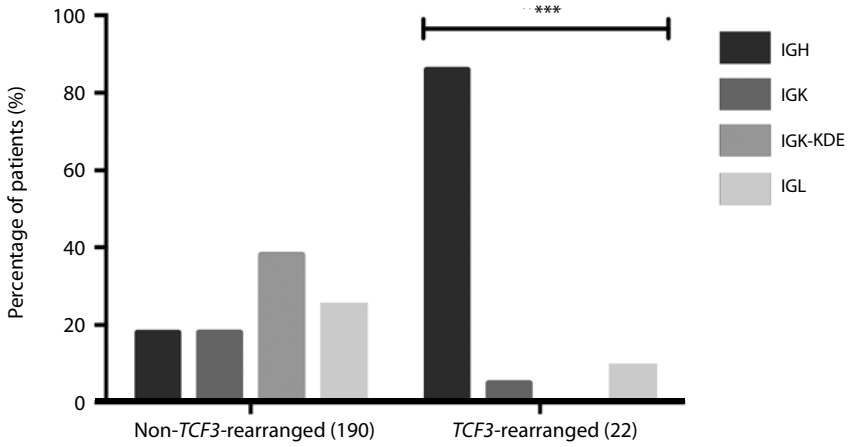

Figure 2 | Distribution of immunoglobulin rearrangement pattern in 22 *TCF3*-rearranged and 190 non-*TCF3*-rearranged BCP-ALL cases. IGH group contains samples with IGH rearrangement only. IGK group consists of cases with V_{κ}-J_{κ} rearrangements without IGK-Kde or V_{λ}-J_{λ}; IGK-Kde group contains IGK-deleted cases without V_{λ}-J_{λ} rearrangements. IGL group contains cases with V_{λ}-J_{λ} rearranged IGL locus.

***: p<0.001, chi-square test for distribution of rearrangements between *TCF3*-rearranged and non-*TCF3*-rearranged group.

The pre-BCR complex of rearranged *IGH* and surrogate light chain is known to activate the downstream effectors ZAP70, SYK, LYN and SLP65 (BLNK). The linker protein SLP65 activates BTK which in turn activates PLCg2 and PI3K-p110δ. This cascade results in MEK-ERK-mediated proliferation of pre-B-II large cycling cells and in activation of the transcription factor IRF4. IRF4 negatively regulates pre-BCR signaling and induces the rearrangement of the immunoglobulin light chain in pre-B-II small resting cells (**Supplementary Figure S1**).[11,28,29] Protein profiling of pre-BCR pathway components showed that these proteins were significantly higher expressed in 19 *TCF3*-rearranged compared to 113 non-*TCF3*-rearranged cases, i.e. ZAP70 1.3-fold (p<0.001), SLP65 3.4-fold (p=0.002), BTK 1.5-fold (p<0.001), PI3K-p110δ 1.2-fold (p=0.02) and IRF4 5.2-fold (p<0.001) (**Figure 3**). In addition, the expression of the *TCF3*-target gene EBF1 was 2.7-fold increased in *TCF3*-rearranged BCP-ALL cases (p<0.001). The variation in expression of these proteins in different (cytogenetic) subtypes of BCP-ALL is shown in **Supplementary Figure S2**. *ETV6-RUNX1*- or *BCR-ABL1*-translocated cases and cases belonging to the newly identified *BCR-ABL1-like* subtype did not differentially express these

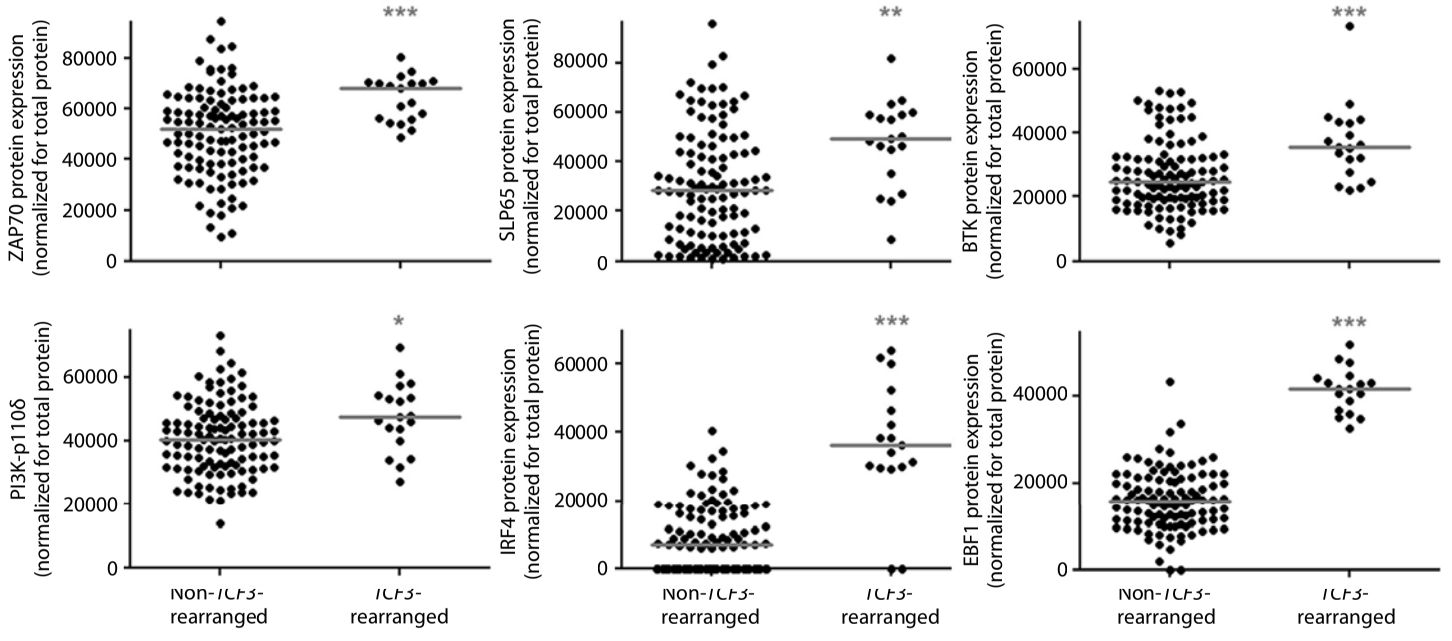

Figure 3 | Expression level of proteins involved in pre-BCR signaling in 19 *TCF3*-rearranged and 113 non-*TCF3*-rearranged BCP-ALL samples. Expression levels were measured by reverse phase protein arrays and levels were normalized for total protein levels in each cell lysate. Red line indicates the median expression level. See also **Supplemental Figure S1** for analysis by known (cytogenetic) subtypes of BCP-ALL.

***: p<0.001; **: p<0.01; *: p<0.05; Mann-Whitney-U.

proteins compared to non-translocate and non-*BCR-ABL1-like* BCP-ALL cases. Hyperdiploid cases expressed more SLP65 (1.8-fold, p<0.001) and BTK (1.3-fold, p<0.001) than non-hyperdiploid cases. These genes are located on chromosome 10 and X, respectively, representing chromosomes which are often gained in hyperdiploid BCP-ALL[30] and therefore this elevated expression level may reflect a gene-dosage effect.

Growth inhibition by ibrutinib

The proliferation rate of the *TCF3*-rearanged cell line MHH-CALL3 decreased upon exposure to increasing concentrations of the BTK inhibitor ibrutinib (**Figure 4A**). The growth inhibitory concentration (GI50) for ibrutinib in MHH-CALL3 was 43.5 μM whereas no GI50 was observed in the other *TCF3*-rearranged cell line 697. Exposure to the highest concentration of ibrutinib also did not result in a decreased proliferation rate of the non-*TCF3*-rearranged cell lines Nalm6 and MHH-CALL4 (**Figure 4A**). Furthermore, the level of phosphorylated ERK1/2, which is an important downstream protein of BTK involved in cell survival, decreased upon treatment with ibrutinib in MHH-CALL3. Importantly, this phosphorylation level of ERK did not decrease in the non-*TCF3*-rearranged cell lines Nalm6 and MHH-CALL4 after exposure to ibrutinib (**Supplementary Figure S3**).

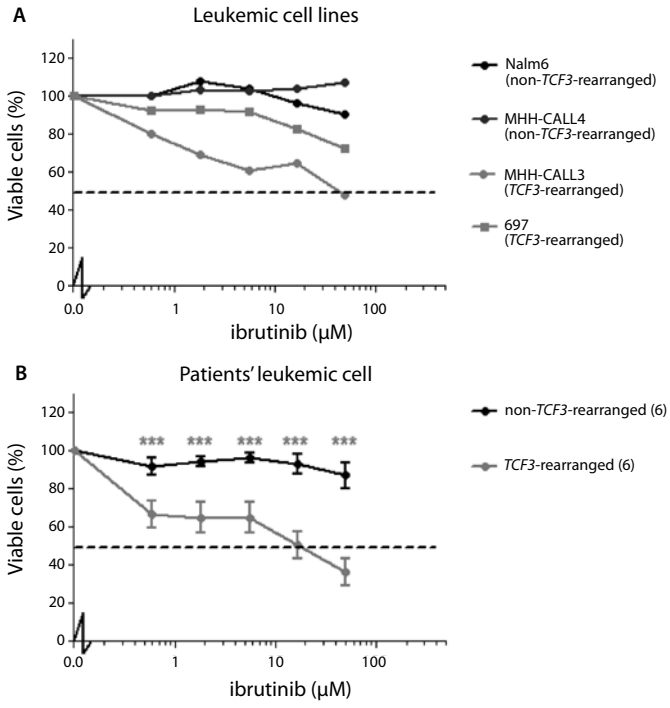

Figure 4 | Sensitivity of leukemic cells to the BTK-inhibitor ibrutinib. A) Leukemic cell lines. **B**) Patients' leukemic cells. Experiments were performed in duplicate. Dashed line indicates concentration lethal to 50% of cells. Bars indicate 95% confidence interval.

***: p<0.001; Mann-Whitney-U

Ibrutinib was also tested in six *TCF3*-rearranged and six non-rearranged leukemic cell samples taken at initial diagnosis of BCP-ALL. The six non-*TCF3*-rearranged samples comprised two hyperdiploid, two *ETV6-RUNX1*-translocated, one *BCR-ABL1-like* and one *BCR-ABL1*-positive case. Ibrutinib significantly reduced cell viability of *TCF3*-rearranged cases at all tested drug concentrations compared to non-rearranged cases ($p<0.001$). The concentration of ibrutinib lethal to 50% of the primary leukemic cells (LC50) was 16.7 μM for *TCF3*-rearranged cases whereas virtually no cell death was induced up to 50.0 μM of ibrutinib in leukemic samples of non-*TCF3*-rearranged patients ($p<0.001$) (**Figure 4B**, **Supplementary Table S3**).

DISCUSSION

TCF3-rearranged ALL is characterized by an immature Cylgμ-positive precursor B-cell immunophenotype.[6,9] This Cylgμ-positive feature is a consequence of an in-frame rearrangement of VDJ genes of the *IGH* locus. In normal B-cell development Cylgμ and the surrogate light chain (composed of VpreB1/CD179a and lambda-like/CD179b) together form a functional pre-BCR complex.[12,13] Activation of this complex triggers the temporary proliferation of pre-B-II large cells in the bone marrow followed by further processing of the *IGK* and *IGL* light chain loci in pre-B-II small resting cells.[10,11,31]

In the present study we observed that the vast majority (86.4%) of *TCF3*-rearranged cases have functional *IGH* rearrangements without further rearrangements of the immunoglobulin light chains *IGK* and *IGL*. In agreement with an early arrest in pre-B cell development, the frequency of Cylgμ-positivity was high in *TCF3*-rearranged cases confirming observations made by others.[6,9,32] Furthermore, we observed that *TCF3*-rearranged BCP-ALL cases constitutively express higher protein levels of the pre-BCR pathway components ZAP70, SLP65, BTK, PI3K-p110δ, IRF4 as well as the *TCF3*-target gene EBF1 known for its role as B-cell transcription factor. Remarkably, in normal B-cell development IRF4 triggers the rearrangement of the immunoglobulin light chain genes *IGK* and *IGL*[28] whereas we here show that *TCF3*-rearranged BCP-ALL cases generally do not rearrange *IGK* and *IGL*. Inhibition of *TCF3* is essential for the successful production of high-affinity immunoglobulins, a process that is mediated via a negative feedback loop of the mature B-cell receptor.[33] Altogether, our data indicate that *TCF3*-rearranged BCP-ALL is arrested at an early stage, resembling pre-B-II-large cells. The selective sensitivity of *TCF3*-rearranged compared to non-*TCF3* rearranged BCP-ALL cases for the BTK-inhibitor ibrutinib -without need for external stimuli – suggests that the pre-BCR pathway is constitutively activated and facilitates proliferation at the expense of immunoglobulin maturation and hence B-cell differentiation. This selective effect is further exemplified by the growth inhibitory effect of ibrutinib on the *TCF3*-rearranged cell line MHH-CALL3. In addition exposure to ibrutinib results in a dephosphorylation of the downstream ERK in specifically MHH-CALL3. This effect is not observed in the non-*TCF3*-rearranged cell line Nalm6 and importantly also not in the *TCF3*-rearranged cell line 697. This can be explained by the fact that both MHH-CALL3 and Nalm6 are characterized by a high BTK activation, whereas 697 has a low level of BTK activation.[34]

These new biological findings warrant clinical studies with a BTK-inhibitor in *TCF3*-rearranged BCP-ALL with high BTK expression. Other biological studies indicated that the BTK-inhibitor ibrutinib effectively affected the viability of chronic lymphoblastic leukemia and multiple myeloma cells and interfered with the downstream phosphorylation level of ERK1/2.[20,22,23] Ibrutinib induced an objective response rate in 60-75% of patients with relapsed or refractory B-cell malignancies.[21,35] A phase II study with ibrutinib in patients with CLL indicates that monotherapy with ibrutinib might be effective even in high-risk cases with limited adverse effects.[35] These early clinical trial observations together with the findings presented in this study strongly implies that introduction of a BTK-inhibitor may be beneficial in the treatment of *TCF3*-rearranged BCP-ALL with high BTK expression.

In conclusion, *TCF3*-rearrangement BCP-ALL is characterized by IGH rearrangements in the absence of light chain rearrangements, and high expression of cytoplasmic Igμ, and pre-BCR pathway components, which was not found for non-*TCF3* rearranged BCP-ALL. Targeting this pre-BCR pathway by the BTK-inhibitor ibrutinib effectively and specifically killed *TCF3*-rearranged BCP-ALL cells with high BTK expression and reduced the phosphorylation level of downstream ERK1/2. The present study therefore provides a strong rationale to design clinical trials with agents interfering with pre-BCR signaling (e.g. ibrutinib) in children with *TCF3*-rearranged BCP-ALL.

CONTRIBUTORS

AVDV, VHJVD, RP, and MLDB designed the study and experiments. AVDV and MEW processed patient's samples and performed inhibitor assays. VHJVDV, and PGH determined immunoglobulin rearrangement pattern. AVDV, MEW, MLDB and EFP contributed to reverse-phase protein array experiments. BHB performed *TCF3* FISH. GE and MAH provided patient's samples. Data were analyzed by AVDV, VHJVDV and MLDB. Manuscript was written and approved by all authors.
All authors declare to have no conflict of interest.

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CHAPTER 6

General discussion and conclusions

DISCUSSION

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy during childhood.[1] The outcome of childhood B-cell precursor ALL (BCP-ALL) improved enormously during the past decades. The actual survival rate is approximately 80%,[2] owing to the improvement of supportive care and the introduction of relapse risk adapted therapy.[3] Patients with a high relapse risk receive more intensive therapy than patients with a low relapse risk. However, ¾ of relapses of DCOG ALL-10 treated patients occur in patients that were initially not considered as high risk for relapse.[4-6] This demonstrates the need for new stratification markers. In addition, these new stratification markers might also be used for the identification of new targets for therapy. The currently used drugs were introduced in the sixties of the past century,[5-7] do not have a specific target and lead to many side effects and long-term co-morbidity.[8,9] Introduction of targeted therapy may lead to a dosage reduction of current therapy, resulting in less toxicity and long term co-morbidity on one hand and to more effective anti-leukemic therapy on the other hand.

Gene expression profile as stratification marker

In solid tumors, such as lung cancer and breast cancer, gene-expression signatures were demonstrated as an accurate tool for relapse prediction.[10-13] These prediction signatures were first tested in a training cohort and validated in independent validation cohorts. In breast cancer, the 70-probesets containing classifier demonstrated the accuracy of these gene-expression classifiers.[13-17] This poor prognostic signature was independent of conventional prognostic features and was related to a 5-fold increased risk for an event.[17] The use of this signature may result in a reduction of over treatment with chemotherapy in women with breast cancer[15,17] and an improved outcome.[16] These promising findings resulted in FDA approval of this classifier and implementation in treatment protocols.

The prognostic relevance of gene-expression profiling was also tested in hematological malignancies. In pediatric and adult acute myeloid leukemia (AML) gene expression studies revealed a classifier that accurately predicted recurrent prognostic cytogenetic groups[18-20] and identified the good prognostic double mutant C/EBPα group.[20] Recently, a 24-probesets gene-expression classifier was identified and tested in 6 independent sets of patients. Patients identified by this gene-expression signature were at a 1.7-fold risk for an event.[21]

Various research groups composed gene-expression classifiers that accurately predicted prognostic cytogenetic subtypes of ALL in children.[22-24] Two research groups independently identified cases that co-clustered together with poor risk *BCR-ABL1*-positive BCP-ALL, lacking the *BCR-ABL1*-translocation and other known genetic aberrations.[22,25-27] These cases were previously cytogenetically unclassified (B-other).[22,27] We found that this so-called *BCR-ABL1-like* group was related to an unfavorable outcome with a 5-years cumulative incidence of relapse (CIR) of 31% and compared to 11% 5-years CIR in non *BCR-ABL1-like* cases, which implies a hazard ratio of 3.7 for a leukemia related event.27 The *BCR-ABL1-like* group comprises between 15 and 20% of all childhood BCP-ALL cases,[22,25,26] whereas the frequency of previously identified poor risk genetic features, such as the MLL-rearrangements and *BCR-ABL1*-translocations, is 5% each.[3] The clinical importance of a

BCR-ABL1-like signature is furthermore demonstrated by the fact that this signature is present in 50% of relapsed BCP-ALL cases.[27]

The American Children's Oncology Group (COG) identified *BCR-ABL1-like* cases by a recognition of outliers by sampling ends (ROSE) clustering approach (so-called R8 cluster),[25,28] or a prediction analysis for microarrays (PAM) classifier model.[28,29] The probesets for this approach were dynamic and varied between study cohorts[22] in contrast to the technique described by our group.[25,26,28,29] We composed a fixed 110-probesets classifier that was conducted in a double-loop cross validation method and then again tested in independent cohorts of childhood ALL cases and that was demonstrated to be reproducible.[22] In this thesis, we describe the use of this 110-probesets classifier on newly arrayed samples to identify poor prognostic *BCR-ABL1-like* cases.[22,27] The presence of the *BCR-ABL1-like* signature appeared to be related to an unfavorable outcome in various treatment protocols and risk groups, independent from conventional stratification markers, and minimal residual disease levels.[27]

Treatment of *BCR-ABL1-like* BCP-ALL

Theoretically, the prognosis of *BCR-ABL1-like* cases could be improved by intensification of current therapy, although there are counter facts. In vitro data demonstrated that leukemic cells from *BCR-ABL1-like* cases are more resistant to L-Asparaginase.[22] Current treatment protocols already use intensive administration of L-Asparaginase and therefore further intensification with L-Asparaginase will not be effective.[30] Secondly, *BCR-ABL1-like* leukemic cells were also in vitro more resistant to daunorubicin.[22] Intensification of therapy by increasing the dosages of antracyclines is not a therapeutic option, because of expected long-term effects on cardiac function.[31,32] Concluding, intensification with conventional cytostatic drugs is not an option for *BCR-ABL1-like* cases. Therefore current research is aimed to identify new targets for drugs.

To discover potential new drugable targets, fifteen *BCR-ABL1-like* samples were analyzed by next generation sequencing by the COG/TARGET-group. In these cases, a gene rearrangement (involving *ABL1*, *JAK2*, *PDGFRB*, *CRLF2* or *EPOR*) or a sequence mutation activating tyrosine kinase and cytokine receptor signaling was found and suggested that *BCR-ABL1-like* cases are characterized by activating genetic alterations in tyrosine kinases.[28] Some of these genetic aberrations were also identified in non-*BCR-ABL1-like* ALL, like the *IGH@-CRLF2* and *NUP214-ABL1* translocations,[28,33,34] *IL7R*[28,35] and *FLT3*[28,36] sequence mutations. The tyrosine kinase inhibitor imatinib was used as a first candidate for targeted therapy in individual cases with *BCR-ABL1-like* BCP-ALL. Two *BCR-ABL1-like* cases refractory to induction therapy harbored an *EBF1-PDGFRB* translocation and were both successfully treated with this imatinib on top of conventional chemotherapy.[37,38] We observed no elevated phosphorylated ABL1 levels nor elevation of potential target proteins for imatinib (e.g. PDGFRB and SRC) in *BCR-ABL1-like* BCP-ALL. This suggests that *BCR-ABL1-like* cases as a group should not be treated with imatinib, but this therapy should only be used as personalized treatment based on further evidence for the presence of a target for imatinib. Our group also investigated the micro-RNA signature of *BCR-ABL1-like* cases. These cases showed heterogeneity in micro-RNA expression pattern without clear evidence for protein-coding genes being affected by the micro-RNA's.[39] Therefore further biological research for drugable targets among *BCR-ABL1-like* cases is warranted. The design and acceptance

of new drugs can take decades. For example, the period between the discovery of the *BCR-ABL1*-translocation in adult chronic myeloid leukemia (CML) and the approval of imatinib was 40 years.[40-42] Although nowadays scientific standards will lead to faster introduction of such medicines, targeted therapy for all *BCR-ABL1-like* cases is not available on short tem. But there are registered TKI's that might be useful in the future. Sporadic *BCR-ABL1-like* cases with an *EBF1-PDGFRB*, or *NUP214-ABL1* translocation may be treated with dasatinib or imatinib, whereas *BCR-JAK2* translocated cases may be treated with the JAK2-inhibitor ruxolitinib.[28] Statistical testing in a clinical trial for the efficiency of such drugs in *BCR-ABL1-like* BCP-ALL would demand an international collaborative study, since the frequency of these translocations separately is below 10% of all *BCR-ABL1-like* cases.[28] An alternative approach is to use personalized therapy for patients who have one of these aberrations and do not respond sufficiently to standard chemotherapy. A different option would be elongation of therapy for all *BCR-ABL1-like* cases.

Genomic deletions in *IKZF1* as stratification marker

The frequency of *IKZF1* deletions is approximately 15% in *BCR-ABL1*-negative BCP-ALL[22,26,27,43-45] and is remarkably lower than in *BCR-ABL1*-positive BCP-ALL (approximately 70%).[22,34,46,47] Various groups demonstrated that *BCR-ABL1*-negative BCP-ALL patients with such a deletion have a poor prognosis.[26,27,43-45] A high frequency (>40%) of *IKZF1* deletions was described in *BCR-ABL1-like* patients.[22,25,26] Hypothetically the poor outcome of *BCR-ABL1-like* and *BCR-ABL1*-positive BCP-ALL may be confounded by underlying *IKZF1* deletions. In this thesis we demonstrate in an interaction model that the prognosis of *BCR-ABL1-like* cases is independent of the *IKZF1* status. In contrast, *IKZF1* status predicts the prognosis in hyperdiploid BCP-ALL cases and B-other ALL cases. These findings indicate that both *BCR-ABL1-like* signature and *IKZF1* deletion are important new prognostic features in childhood BCP-ALL. This is further underscored by the fact that 63% of the children who relapsed during or after treatment had an *IKZF1* deletion or a *BCR-ABL1-like* signature.

We found that approximately 70% of childhood *BCR-ABL1*-positive BCP-ALL cases has a deletion in *IKZF1*,[27,34] consistent with other studies.[22,46,47] A study with a monozygotic twin with prenatal *BCR-ABL1*-positive BCP-ALL suggested that an *IKZF1* deletion resulted in a more aggressive leukemia.[48] The twin-brother without the *IKZF1* deletion survived after conventional therapy, whereas his twin-brother with the *IKZF1* deletion had an early relapse.[48] Secondly, an in vitro study demonstrated that the proliferation of *BCR-ABL1*-positive CD34+ hematopoietic cells increased after the introduction of a deleted variant of *IKZF1*.[49] Finally, the survival time for *BCR-ABL1*-positive BCP-ALL adult cases with an *IKZF1* deletion was three times shorter than for wild-type cases.[50] Our international collaborative study demonstrates that before the structural implementation of imatinib in treatment protocols, *IKZF1* deletions were strongly related to an unfavorable outcome in childhood *BCR-ABL1*-positive BCP-ALL. After the introduction of imatinib, *IKZF1* deletions predicted an unfavorable outcome only in good-risk stratified cases.

Current studies combine all *IKZF1* deletion variants into one group to study the prognostic value.[22,26,27,34,43-45,50] Biological studies demonstrate that especially the variant that lacks the DNA-binding part due to a deletion in exon 4 to 7, isoform 6, is associated with childhood ALL.[51,52] Introduction of this variant in *BCR-ABL1*-positive BCP-ALL cells, results in an increase of cell

proliferation.[49] We found that approximately 25% of al deleted BCP-ALL patients had such a deletion that results into isoform 6.[27] The remaining 75% of deletions were not tested in functional biological studies. *IKZF1* deletions in exon 1 to 8 resulted in a different gene-expression profile compared with *IKZF1* deletions in exon 4 to 7.[53] This suggests a different biology between different variants and therefore a possible difference in clinical outcome. However, our further analysis demonstrated evidence for an unfavorable prognosis of all *IKZF1* deletion variants. This poor prognosis was independent of treatment protocol, stratification arm, cytogenetic group, age, and WBC count. Further studies about the causes and consequences of types of deletions are still needed. Since *IKZF1* deletions are not related to an altered level of Ikaros expression,[54] it may be hypothesized that the poor prognosis is due to an altered function of Ikaros or that *IKZF1* deletions are a reflection of general genomic instability such as recently shown for *ETV6-RUNX1* positive ALL.[55]

Treatment of *IKZF1*-deleted BCP-ALL

Approximately 90% of *IKZF1*-deleted cases are enrolled in non-high risk arms of current treatment protocols,[27] demonstrating that their unfavorable prognosis is independent of conventional features and MRD-level. *IKZF1*-deleted cases had an unfavorable 5-years CIR (~25%) compared with wild-type cases (~8%). These observations suggest that *IKZF1* deletions can be implemented as a high risk stratification feature. However, treatment of cases in high risk arms will additionally result in an increase of toxic side effects, demonstrated by the observation that 5−10% of high risk treated patients with intensive chemotherapy and/or hematopoietic stem cell transplantation (HSCT) died of toxicity.[56] Therefore, it was decided not to use this high risk protocol but to elongate maintenance therapy with one year for *IKZF1*-deleted cases in the current DCOG ALL-11 protocol (**Figure 1**).

Since the implementation of imatinib in treatment protocols,[57,58] good-risk *BCR-ABL1*-positive patients with *IKZF1* wild-type have a remarkable good prognosis with a 4-years event-free survival of 75%. Because approximately 70% of the European *BCR-ABL1*-positive BCP-ALL cases receives a hematopoietic stem cell transplantation (HSCT),[34,57] it may be suggested to reduce the intensity of therapy for this group by omitting HSCT's. This would results in less toxicity. Furthermore the interaction between *IKZF1* status and MRD-levels should be investigated. It was demonstrated that low MRD levels are related to a favorable outcome in imatinib-treated *BCR-ABL1*-positive BCP-ALL,[59] suggesting a correlation between low MRD-levels and wild-type *IKZF1*. The treatment intensity for *BCR-ABL1*-positive BCP-ALL cases is high. Therefore in *IKZF1* deleted *BCR-ABL1*-positive cases with an unfavorable prognosis, a search for alternative therapy is warranted, since further intensification of therapy is no feasible option.

Promising recent studies demonstrate that both haplo-insufficient and dominant-negative *IKZF1* deletions result in an overexpression of THY1, which is a target of Ikaros repression.[60] Synthetical lethal screening focused attention on retinoid acid to restore the normal phenotype. This drug was successfully validated in a *BCR-ABL1*-positive murine model and improved the sensitivity of leukemic cells to imatinib.[60] This effect should be evaluated in independent studies. Furthermore, the chemo sensitizing effect of retinoid acid for conventional therapy should be investigated in *BCR-ABL1*-negative BCP-ALL with an *IKZF1* deletion.

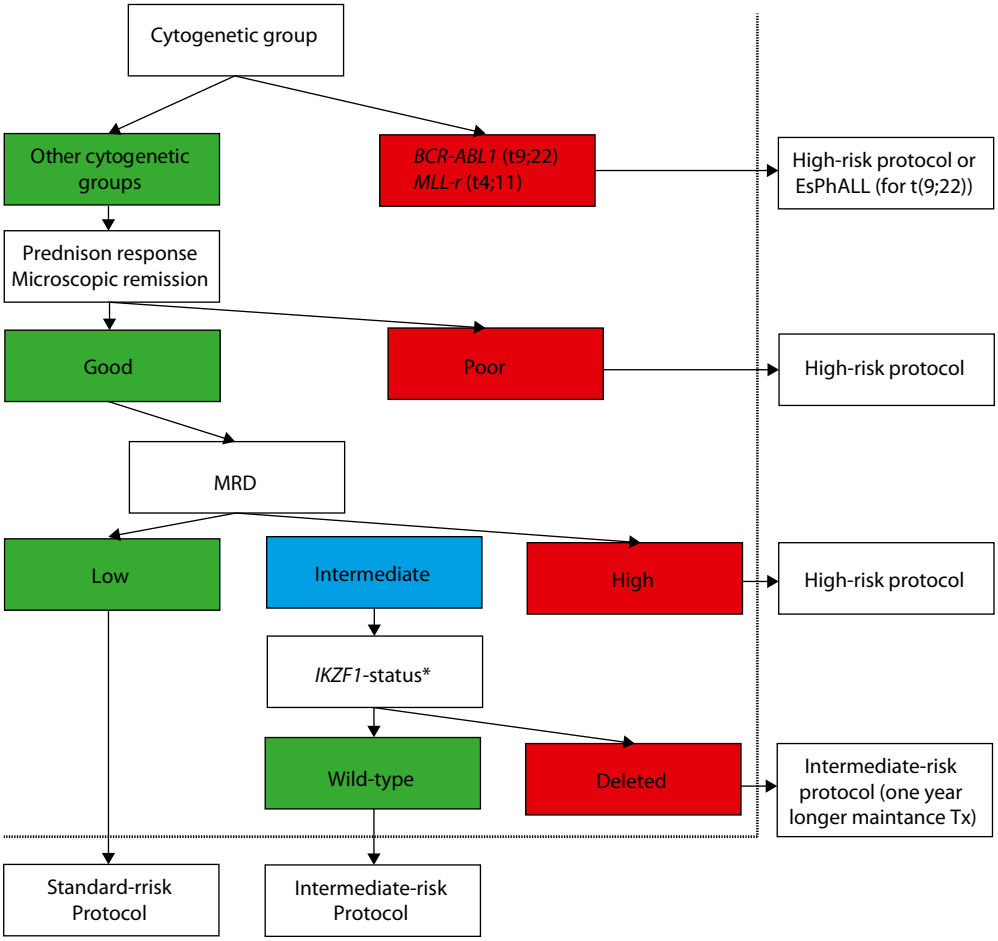

Figure 1 | Currently used stratification markers for childhood ALL cases in DCOG ALL-11 treatment protocol. Red boxes indicate unfavorable risk features. Green indicates favorable risk and blue intermediate risk. Microscopic remission is measured 33 days after initial diagnosis. Minimal residual disease levels (MRD) are measured at day 33 and day 79 of therapy. *BCR-ABL1*-positive case are enrolled in the EsPhALL protocol. * *ETV6-RUNX1* positive cases with *IKZF1*-deleted will not receive elongation of therapy.

BCP-ALL cells that predominately expressed *IKZF1* Isoform 6 had a lower expression of AIOLOS and HELIOS when compared with cells that expressed the normal IKAROS variants.[61] These transcription factors form homo- and hetero dimers.[62] Since a recently published study demonstrated that overexpression of AIOLOS resulted in down regulation of IKZF1 and inhibited cell proliferation the Nalm6 BCP-ALL cell line,[63] it may be hypothesized that interference in this IKAROS/AIOLOS/HELIOS balance offers therapeutic options for *IKZF1*-deleted cases.

Targeted therapy in *TCF3*-rearranged BCP-ALL

In this thesis we found that *TCF3*-rearranged cases were characterized by a homogenous expression of cytoplasmic immunoglobulin heavy chain (CyIgμ) and a low incidence of light chain rearrangements relatively to the other cytogenetic groups. Light chain rearrangements occur during normal B-cell development as a consequence of pre-B cell receptor (pre-BCR) stimulation.[64,65] Igμ and the surrogate light chain (SLC) together constitute this pre-BCR.[66] Analysis of protein data reveals a constitutively activated pre-BCR pathway and a relatively high IRF4 expression in *TCF3*-rearranged BCP-ALL cells. Remarkably, during normal B-cell development, IRF4 regulates light chain rearrangements,[67,68] indicating that B-cell development is specifically impaired in *TCF3*-rearranged BCP-ALL. Based on the absence of light chain rearrangements and the expression of pre-BCR signaling related proteins we concluded that *TCF3*-rearranged BCP-ALL is arrested at an early stage in B-cell development, presumably resembling pre-B-II-large cells. These data suggest that this pre-BCR receptor pathway may be used as a target for therapy in *TCF3*-rearranged BCP-ALL. A possible new target in *TCF3*-rearranged BCP-ALL may be IRF4. Lenalidomide resulted in down regulation of IRF4 and a reduction in cell proliferation in multiple myeloma cells.[69] This drug may be tested in *TCF3*-rearranged BCP-ALL cells.

A second candidate target for therapy may be BTK, because it is involved in the pre-BCR pathway[70] and is highly expressed in *TCF3*-rearranged BCP-ALL cells. We therefore tested the BTK-inhibitor ibrutinib in BCP-ALL cells. *TCF3*-rearranged BCP-ALL cells were specifically sensitive for ibrutinib. Before clinical trials are started with ibrutinib for *TCF3*-rearranged BCP-ALL, the role of the pre-BCR should be validated by the inhibition of other pre-BCR related proteins such as PI3Kinase and SYK. The specificity and and-leukemic effect of these drugs could be further studied in healthy mice, mice with non-*TCF3* and *TCF3*-rearranged BCP-ALL. Future clinical studies may be done with ibrutinib, since this drug is safe in adults with B-cell malignancies in adults and now FDA approved.[71-74]

CONCLUSIONS

The need for new prognostic stratification markers is demonstrated by the fact that approximately ¾ of relapsed BCP-ALL cases were not recognized as high risk cases at initial diagnosis. This thesis provides evidence for the prognostic power of biological aberrations by the use of relatively new diagnostic techniques. The *BCR-ABL1-like* signature allows identifying poor risk cases by the use of gene-expression arrays. In addition, the use of the highly reproducible Multiplex Ligation Probe-dependent Assays[75] allows the identification of unfavorable prognostic *IKZF1* deletions in childhood ALL. Importantly, this thesis demonstrates that both *IKZF1* deletions and *BCR-ABL1-like* signature have an independent prognostic value and occur in 2/3 of relapsed BCP-ALL patients and therefore may be used as a relapse risk stratification marker. Based on these findings it was decided to elongate maintenance therapy for intermediate risk BCP-ALL patients with an *IKZF1* deletion to three years in the DCOG ALL-11 protocol (**Figure 1**). The effect of this lengthening should be evaluated during this trial. Furthermore, our findings will result in a debate in the European Study for Philadelphia-positive ALL (EsPhALL) group whether a potential toxic HSCT should be omitted for good-risk *BCR-*

ABL1 -positive BCP-ALL patients with *IKZF1* wild-type. For *BCR-ABL1-like* cases it should be decided to treat cases with a drugable target with personalized medicine or to uniformly elongate therapy for all *BCR-ABL1-like* cases in parallel to *IKZF1*-deleted cases.

The role of *BCR-ABL1-like* signature and *IKZF1* deletions in the pathobiology of BCP-ALL is yet largely unknown and is focus for current research. Next generation sequencing may identify common alterations in these groups and together with protein expression data this could result in the discovery of new targets for therapy, such as identified by us in *TCF3*-rearranged ALL. Introduction of drugs against these targets could further improve the survival of the children with ALL and therefore should be the higher goal of research.

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CHAPTER 7

Summary

Nederlandse samenvatting

SUMMARY

Despite the improvement in prognosis of childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) due to the introduction of relapse risk adapted therapy, 75% of relapses still occurs in cases that were initially not considered at high risk for treatment failure. Furthermore, current conventional therapy results in toxicity and mortality due to non-specifically acting drugs. These observations highlight the need for new stratification options and novel alternative therapy. New stratification markers may be identified by genomic techniques. Biological research of old and new genetic subtypes may result in the identification of new targets for therapy. Application of targeted drugs may improve the outcome by a reduction in relapses, but also by a reduction of conventional cytostatic drugs, which will reduce short- and long-term side effects. In this thesis we specifically focused on the prognostic value of gene-expression signatures, genomic alteration in B-cell development genes, and the identification of targeted therapy based on biological characteristics of leukemic cells.

In **chapter 2** we determined the co-occurrence and independent prognostic relevance of three recently identified prognostic features: *BCR-ABL1-like* gene signature, deletions in *IKZF1* and high *CRLF2* mRNA expression (*CRLF2*-high). These new features were recently discovered in different study cohorts using different techniques. However, an overlap may exist in the presence of two or more of the newly identified features, which may hamper to demonstrate the independent prognostic value of each new feature. We used a cohort that represented 1128 children with *BCR-ABL1*-negative, non *MLL*-rearranged ALL from four different treatment protocols. The *BCR-ABL1-like* signature was specifically identified in cytogenetically unclassified B-other group and occurred in 16% of BCP-ALL cases. 40% of the *BCR-ABL1-like* cases harbored an *IKZF1* deletion, whereas the whole BCP-ALL group had an *IKZF1* deletion in only 17% of the cases. Cumulative incidence of relapse (CIR) curves and a multivariate Cox regression model demonstrated that both *BCR-ABL1-like* signature and *IKZF1* deletions were independently prognostic in childhood BCP-ALL. In contrast, *CRLF2*-high was not correlated to an unfavorable outcome. In children, altogether, 63% of all relapses occurred in cases with *BCR-ABL1-like* signature and/or *IKZF1* deletion. Based on these findings we concluded that the *BCR-ABL1-like* signature and an *IKZF1* deletion, but not *CRLF2*-high expression, may serve as new prognostic markers in childhood ALL. In this chapter we suggested to implement these features for relapse risk stratification and to focus biological studies on these groups to identify potential drugable targets.

The unfavorable prognostic *BCR-ABL1*-positive BCP-ALL group is characterized by *IKZF1* deletions in approximately 70% of the cases. In **chapter 3** we aimed to clarify the prognostic value of *IKZF1* deletions in *BCR-ABL1*-positive BCP-ALL before and after the introduction of the tyrosine kinase inhibitor (TKI) imatinib. Patients after the introduction of imatinib were enrolled in good-risk or poor-risk stratification arms based on their early clinical response after 7 days of prednisolone administration. The international study cohort consisted of 192 cases. In the pre-TKI cohort, cases with *IKZF1*-deleted had an unfavorable outcome when compared with wild-type cases. After the introduction of imatinib, *IKZF1* deletions had poor prognostic value only in good-risk stratified

patients. Strikingly, the prognosis for *IKZF1* wild-type good-risk patients was good and comparable to *BCR-ABL1*-negative BCP-ALL. Since the intensity of treatment protocols for *BCR-ABL1*-positive BCP-ALL is high, further intensification for *IKZF1*-deleted cases is not an option. Therefore, we concluded that for this group alternative therapy should be designed. For good-risk *BCR-ABL1*-positive BCP-ALL with *IKZF1* wild-type we suggested a reduction in therapy by avoiding the toxic hematopoietic stem cell transplantations.

Different types of genomic deletions in *IKZF1* occur in childhood BCP-ALL varying from exons to the complete gene. Only for deletions in exon 4 to 7 (DEL 4-7) biological studies demonstrate an evident role in leukemogenesis. Also for deletions in exon 1 to 8 (DEL 1-8) there is strong statistical evidence for a poor outcome in BCP-ALL. These two deletions represent ~65% of all cases with *IKZF1* deletions. Since the incidence of all remaining deletion variants is low, studies combine all variants of *IKZF1* deletions to study their prognostic value. The prognosis of each of the rare *IKZF1* deletions is therefore yet unknown. In **chapter 4** we describe an international collaborative study that addresses prognostic value of each of these variants. Each case with a rare deletion was matched to three wild-type controls. Matching was based on date of diagnosis, treatment protocol, risk stratification arm, cytogenetic group, WBC, and age. Each of these rare deletion variants appeared to be predictive for unfavorable outcome based on CIR analysis and a matched pair event-free survival (EFS). Based on these results, we recommended implementing all variants of *IKZF1* deletions as a stratification factor in the treatment of childhood BCP-ALL.

An option for targeted drugs is described in **chapter 5**. *TCF3*-rearranged BCP-ALL cells are characterized by cytoplasmic IgH (CyIgμ) expression. This suggests a role for the pre-B-cell receptor (pre-BCR) in this type of childhood BCP-ALL. We discovered that *TCF3*-rearranged leukemia is characterized by an immature immunoglobulin rearrangement pattern with a completely rearranged immunoglobulin heavy chain, but lacking immunoglobulin light chain rearrangements. Proteins linked to the pre-BCR were higher expressed in *TCF3*-rearranged BCP-ALL compared with other types of BCP-ALL. We furthermore demonstrated that inhibition of this pre-BCR signaling by ibrutinib resulted in cell death in specifically *TCF3*-rearanged BCP-ALL cells. Since ibrutinib is a promising drug in adult B-cell malignancies, these experiments warrant further clinical studies in childhood *TCF3*-rearanged BCP-ALL.

The general discussion and conclusion are described in **chapter** 6. This thesis provides evidence for the poor prognostic power of both *BCR-ABL1-like* signature and *IKZF1* deletions in childhood BCP-ALL by the use of accurate and relatively new molecular techniques. Due to our results, *IKZF1* deletions are now implemented in the DCOG ALL-11 treatment protocol as relapse risk stratification marker. Intermediate risk stratified patients with such a deletion now receive one year longer maintenance therapy. The treatment for *BCR-ABL1-like* cases is not adapted yet. Based on pre-clinical research and case-reports, a small selection of these cases may be treated by personalized medicine in case of a drugable target. A different option may be to elongate therapy in parallel to *IKZF1*-deleted cases. Because of the good outcome of *BCR-ABL1*-positive patients with *IKZF1* wild-type, the role of HSCT for these cases is now on debate. Current research is now focusing on unraveling the biology underlying these two features and will hopefully result in drugs that further improve the outcome of children with ALL.

NEDERLANDSE SAMENVATTING

Ondanks dat de prognose van kinderen met B-cel voorloper acute lymfatische leukemie (BCV-ALL) verbeterd is door de introductie van therapie aangepast aan recidief risico, is 75% van de kinderen die een recidief krijgt initieel niet ingeschat als hoog-risico. Bovendien leidt de huidige therapie tot toxiciteit en mortaliteit als gevolg van niet-leukemie specifieke medicijnen. Deze observaties tonen aan dat er behoefte is aan nieuwe risico stratificatie markers en nieuwe therapie opties. Nieuwe stratificatie markers kunnen mogelijk geïdentificeerd worden door middel van nieuwe genetische technieken. Door middel van moleculair biologisch onderzoek kunnen mogelijkheden voor nieuwe leukemie-specifieke medicijnen geïdentificeerd worden binnen oude maar ook nieuwe genetische groepen in BCV-ALL. Deze leukemie-specifieke medicijnen kunnen de prognose van BCV-ALL verbeteren door recidieven te reduceren, maar ook door de hoeveelheid conventionele niet-leukemie-specifieke medicijnen te verminderen. Dit laatste zal leiden tot minder bijwerkingen op lange en korte termijn. In dit proefschrift hebben we ons gericht op de prognostische waarde van gen-expressie profielen, afwijkingen in B-cel ontwikkelingsgenen en de identificatie van leukemie-specifieke medicijnen gebaseerd op biologische karakteristieken van de leukemie cellen.

In **hoofdstuk 2** beschrijven we de overlap en de onafhankelijk prognostische waarde van recentelijk gevonden prognostische factoren in BCV-ALL: het *BCR-ABL1-like* gen expressie profiel, deleties in *IKZF1* en hoge *CRLF2* mRNA expressie (*CRLF2*-hoog). Deze markers zijn voorheen geïdentificeerd door middel van verschillende technieken en in afzonderlijke behandelgroepen. De data suggereren overlap waardoor de onafhankelijke prognostische waardes van deze markers onbekend is. In onze studie includeerden we uit vier onafhankelijke behandelgroepen 1128 kinderen met BCV-ALL zonder *BCR-ABL1*-translocatie en zonder *MLL*-herschikking. Het *BCR-ABL1-like* gen-expressie profiel werd alleen gevonden in kinderen met BCV-ALL die geen bekende cytogenetische afwijking hadden in hun leukemie cellen. 16% van alle kinderen met BCV-ALL behoort nu tot de *BCR-ABL1-like* groep. Van deze kinderen met het *BCR-ABL1-like* gene expressie profiel had 40% een *IKZF1* deletie, terwijl in de hele groep kinderen met BCV-ALL deze *IKZF1* deletie gevonden werd in 17% van de gevallen. Cumulatieve incidentie van recidieven (CIR) en multivariate Cox regressie modellen toonden aan dat zowel *BCR-ABL1-like* gen-expressie profiel als *IKZF1* deleties onafhankelijk van elkaar slecht prognostische factoren zijn voor kinderen met BCV-ALL. *CRLF2*-hoog bleek niet gerelateerd te zijn met een slechte prognose. We stelden vast dat 62.9% van de kinderen met recidief BCV-ALL, bij initiële diagnose of een *BCR-ABL1-like* gen-expressie of een deletie in *IKZF1* had in zijn leukemie cellen. Op basis van deze gegevens concludeerden we dat *BCR-ABL1-like* gen-expressie profiel en deleties in *IKZF1*, maar niet *CRLF2*-hoog, gebruikt kunnen worden als prognostische factoren bij BCV-ALL. Daarom stelden wij voor om beide factoren te implementeren in behandelprotocollen en binnen deze groepen op zoek te gaan naar de mogelijkheden voor leukemie-specifieke medicijnen.

De historisch gezien slecht prognostische *BCR-ABL1*-positieve BCV-ALL groep wordt gekarakteriseerd door een *IKZF1* deletie in 70% van de gevallen. In **hoofdstuk 3** hadden wij als doel de prognostische waarde van *IKZF1* deleties te beschrijven in *BCR-ABL1*-positieve BCV-ALL voor de introductie van de tyrosine kinase inhibitor (TKI) imatinib in de behandelprotocollen en na de

introductie hiervan. Deze internationale studie bevatte 192 kinderen. Voor de introductie van TKI hadden alle kinderen met een *IKZF1* deletie in hun leukemie cellen een slechte prognose vergeleken met gevallen met een intact *IKZF1*. Na de introductie van TKI werd er alleen een discriminerende slechte prognose voor kinderen met *IKZF1* deleties gevonden in gunstig-risico gestratificeerde *BCR-ABL1*-positieve gevallen. Opvallend was de goede prognose van gunstig-risico ingeschatte BCV-ALL gevallen met een intact *IKZF1*. Deze prognose was vergelijkbaar met *BCR-ABL1*-negatieve BCV-ALL. Omdat de intensiteit in huidige behandelprotocollen voor *BCR-ABL1*-positieve BCV-ALL hoog is, is verdere intensivering van therapie geen optie. Daarom concludeerden we dat voor *BCR-ABL1*-positieve BCV-ALL met een *IKZF1* deletie alternatieve therapie gevonden moet worden. Voor gunstig-risico *BCR-ABL1*-positieve BCV-ALL met een in tact *IKZF1* suggereerden we een reductie in therapie door af te zien van hematopoeitische stam cel transplantaties.

De deleties in *IKZF1* die gevonden zijn, variëren van enkele exonen tot het gehele gen. Alleen voor deleties van exon 4 tot en met 7 is in biologische studie de rol in leukemie beschreven. Voor deleties van exon 1 tot en met 8 is epidemiologisch bewezen als slecht prognostische in BCV-ALL. 65% van de *IKZF1* deleties behoort tot een van deze twee varianten. Omdat deze incidentie van alle andere afzonderlijke varianten laag is, worden alle varianten in een groep geanalyseerd. Daarom is de prognose van iedere variant afzonderlijk onbekend. In **hoofdstuk 4** beschrijven we een internationale studie waarin de prognostische waarde van deze varianten afzonderlijk geëvalueerd wordt. Voor iedere case met een *IKZF1* deletie variant werden 3 controles met een in tact *IKZF1* geselecteerd (1:3). Deze selectie werd gedaan op basis van datum van diagnose, behandelprotocol, risico stratificatie arm, cytogenetische groep, leukocyten getal bij diagnose en leeftijd. Alle varianten waren voorspellend voor slechte uitkomst. Gebaseerd op deze bevindingen, adviseerden we implementatie van alle soorten van *IKZF1* deleties in de behandelprotocollen voor kinderen met BCV-ALL.

Een mogelijkheid voor leukemie specifieke medicatie wordt beschreven in **hoofdstuk 5**. *TCF3*-herschikte BCV-ALL cellen worden gekarakteriseerd door cytoplasmatisch IgH (Cylgμ) expressie. Dit suggereert een rol voor de pre-B-cel (pre-BCR) in dit type leukemie. Wij ontdekten dat *TCF3*-herschikte leukemie wordt gekarakteriseerd door een immatuur immunoglobuline herschikkingspatroon met een compleet herschikte zware keten, maar zonder lichte keten herschikkingen. Ook de pre-BCR geassocieerde eiwitten kwamen hoger tot expressie in *TCF3*-herschikte BCV-ALL vergeleken met andere types van BCV-ALL. Bovendien demonstreerden we dat inhibitie van de pre-BCR pathway met ibrutinib resulteert in cel dood in specifiek *TCF3*-herschikte BCV-ALL cellen. Omdat ibrutinib nu al toepasbaar is in andere B-cel maligniteiten, geeft ons onderzoek aanleiding tot klinisch testen van deze drug in *TCF3*-herschikte BCV-ALL.

De discussie en conclusies van dit proefschrift zijn beschreven in **hoofdstuk 6**. Dit proefschrift toont dat zowel *BCR-ABL1-like* gen-expressie profiel als *IKZF1* deleties onafhankelijke prognostische factoren zijn in BCV-ALL. De factoren zijn goed te testen in een diagnostische setting met relatief nieuwe moleculaire biologische technieken. Onder andere op basis van de resultaten beschreven in dit proefschrift zijn *IKZF1* deleties geïmplementeerd in het nieuwe DCOG ALL-11 behandelprotocol als recidief risico stratificatiemarker. De therapie voor patiënten met een *BCR-ABL1-like* profiel is nog niet aangepast. Gebaseerd op pre-klinische studies kan een kleine selectie hiervan behandeld

worden met reeds beschikbare leukemie-specifiek medicijnen. Een andere optie is om *BCR-ABL1-like* patiënten langer te behandelen zoals vanaf nu voor patiënten met een *IKZF1* deletie geldt. De resultaten in de proefschrift hebben verder geleid tot een discussie over het nut van een hematopoeitische stamcel transplantatie voor kinderen met *BCR-ABL1*-positieve BCV-ALL en een in tact *IKZF1*, omdat we hebben laten zien dat deze groep een hele goede prognose heeft. Het huidige onderzoek richt zich op de biologie die onderliggend is aan *IKZF1* deleties en *BCR-ABL1-like* gen-expressie profiel. Hopelijk resulteert dit in de ontwikkeling van nieuwe leukemie-specifieke medicatie en verder verbetering van de prognose voor kinderen met BCV-ALL.

CHAPTER 8

List of publications

Curriculum vitae

PhD portfolio

Affiliations of co-authors

Dankwoord

LIST OF PUBLICATIONS

1. **Arian van der Veer**, Judith M. Boer, Dimitris Rizopoulos, Marta Fiocco, Hester de Groot, Roland P. Kuiper, Udo zur Stadt, Jan Trka, Maria S. Pombo-de-Oliveira, Tomek Szczpanski, Sabine Strehl, Rosemary Sutton, Gianni Cazzaniga, Martin Zimmermann, Anthony Moorman, Rob Pieters, Monique L. den Boer. Prognostic value of rare *IKZF1* variants in childhood B-cell precursor acute lymphoblastic leukemia: an international collaborative study. *In progress.*
2. Lieke C.J. van den Berk, Vincent H. van der Velden, Myrte J.G.A. Theeuwes, **Arian van der Veer**, Marieke E. Willemse, Rob Pieters, and Monique L. den Boer. Altered signaling and cytokine expression in mesenchymal stromal cells derived from pediatric patients affected by acute lymphoblastic leukemia. *In progress.*
3. Lieke C. J. van den Berk, **Arian van der Veer**, Marieke E. Willemse, Myrte J.G.A. Theeuwes, Mirjam W. Luijendijk, Wing H. Tong, Inge M. van der Sluis, Rob Pieters, Monique L. den Boer. Important role for the CXCR4/CXCL12 axis in pediatric precursor B-cell acute lymphoblastic leukemia. *Br J Haematol 2014, accepted*
4. **Arian van der Veer**, Vincent H. J. van der Velden, Marieke E. Willemse, Patricia G. Hoogeveen, Emanuel F. Petricoin, H. Berna Beverloo, Gabriele Escherich, Martin A. Horstmann, Rob Pieters and Monique L. den Boer. Interference with pre-B-cell receptor signaling offers a therapeutic option for *TCF3*-rearranged childhood acute lymphoblastic leukemia. *Blood Cancer 2014, Feb 14;4:e181*.
5. Akbari Moqadam F, Lange-Turenhout EA, **van der Veer A,** Marchante JR, Boer JM, Pieters R, Den Boer M. MiRNA signature in *BCR-ABL1-like* and *BCR-ABL1*-positive childhood acute lymphoblastic leukemia, similarities and dissimilarities. *Leuk Lymphoma 2014, Epub ahead of print*.
6. **Arian van der Veer**§, Marketa Zaliova§, Federica Mottadelli§, Paola De Lorenzo, Gertruuy te Kronnie, Christine J. Harrison, Hélène Cavé, Jan Trka, Vaskar Saha, Martin Schrappe, Rob Pieters, Andrea Biondi, Maria Grazia Valsecchi, Martin Stanulla§, Monique L. den Boer§, Giovanni Cazzaniga§. *IKZF1* status as a prognostic feature in *BCR-ABL1*-positive childhood ALL. *Blood 2014 Epub ahead of print.* §**Shared first** and last authorship
7. **Arian van der Veer**§, Esmé Waanders§, Rob Pieters, Marieke E. Willemse, Simon V. Van Reijmersdal, Lisa J. Russell, Christine J. Harrison, William E. Evans, Vincent H.J. van der Velden, Peter M. Hoogerbrugge, Frank Van Leeuwen, Gabriele Escherich, Martin A. Horstmann, Leila Mohammadi Khankahdani, Dimitris Rizopoulos, Hester A. De Groot-Kruseman, Edwin Sonneveld, Roland P. Kuiper§, Monique L. Den Boer§. Independent prognostic value of *BCR-ABL1-like* signature and *IKZF1* deletion, but not high *CRLF2* expression, in children with B-cell precursor ALL. *Blood. 2013,122(15):2622-9.* §**Shared first** and last authorship
8. Buitenkamp TD, Pieters R, Gallimore NE, **van der Veer A**, Meijerink JP, Beverloo HB, Zimmermann M, de Haas V, Richards SM, Vora AJ, Mitchell CD, Russell LJ, Schwab C, Harrison CJ, Moorman AV, van den Heuvel-Eibrink MM, den Boer ML, Zwaan CM. Outcome in children with Down's syndrome and acute lymphoblastic leukemia: role of *IKZF1* deletions and *CRLF2* aberrations. *Leukemia. 2012;26(10):2204-11*.
9. **Arian van der Veer**, Saskia de Pont, Marc van Milligen de Wit. Crohn's disease and Listeria meningitis: just a coincidence? *Magma 2008: 42-44.*

CURRICULUM VITAE

Arian van der Veer werd geboren op 7 november 1980 te Vlaardingen. Hij groeide op in Maassluis en heeft het gymnasium (Interconfessioneel Scholengemeenschap Westland, Naaldwijk) in 1998 afgerond. Hij heeft zijn propedeuse Biologie gehaald aan de Universiteit Leiden (2001). Daarna kreeg hij de mogelijkheid om Geneeskunde te studeren aan de Erasmus Universiteit Rotterdam. Dit heeft geresulteerd in het *cum laude* behalen van zijn artsdiploma in 2007. Vervolgens is hij 1 jaar werkzaam geweest als arts-assistent niet in opleiding tot specialist (ANIOS) kindergeneeskunde in het Amphia Ziekenhuis te Breda (opleidster dr. A. A. P. H. Vaessen-Verberne). Hij zette de stap onderzoek te gaan doen in de kinderoncologie onder leiding van dr. M. L. den Boer en prof. dr. R. Pieters. Hij onderzocht tussen 2008 en 2012 de prognostisch en therapeutische waarde van verworven genetische afwijkingen in kinderleukemie. De stichting kinderoncologie Nederland (SKION) besloot mede op basis van zijn resultaten een van deze afwijkingen, namelijk *IKZF1* deleties, te implementeren in het nieuwe behandelprotocol voor acute lymfatische leukemie. De resultaten van Arians onderzoek zijn beschreven in dit proefschrift en werden in 2013 bekroond met de Kika Tom Voûte Award. In 2013 hervatte hij het klinische werk in de kindergeneeskunde in het Erasmus MC - Sophia kinderziekenhuis te Rotterdam als ANIOS (opleider prof. M. de Hoog) en maakte in 2014 de overstap naar het ZNA – Paola kinderziekenhuis te Antwerpen (opleider dr. M. Wojciechowski, stagemeester dr. A. Bael) als ANIOS. Per 1 juni 2014 start hij als arts in opleiding tot kinderarts in het academische ziekenhuis Maastricht (opleider dr. A. L. M. Mulder en dr. Q. Jöbsis). Hij is vijf jaar vrijwilliger geweest bij de Zuiderzeevaart, een vakantiekamp van de Stichting Kinderoncologische Vakanties (SKOV). Drie jaar hiervan was hij bestuurslid. Arian is getrouwd met Marjolein van der Veer-Meerkerk en heeft 3 kinderen: Anne (2008), Floris (2010) en Lieve (2013).

PHD PORFOLIO

Summary of PhD training and research

Name PhD student:	Arian van der Veer
Erasmus MC department:	Sophia Children's Hospital-Department of Pediatric Oncology
Research school:	Molecular Medicine
PhD period:	1 November 2008 – 1 November 2012
Promotor:	Prof.dr. R. Pieters
Co-promotor:	Prof.dr. M.L. den Boer

1. Ph Dtraining	Year	Workload ECTS
General courses		
Basis Cursus Oncologie	2010	1.4
Classical Methods for Data Analysis (CCO2) (NIHES)	2010	5.7
Biomedical English Writing and Communication	2009	4.0
Specific courses		
Statistics for genomic data analysis (K77) (EpiDM)	2011	1.4
Intense R course (LUMC)	2008	1.4
Biomedical Research Techniques (MM)	2009	1.4
Basic and Translational Oncology (MM)	2009	1.4
Seminars and workshops		
Annual Molecular Medicine day (two poster presentations, one oral)	2009-2011	1.7
Annual pediatric reseach day, Erasmus MC, Rotterdam	2008-2011	0.4
Annual DCOG shared care day, Utrecht	2010, 2011	0.6
NVVO milestone dag, jongeren en kanker, Amsterdam	2010	0.2
Presentations		
See also "Seminars and Workshops" and " (Inter)national conferences" presentation		
18th EH Aannual Meeting, Stockholm, Sweden (preparation poster and oral presentation)	2013	0.8
Annual meeting for parents, Sophia Children's Hospital, Rotterdam, the Netherlands	2011	0.6
7 oral presentations at weekly Pediatric Oncology Research meeting and Pediatric Research meeting	2008-2012	4.0

1. Ph Dtraining *(Continued)*	**Year**	**Workload ECTS**
(Inter)national conferences		
45th SIOP annual Meeting, Hong Kong, Hong Kong (poster and oral presentation)	2013	2.0
54th annual ASH Meeting, Atlanta, USA (oral presentation)	2012	2.0
17th annual EHA Meeting, Amsterdam, the Netherlands (oral presentation)	2012	2.0
23th annuall-BFM Meeting, Santiago de Chile, Chile (oral presentation)	2012	2.0
8th bi-annual CLS Meeting, Santiago de Chile, Chile (poster presentation)	2012	1.6
53th annual ASH Meeting, San Diego, USA (poster presentation)	2011	1.6
4th Dutch Hematology Congress, Arnhem, the Netherlands	2010	0.6
ECCO15-34ESMO Meeting, Berlin, Germany (oral presentation)	2009	2.0
Other		
KiKa Tom Voûte Award (EURO 2500,=)	2013	1.0
Travel Grant for the 54th annual ASH meeting by ASH committee	2012	0.0
Travel Grant for 53th and 54th annual ASH meeting by Erasmus Trustfonds	2011-2012	0.2
SSWO Grant Application (grant for technician, EURO 120.000,=)	2010	4.0
Travel Grant for ECCO15-34ESMO by ECCO-ESMO committee	2009	0.2

2. Teaching	**Year**	**Workload ECTS**
Supervising Archana Maharba, HLO bachelor thesis, Hogeschool Rotterdam The role of BT Kinchildhood leukemia	2010-2011	10

3. Extracurricular	**Year**	**Workload ECTS**
Medical management for 'Zuiderzeevaart'. Sailing camp for children with amalignancy	2010-2012	N/A

Total	**54.2**

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Mijn B-ALL groepsgenoten: **Farhad**, **Stefanie**, **Ingrid**, **Bob** en **João**, **Lieneke**, **Ellen**, **Marieke**, **Rosanne**, **Fienke** en **Cesca**: ontzettend bedankt! **Ingrid**, zet hem op strakjes in de VS. ik moet bekennen dat er heel wat momenten zijn geweest waarin ik iets meer van jouw eigenschappen wilde hebben; petje af voor je onderzoek. **Farhad**: I hope that you will be succesful in research, medicine and your familiy live. **Stefanie**: zet hem op met afronden van je promotie onderzoek! **Bob**: Je bent met een leuk onderwerp bezig en ik weet zeker dat jouw enthousiasme en kennis zal leiden tot veel nieuwe publicaties! **João**: Thank you so much for your ideas, your humor and soccer matches! **Lieneke**: veel plezier en succes de komende jaren! **Ellen**: Ik heb veel respect voor de dingen die je doet, hoe je als mens bent. Ontzettend bedankt voor de afgelopen jaren! **Rosanne** en **Fienke**: de nieuwe spil van onze groep voor praktisch werk: veel plezier en succes toegewenst. **Judith**: bedankt voor je enthousiaste inbreng tijden werkbesprekingen en je geduld dat je opbracht bij het maken van het vierde hoofdstuk in dit boekje. **Cesca**: jouw impulsieve uitspraken waren soms op het randje, maar brachten mij vaak plezier. **Myrthe**, dankzij jou is mijn kennis van het Brabants (spreken en horen) sterk verbeterd: dank daarvoor! Lijkt Limburgs eigenlijk een beetje op Brabants?

Speciale dank voor mijn drie rechter handjes: **Marieke**, mijn paranimf. Jij kwam mijn onderzoek versterken drie maanden nadat ik gestart was. Ik heb in praktische zin veel van je geleerd. Ik waardeer je ontzettend in hoe je bent en hoe je doet. Ik realiseer me dat je een belangrijke rol hebt gespeeld in dit onderzoek en dit boekje; ik wil je ontzettend bedanken voor je inbreng in dit boekje. Ik denk dat de baan die je nu begonnen bent bij Inekee, uitermate geschikt is, zet hem op! En wanneer ik toch weer overweeg om vrachtwagenchauffeur te worden, bel ik je op. **Lieke**, mijn andere paranimf, ook voor jou heb ik heel veel respect. En eigenlijk had ik dat al toen je voor het eerst solliciteerde: ik durfde je niet te tutoyeren en sprak je aan met Doctor; sindsdien sprak je mij aan met arts-onderzoeker. In de afgelopen jaren heb ik je leren kennen als heel eerlijk, recht door zee en als een goed onderzoekster. Jij hebt mij geleerd hoe je een experiment met de juiste controles op moet zetten. Ik denk dat je met de juiste beslissingen in je carrière een heel eind zal komen. Ontzettend bedankt voor alles, ook voor jouw inbreng in dit boekje.

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Ook wil ik graag de leden van de sequence dienst bedanken: **Jessica**, **Marcel** en **Ellen**: ik vond deze lab-taak altijd een leuk klusje en waardeerde het ontzettend dat we niet te beroerd waren om elkaars problemen op te lossen. Speciaal wil ik **Marcel** bedanken: je hebt een loopbaan doorlopen van analist tot vervanger van **Ingrid L.**: normaal functioneer ik altijd als het luisterend oor voor anderen, maar in jou heb ik ook een luisterend oor gevonden; heel prettig, bedankt daarvoor.

De oncologen van het Sophia kinderziekenhuis, **Auke**, **Inge**, **Michel**, **Erna**, **Max**, **Marry** en fellow **Martine** waren altijd geïnteresseerd in mijn onderzoek en mijn loopbaan. **Max**, **Auke**, **Martine** en **Marry** hebben mij verschillende malen geadviseerd om niet op te geven en mijn doel voor ogen te houden. Ik ben blij dat jullie mij op deze manier ondersteunden, omdat ikzelf het vertrouwen in een goede afloop van promotie en opleidingsplaats kindergeneeskunde weleens kwijt was: jullie hadden gelijk!

Omdat ik een plezierige maand in het Paola kinderziekenhuis in Antwerpen heb beleefd, wil ik de opleider pediatrie van de UZA, **Dr. Wojchiechowski**, mijn stagemeester, **Dr. Bael**, en mijn collega arts-assistenten bedanken voor de gastvrijheid en de inspanning voor een opleidingplaats in België. Ik vertrek alweer uit België en krijg nu een opleidingsplaats in het Academisch Ziekenhuis Maastricht. Omdat ik deze mogelijkheid krijg, wil ik de opleiders kindergeneeskunde, **dr. Mulder** en **dr. Jöbsis** bedanken voor het vertrouwen in mij. Ik heb er ontzettend zin in om in het mooie Limburg te gaan wonen en te werken: tot snel!

Als laatste, maar wel heel belangrijk voor mij, wil ik mijn **gezin** bedanken. Ik realiseer me dat ik de laatste tijd (= jaren) jullie te weinig aandacht heb gegeven. Het is regelmatig voorgekomen dat ik er misschien wel fysiek was, maar met mijn gedachten niet. **Marjolein**, liefje, bedankt dat je zoveel wilde inspringen. Ik vind het heel knap hoe je er voor ons bent, een goede dokter bent en een wetenschapper. Natuurlijk hebben onze ouders ook heel veel bijgedragen door een luisterend oor te zijn en op de jongens te passen. **Desiree**, ook jij maakt naar mijn gevoel deel uit van ons gezin. Zonder jou was het onmogelijk geweest om mijn ambities waar te maken. We hadden graag gehad dat je mee was gegaan naar Limburg, maar dat gaat helaas niet en dat begrijpen we: bedankt voor je hulp en succes verder! **Anne** en **Floris** en **Lieve**, jullie zullen dit waarschijnlijk pas lezen als jullie ouder zijn en nog knapper. De momenten die ik met jullie samen heb, geniet ik intens van jullie en is echt ontspanning; ik hoop dat jullie het ook zo ervaren hebben. Ik hou ontzettend veel van jullie!

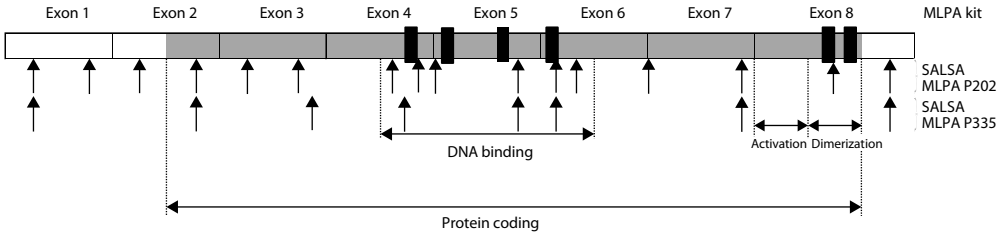
CHAPTER 9

Supplemental information

Supplemental data for:

Chapter 4

Prognostic value of rare *IKZF1* variants in childhood B-cell precursor acute lymphoblastic leukemia: an international collaborative study



Supplemental Figure S1 | Localization of MLPA-probes. *IKZF1*-gene is depicted in bars. Transparant bars represent non-coding sequences. Gray bars indiciate amino-acid coding sequences. Zinc fingers are depicted in black. The DNA-binding, activation and dimerization regions are assigned by double arrows. Normal arrows indicate the localization of SALSA MLPA P202 and SALSA MLPA P335 probes respectively.

Supplemental Table S1 | Characteristics at initial diagnosis of *IKZF1*-deleted cases and their matched wild-type controls split in the different variants of deletions

		All wild-type			All rare DEL			*p*-value
		Absolute	n	%	Absolute	n	%	
Age (years)	Median	5.3			7.0			NS
	<10		297	74%		99	74%	NS
	≥10		105	26%		35	26%	
Gender								
	Male		234	58%		69	51%	NS
	Female		168	42%		65	49%	
White blood cell count	Median	15.1			20.0			NS
(cells/nl)	<50		291	72%		97	72%	NS
	≥50		111	28%		37	28%	
	missing		0			1		
Stratification								
	standard risk		168	42%		56	42%	NS
	intermediate risk		99	25%		33	25%	
	high risk		135	34%		45	34%	
Subtype								
	ETV6-RUNX1		36	9%		12	9%	NS
	Hyperdiploid		63	16%		21	16%	
	B-other		264	66%		88	66%	
	Undetermined		39	10%		13	10%	

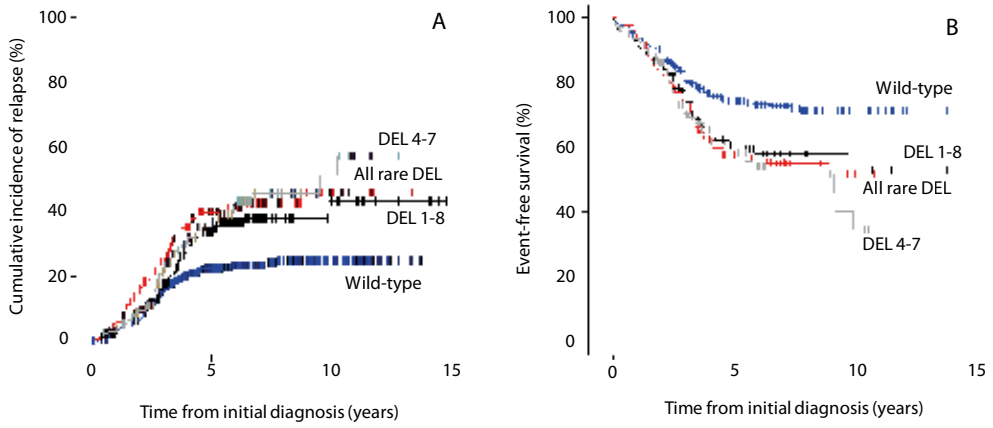
		Wild-type			DEL 2-3			*p*-value
		Absolute	**n**	**%**	**Absolute**	**n**	**%**	
Age (years)	Median	4.0			7.0			NS
	<10		66	85%		22	85%	NS
	≥10		12	15%		4	15%	
Gender								
	Male		102	58%		17	49%	NS
	Female		73	42%		18	51%	
White blood cell count	Median	8.5			12.3			NS
(cells/nl)	<50		75	96%		25	96%	NS
	≥50		3	4%		1	4%	
Stratification								
	standard risk		51	65%		17	65%	NS
	intermediate risk		12	15%		4	15%	
	high risk		15	19%		5	19%	
Subtype								
	ETV6-RUNX1		21	27%		7	27%	NS
	Hyperdiploid		18	23%		6	23%	
	B-other		30	38%		10	38%	
	Undetermined		9	12%		3	12%	

		Wild-type			DEL 2-7			*p*-value
		Absolute	**n**	**%**	**Absolute**	**n**	**%**	
Age (years)	Median	6.8			7.3			NS
	<10		69	72%		23	72%	NS
	≥10		27	28%		9	28%	
Gender								
	Male		52	54%		18	56%	NS
	Female		44	46%		14	44%	
White blood cell count	Median	20.1			21.9			NS
(cells/nl)	<50		63	66%		21	66%	NS
	≥50		33	34%		11	34%	
Stratification								
	standard risk		30	31%		10	31%	NS
	intermediate risk		21	22%		7	22%	
	high risk		45	47%		15	47%	
Subtype								
	ETV6-RUNX1		0	0%		0	0%	NS
	Hyperdiploid		15	16%		5	16%	
	B-other		69	72%		23	72%	
	Undetermined		12	13%		4	13%	

		Wild-type			DEL 2-8			*p*-value
		Absolute	**n**	**%**	**Absolute**	**n**	**%**	
Age (years)	Median	11.0			11.0			NS
	<10		18	40%		6	40%	NS
	≥10		27	60%		9	60%	
Gender								
	Male		28	62%		7	47%	NS
	Female		17	38%		8	53%	
White blood cell count	Median	16.1			19.5			NS
(cells/nl)	<50		33	73%		11	73%	NS
	≥50		12	27%		4	27%	
Stratification								
	standard risk		21	47%		7	47%	NS
	intermediate risk		6	13%		2	13%	
	high risk		18	40%		6	40%	
Subtype								
	ETV6-RUNX1		6	13%		2	13%	NS
	Hyperdiploid		3	7%		1	7%	
	B-other		33	73%		11	73%	
	Undetermined		3	7%		1	7%	

		Wild-type			DEL 4-8			*p*-value
		Absolute	**n**	**%**	**Absolute**	**n**	**%**	
Age (years)	Median	5.0			6.0			NS
	<10		57	70%		19	70%	NS
	≥10		24	30%		8	30%	
Gender								
	Male		50	62%		12	44%	NS
	Female		31	38%		15	56%	
White blood cell count	Median	28.9			32.6			NS
(cells/nl)	<50		45	56%		15	56%	NS
	≥50		36	44%		12	44%	
Stratification								
	standard risk		30	37%		10	37%	NS
	intermediate risk		21	26%		7	26%	
	high risk		30	37%		10	37%	
Subtype								
	ETV6-RUNX1		6	7%		2	7%	NS
	Hyperdiploid		12	15%		4	15%	
	B-other		57	70%		19	70%	
	Undetermined		6	7%		2	7%	

		Wild-type			DEL-Other			*p*-value
		Absolute	**n**	**%**	**Absolute**	**n**	**%**	
Age (years)	Median	4.0			4.0			NS
	<10		87	85%		29	85%	NS
	≥10		15	15%		5	15%	
Gender								
	Male		59	58%		21	62%	NS
	Female		43	42%		13	38%	
White blood cell count	Median	15.8			18.9			
(cells/nl)	<50		75	74%		25	74%	NS
	≥50		27	26%		9	26%	
Stratification								
	standard risk		36	35%		12	35%	NS
	intermediate risk		39	38%		13	38%	
	high risk		27	26%		9	26%	
Subtype								
	ETV6-RUNX1		3	3%		1	3%	NS
	Hyperdiploid		15	15%		5	15%	
	B-other		75	74%		25	74%	
	Undetermined		9	9%		3	9%	



Supplemental Figure S2 | CIR (A) and EFS (B) of all rare *IKZF1*-deleted cases together in relation to matched wild-type controls, unmatched DEL 4-7 cases and unmatched DEL 1-8 cases. DEL 4-7 and DEL 1-8 cases were extracted from the same treatment protocols as rare deleted cases and controls..

Supplemental Table S2 | Toxicity related deaths and relapse per *IKZF1* deletion variant group compared with their matched wild-type controls

Variant			Death			Relapse		
		total	number	%	*p*-value	number	%	*p*-value
All rare								
	Wild-type	**402**	**14**	**3%**	NS	**87**	**22%**	<0.001
	Deleted	**134**	**3**	**2%**		**52**	**39%**	
DEL 2-3								
	Wild-type	**78**	**3**	**4%**	NS	**12**	**15%**	0.08
	Deleted	**26**	**0**	**0%**		**8**	**31%**	
DEL 2-7								
	Wild-type	**96**	**3**	**3%**	NS	**18**	**19%**	0.07
	Deleted	**32**	**1**	**3%**		**11**	**34%**	
DEL 2-8								
	Wild-type	**45**	**2**	**4%**	NS	**14**	**31%**	0.01
	Deleted	**15**	**0**	**0%**		**10**	**67%**	
DEL 4-8								
	Wild-type	**81**	**5**	**6%**	NS	**19**	**23%**	NS
	Deleted	**27**	**0**	**0%**		**8**	**30%**	
DEL-Other								
	Wild-type	**102**	**1**	**1%**	0.09	**24**	**24%**	<0.001
	Deleted	**34**	**2**	**6%**		**15**	**44%**	

Cases with death without relapse were counted as toxicity related death. *P*-values are based on Chi-square tests.

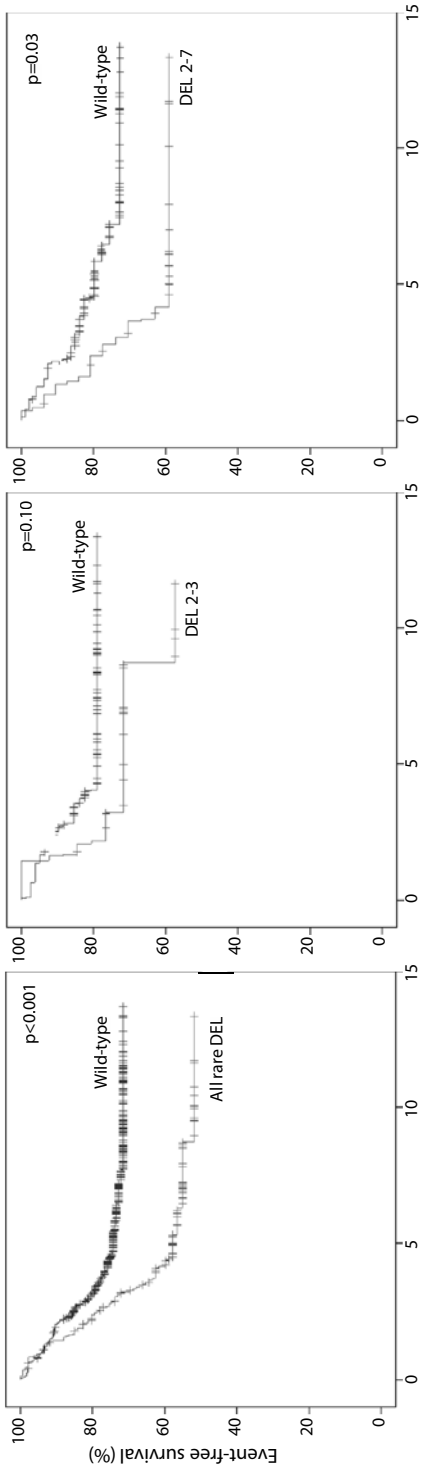
Supplemental Table S3 | Outcome of rare variants compared withreference cases

A				CIR		EFS		
	cases	relapses	events	5-yr CIR	*p*-value	HR	95% CI	*p*-value
DEL 2-3	26	8	8	28%	0.9	0.8	0.4–1.6	0.5
DEL 2-7	32	11	12	38%	0.8	1.0	0.5–1.8	1
DEL 2-8	15	10	10	60%	0.01	2.0	1.0–4.0	0.04
DEL 4-8	27	8	8	34%	0.9	0.8	0.4–1.6	0.5
DEL-Other	34	15	17	45%	0.4	1.3	0.7–2.2	0.4
DEL 4-7	146	49	55	38%	0.3	1.2	0.8–1.7	0.4
DEL 1-8	163	52	60	35%	ref	ref	ref	ref

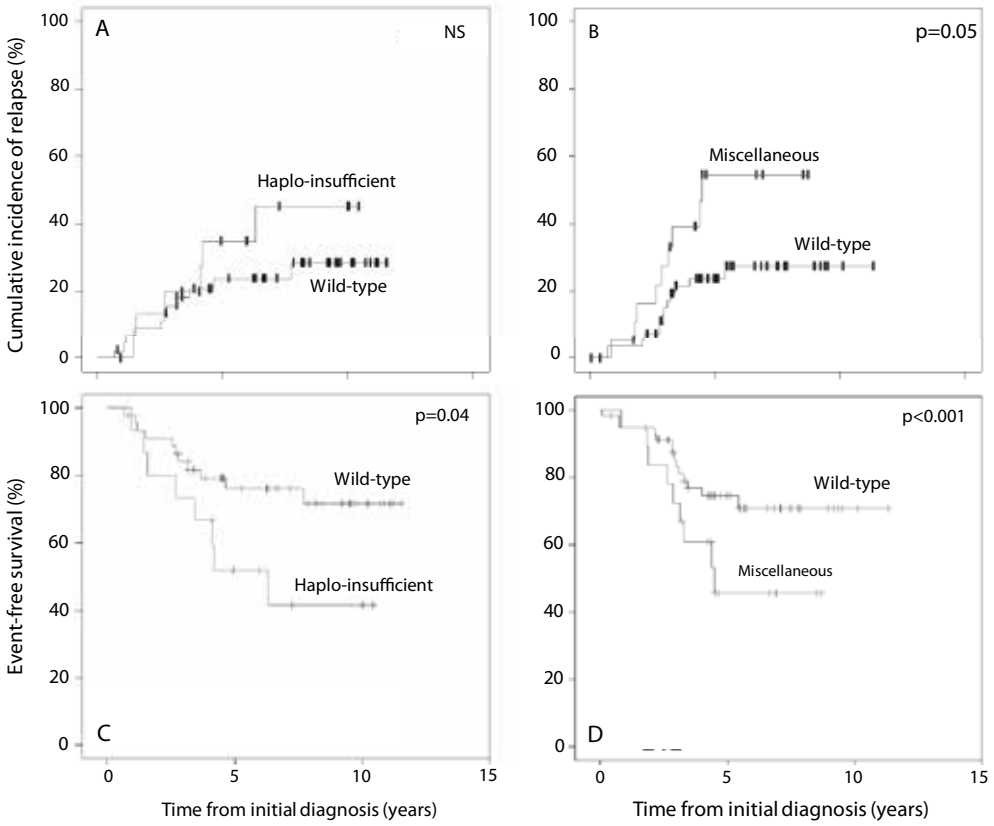
B				CIR		EFS		
	cases	relapses	events	5-yr CIR	*p*-value	HR	95% CI	*p*-value
DEL2-3	26	8	8	28%	0.5	0.6	0.3–1.1	0.3
DEL2-7	32	11	12	38%	0.7	0.8	0.5–1.6	0.6
DEL2-8	15	10	10	60%	0.04	1.8	0.9–3.5	0.1
DEL4-8	27	8	8	34%	0.5	0.6	0.3–1.4	0.3
DEL-Other	34	15	17	45%	0.8	1.1	0.6–1.9	0.7
DEL4-7	146	49	55	38%	ref	ref	ref	ref

C				CIR		EFS		
	cases	relapses	events	5-yr CIR	*p*-value	HR	95% CI	*p*-value
DEL2-3	26	8	8	28%	0.7	0.7	0.3–1.5	0.4
DEL2-7	32	11	12	38%	1	0.9	0.5–1.7	0.8
DEL2-8	15	10	10	60%	0.02	1.9	1.0–3.6	0.05
DEL4-8	27	8	8	34%	0.7	0.7	0.3–1.5	0.4
DEL-Other	34	15	17	45%	0.5	1.2	0.7–2.0	0.5
DEL4-7 plus DEL 1-8	309	101	115	36%	ref	ref	ref	ref

Panel **A** compares rare variant with DEL 1-8, **B** with DEL 4-7, and **C** with DEL 1-8 + DEL 4-7



Supplemental Figure S3 | Event-free survival (EFS) of *IKZF1*-deleted variants cases compared with their wild-type controls. Data were summarized in the first panel and split up in the other panels. Relapse and death were considered as event. *P*-values were calculated by a matched pair EFS analysis. Cases with death without relapse were counted as toxicity related death. *P*-values are based on Chi-square tests.



Supplemental Figure S4 | CIR and EFS curves for Haplo-insufficient (A and C) and Miscellaneous (B and D) cases extracted from DEL-Other group compared with matched wild-type controls. *P*-values in CIR curves (**A** and **B**) are based on Gray with death as competing event. *P*-values in EFS curves (**C** and **D**) are based on match pair analysis.

Supplemental Table S4 | Clinical follow up of DEL-Other cases

haplo-insufficiency	no.	Relapses	%	Median time follow up
DEL 1-3	5	2	40%	7.2CCR
DEL 1-5	2	2	100%	5.2R
DEL 1-5 + 7-8	1	0	0%	0.9CCR
DEL 1-7	1	0	0%	6.0CCR
DEL 1-8 + 1	1	0	0%	10.0CCR
DEL 1-8 + 4-6	1	1	100%	1.4CCR
DEL 1-8 + 1-8	4	1	25%	4.9CCR
Total	**15**	**6**	**40%**	**6.0CCR**

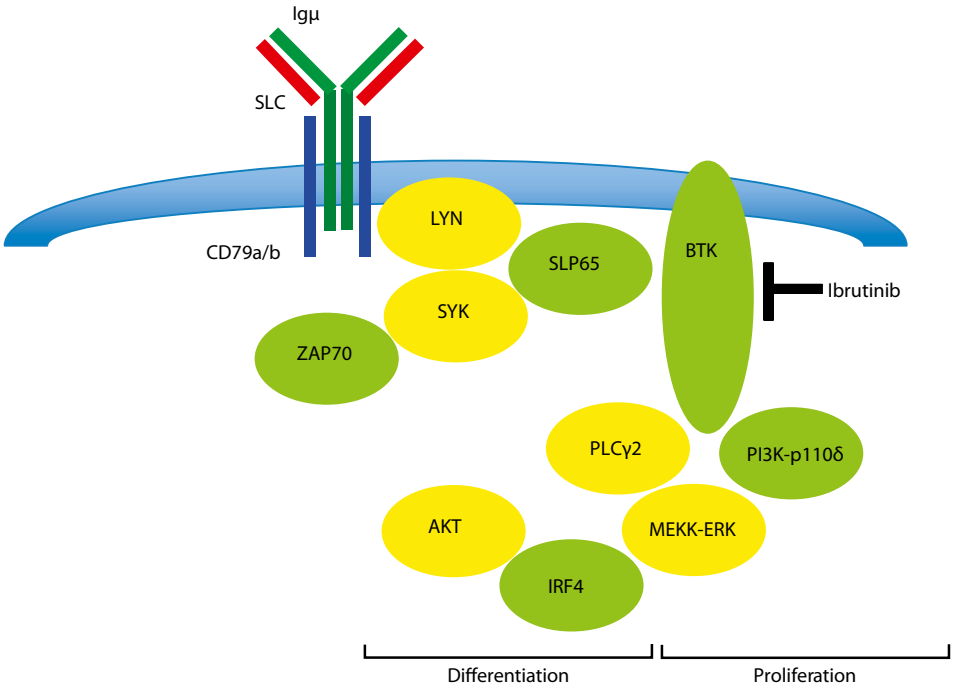
miscellaneous	no.	Relapses	%	Median time follow up
DEL 1	6	3	50%	6.7CCR
DEL 3	3	1	33%	3.1CCR
DEL 3-8	2	2	100%	3.9R
DEL 4	1	1	100%	4.4R
DEL 4-6	1	1	100%	1.9R
DEL 4-7 + 4-7	1	0	0%	4.4CCR
DEL 4-8 + 1	1	0	0%	6.9CCR
DEL 5-7	1	0	0%	4.2CCR
DEL 7	1	0	0%	4.6CCR
DEL 8	2	1	50%	8.7CCR
Total	19	9	47%	4.6CCR

If exon 2 is affected, cases were enrolled in haplo-insufficient group. Remaining cases were enrolled in the miscellaneous group. The median time of follow up is calculated based on the cases in complete continuous remission (CCR) or when the cases relapsed (R) when none of them had a complete continuous remission.

Supplemental data for:

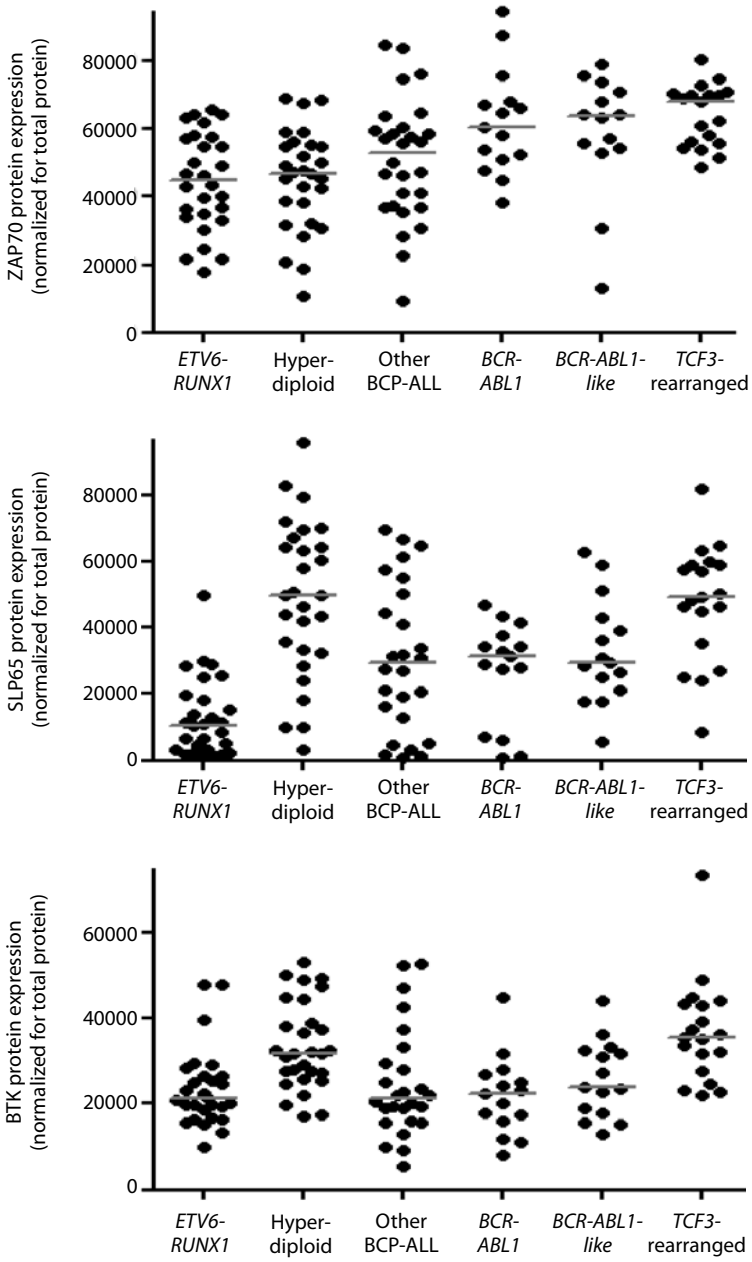
Chapter 5

Interference with pre-B-cell receptor signaling offers a therapeutic option for *TCF3*-rearranged childhood acute lymphoblastic leukemia

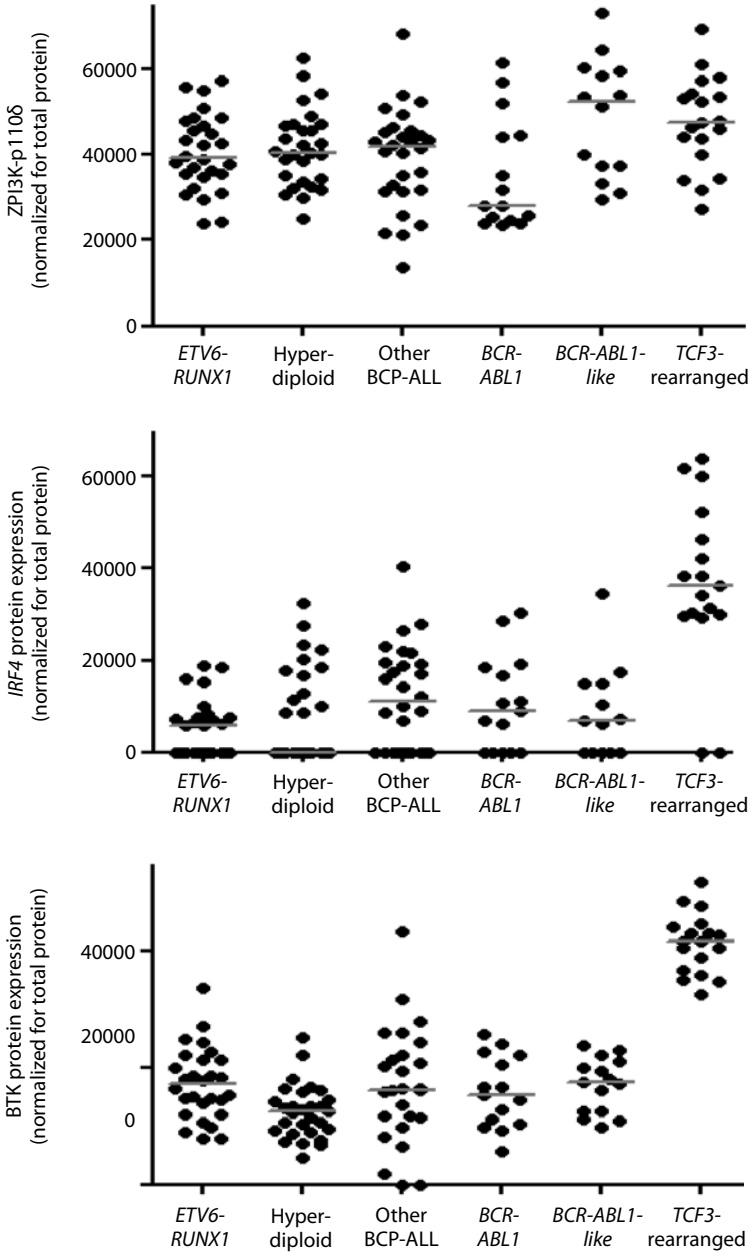


Supplementary Figure S1 | Simplified overview of proteins involved in pre-BCR signalling. Proteins depicted in green were tested by flow cytometry (Igμ) or reverse phase protein arrays (all other proteins) in the present study.

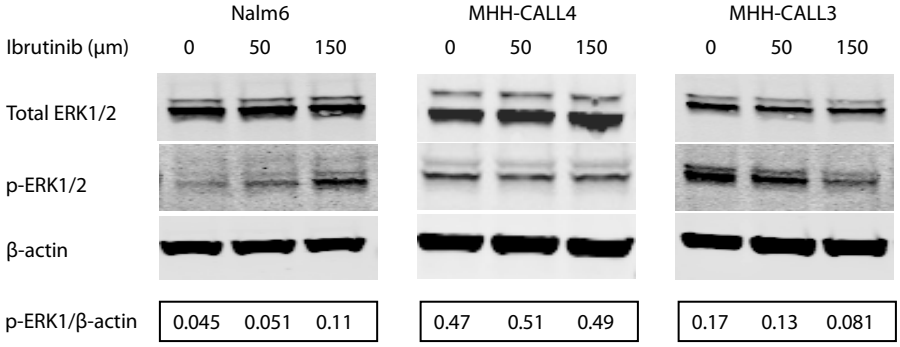
SLC: surrogate light chain. Activation of pre-BCR results in proliferation followed by differentiation.



Supplementary Figure S2 | Expression of proteins involved in pre-BCR pathway signalling in known (cytogenetic) subtypes of childhood BCP-ALL. Expression levels were measured by reverse phase protein arrays and levels were normalized for total protein levels in each cell lysate. Gray line indicates the median expression level.



Supplementary Figure S2 | *Continued*



Supplementary Figure S3 | Effect of ibrutinib on ERK protein levels in leukemic cell lines. Cell lines were exposed to 0, 50 and 150 mμ ibrutinib for 3 days. The level of phosphorylated ERK1/2 (p-ERK1/2) was reduced in *TCF3*-rearranged MHH-CALL3 compared to non-*TCF3*-rearranged Nalm6 and MHH-CALL4 cell lines. β-actin served as a loading control.

P-ERK/β-actin: ratio of the intensities.

Supplementary Table S1 | Frequency of CyIgμ-positive cases in known subtypes of newly diagnosed pediatric BCP-ALL

Subtype	Total	CyIgμ negative		CyIgμ positive	
	n	n	%	n	%
ETV6-RUNX1	202	170	84.2%	32	15.8%
Hyperdiploid	74	54	73.0%	20	27.0%
B-other	21	17	81.0%	4	19.0%
BCR-ABL1-like	11	7	63.6%	4	36.4%
BCR-ABL1	17	9	52.9%	8	47.1%
TCF3-rearranged	13	2	15.4%	11	84.6%

CyIgμ-positive case: >30% of CyIgMμ-positive leukemic cells

Supplementary Table S2 | Frequency of immunoglobulin rearrangements in (cytogenetic) subtypes of BCP-ALL

A

		IGH total		V_κ-J_κ		IGK-Kde		V_λ-J_λ	
Subtype	Total	n	%	n	%	n	%	n	%
ETV6-RUNX1	42	36	85.7%	25	59.5%	37	88.1%	11	26.2%
Hyperdiploid	20	20	100.0%	7	35.0%	4	20.0%	2	10.0%
B-other	18	15	83.3%	11	61.1%	10	55.6%	6	33.3%
BCR-ABL1-like[#]	74	62	83.8%	37	50.0%	46	62.2%	21	28.4%
BCR-ABL1	36	33	91.7%	20	55.6%	19	52.8%	7	19.4%
TCF3-rearranged	22	22	100.0%	1	4.5%	0	0.0%	2	9.1%

B

		IGH		IGK		IGK-Kde		IGL	
Subtype	Total	n	%	n	%	n	%	n	%
ETV6-RUNX1	42	2	4.8%	5	11.9%	24	57.1%	11	26.2%
Hyperdiploid	20	10	50.0%	5	25.0%	3	15.0%	2	10.0%
B-other	18	2	11.1%	4	22.2%	6	33.3%	6	33.3%
BCR-ABL1-like[#]	74	13	17.6%	12	16.2%	27	36.5%	21	28.4%
BCR-ABL1	36	7	19.4%	9	25.0%	13	36.1%	7	19.4%
TCF3-rearranged	22	19	86.4%	1	4.5%	0	0.0%	2	9.1%

A) Frequencies of immunoglobulin heavy and light chain rearrangements in subtypes of ALL. **B**) Frequencies of heavy and light chain rearrangement stadia at which the immunoglobulin rearrangement was uniquely arrested per patient. [#] One *BCR-ABL1-like* sample did not have any rearrangement.

Supplementary Table S3 | Sensitivity of individual samples for ibrutinib

A

	non-*TCF3*-rearranged											
	#C1		#C2		#C3		#C4		#C5		#C6	
	ETV6-RUNX1		*ETV6-RUNX1*		Hyperdiploid		Hyperdiploid		*BCR-ABL1*		*BCR-ABL1-like*	
	IGK-Kde		IGH		IGH		IGL		IGK-Kde		IGH	
50μM	98%	101%	91%	87%	75%	83%	74%	72%	83%	86%	89%	105%
16.7μM	99%	105%	89%	91%	87%	86%	81%	91%	87%	95%	95%	110%
5.5μM	97%	98%	91%	91%	97%	93%	100%	103%	92%	99%	93%	102%
1.8μM	97%	95%	87%	95%	90%	96%	101%	100%	93%	95%	87%	96%
0.6μM	95%	97%	89%	87%	83%	92%	102%	106%	93%	89%	83%	87%
0.0μM	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

B

	TCF3-rearranged											
	#T1		#T2		#T3		#T4		#T5		#T6	
	TCF3-rearranged		*TCF3*-rearranged		*TCF3*-rearranged		*TCF3*-rearranged		*TCF3*-rearranged		*TCF3*-rearrangedd	
	IGH		IGH		IGH		IGL		IGH		IGH	
50μM	30%	38%	45%	46%	22%	25%	58%	40%	41%	45%	24%	27%
16.7μM	49%	51%	65%	75%	40%	43%	64%	46%	38%	45%	43%	47%
5.5μM	49%	59%	80%	87%	56%	61%	84%	71%	56%	57%	55%	64%
1.8μM	59%	60%	103%	72%	59%	64%	63%	57%	62%	62%	55%	64%
0.6μM	58%	62%	82%	92%	61%	67%	63%	62%	71%	73%	51%	61%
0.0μM	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

Cell viability is calculated relatively to drug-free control (%). Experiments were done in duplicate. Subtype and last immunoglobulin rearrangement are indicated per sample. Table **A** shows non-*TCF3*-rearranged BCP-ALL samples and table **B** shows *TCF3*-rearranged samples.Visualization in **Figure 4**.