Clinical Relevance of Genetic Alterations in Acute Lymphoblastic Leukemia in Children with Down syndrome

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Klinische relevantie van genetische afwijkingen in Acute Lymfatische Leukemie bij kinderen met het syndroom van Down.

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"Life isn't about waiting for the storm to pass,
it's learning to dance in the rain."

Voor Martijn & Sterre

CONTENTS

1 General Introduction

NORMAL HEMATOPOIESIS AND LEUKEMIA

Hematopoiesis encompasses the process by which mature peripheral blood cells are generated by differentiation of hematopoietic stem cells (HSC) in the bone marrow (BM). The daughter cells of HSC's, progenitor cells, can each commit to the myeloid or lymphoid differentiation pathways that lead to the production of one or more specific types of mature blood cells. These cells are then released in the peripheral blood. The myeloid lineage generates platelets, erythrocytes, granulocytes (basophils, neutrophils and eosinophils) and monocytes, whereas the lymphoid lineage differentiates into T and B-lymphocytes (Figure 1).[1,2]

Leukemia is a comprehensive term covering a spectrum of malignant hematological diseases originating from hematopoietic precursor cells and affecting the hematopoietic system and extra-medullary sites. The initial lineage of the leukemic cells defines the type of leukemia. In general, leukemic blasts originate from either the lymphoid lineage resulting in lymphoblastic leukemia, or from the myeloid lineage causing myeloid leukemia, although mixed lineage leukemias/bi-phenotypic leukemias exist. Both lymphoid and myeloid leukemia can be further subdivided into acute and chronic leukemias. Acute leukemia is character-

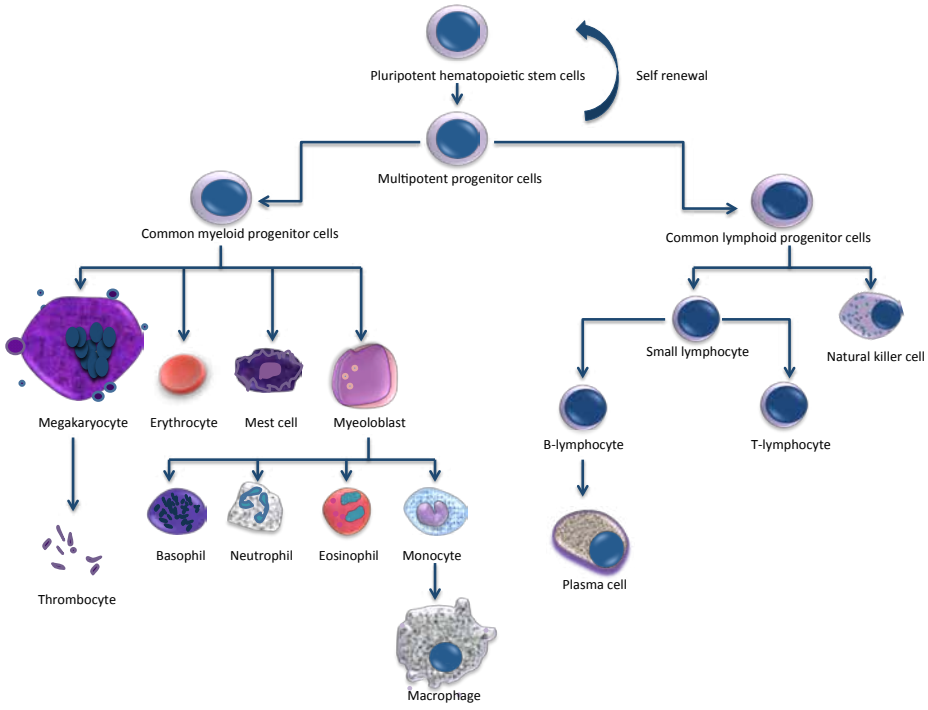

Figure 1. Schematic representation of the different lineages and stages during normal hematopoiesis
The development of blood cells from a bone marrow pluripotent hematopoietic stem cell to mature cells of different lineages.

ized by a maturation arrest, followed by rapid clonal expansion of highly immature malignant precursor cells in the BM and blood. In chronic leukemia, malignant cells accumulate due to hyper-proliferation without a clear maturation arrest, and usually develop more slowly. Subsequently, normal hematopoiesis fails and patients suffer from the absence of normal blood cells resulting in symptoms such as anemia, paleness, fatigue, fever, infections, bruising and petechiae.

Pediatric Acute Lymphoblastic Leukemia

Worldwide, more than 200,000 children are diagnosed with cancer every year.[3] In the Netherlands, 500-600 children are diagnosed with cancer annually. Leukemia is the most common type of cancer in children, comprising approximately 30% of all childhood malignancies (DCOG registration 2006-2011). Acute lymphoblastic leukemia (ALL) accounts for ~75% of all childhood leukemias, and can be subdivided in precursor B-cell ALL (BCP-ALL; ~85%) and in ALL originating from the T-cell lineage (T ALL; ~15%). The proportion of acute myeloid leukemia (AML) is ~18%, and the very rare other myeloid malignancies, such as myelodysplastic syndrome (MDS), juvenile myelomonocytic leukemia (JMML) or chronic myeloid leukemia (CML), represent the remaining cases (Figure 2).[4]

Leukemias in childhood are heterogeneous disorders, which is reflected by differences in clinical presentation, morphology and immunophenotype, and by a variety of acquired (cyto-) genetic aberrations. In precursor B-ALL, different well-established genetic risk groups can be

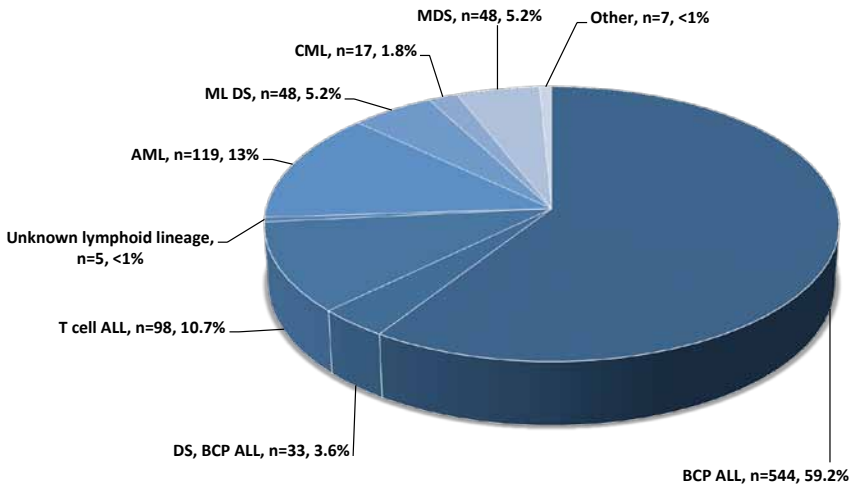

Figure 2. Distribution of types of childhood leukemia in The Netherlands

Numbers are derived from the Dutch childhood oncology group registration 2006-2011. ALL, acute lymphoblastic leukemia; BCP, B-cell precursor; ML DS, myeloid leukemia of down syndrome; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome.

distinguished. The favorable risk group includes cases with abnormalities such as t(12;21) (p13;q22) [*ETV6-RUNX1*] and high hyperdiploidy (HeH) (51-65 chromosomes), especially when extra copies of chromosome 4 and 10 are present.[5-9] The unfavorable abnormalities include translocations such as t(9;22)(q34;q11) [*BCR-ABL1*] and t(4;11)(q21;q23) [*MLL-AF4*].[6-9] The World Health Organisations' (WHO) classification of childhood ALL is defined by these genetic aberrations in combination with an immunophenotypic classification based on the different maturation stages in B lymphocyte development (Table 1).[10] These cytogenetic abnormalities are essential for risk group stratification in treatment protocols.[5]

The remaining cases are patients with genetically unclassified disease and referred to as 'B-Other' ALL, comprising ~25% of pediatric B-cell precursor-ALL.[11-13] Interestingly, the availability of new genome-wide screening techniques led to the discovery of a large new genetic subgroup, referred to as '*BCR-ABL1* like'.[11,69] These leukemias have a similar gene expression profile as *BCR-ABL1* positive ALL, but lack the typical *BCR-ABL1* translocation. Moreover, over 70% of these '*BCR-ABL1* like' cases have abnormalities in B-cell differentiation genes, including the *IKZF1*, *PAX5* and *EBF1 genes*. The prognosis of '*BCR-ABL1* like' patients is poor, as they merely reach an event free survival (EFS) of 50-70%.[11,14]

Treatment of Acute Lymphoblastic Leukemia

In recent decennia, ALL therapy has become very effective, now resulting in an overall survival of ~85% for children diagnosed with ALL and treated with risk-stratified combination chemotherapy.[15-18] In the Netherlands, children with ALL are currently treated according to the Dutch Childhood Oncology Group (DCOG) ALL-11 treatment protocol, which opened in 2013 and followed the previous ALL-10 protocol. Both protocols use a Berlin-Frankfurt-Münster Group (BFM) backbone,[19] consisting of an initial consolidation (protocol 1B) phase, an interim maintenance phase, a reinduction (delayed intensification) phase followed by maintenance therapy.

Table 1. The WHO classification of B cell ALL based on immunophenotype and genotype

Immunophenotype	CD19	CD10	cy μ	sur μ	Genotype
Pro B-cell ALL	+	-	-	-	*MLL-AF4*, *MLL-ENL*
Common B-cell ALL	+	+	-	-	*ETV6-RUNX1*, *BCR-ABL1*
Pre B-cell ALL	+	+	+	-	*E2A-PBX*

WHO, world health organization; CD, cluster of differentiation; Cy, cytoplasmic; sur, surface; ALL, acute lymphoblastic leukemia.

DOWN SYNDROME AND LEUKEMIA

Down syndrome

Down syndrome (DS) is considered one of the most common chromosomal abnormalities in the normal population,[20] with a prevalence of ~320 live births in the Netherlands.[21,22] Despite the increasing age at which women deliver their first child in Europe, this prevalence has decreased over the past decades due to increased screening and termination of DS pregnancies.[23]

DS is caused by trisomy of chromosome 21 (Hsa21), which is the result of abnormal division of chromosomes (non-disjunction) in the first or second meiotic division of the oocyte (80-95%) or the sperm cell (5-20%), or occasionally during mitotic division.[23-25] These DS children (95%) carry trisomy 21 in all their cells. However, ~5% of DS children have phenotypically none or fewer symptoms, which is a result of 1) mosaicism, i.e. that not all cells carry an additional chromosome 21, or 2) due to a Robertsonian translocation, where the participating chromosomes, usually chromosome 14 and 21, break at their centromeres and the long (q) arms fuse to form a single chromosome with a single centromere.[25]

Typically, DS patients have characteristic clinical features, such as an up-slant of the eyes, a protruding tongue, a single crease of the hand palm, and a sandal gap deformity of the feet. Most DS children show cognitive impairment, although this varies in severity.[23] DS children carry an increased risk to encounter a wide variety of complex medical problems including congenital cardiac anomalies, anatomical abnormalities of the gastrointestinal tract, autoimmune phenomena such as hypothyroidism and coeliac disease, a vulnerability to recurrent bacterial and viral complications due to an impaired immune system, and an increased risk of hematological malignancies.[23,26-30] The life expectancy of DS children is primarily dependent on the risk of morbidity and mortality in the first year of life.[23]

Leukemia in Down syndrome

DS children have an increased risk of developing leukemia as compared to non-DS children, which was already recognized in the 1930s.[31-34] This increased risk of leukemia includes both the risk for ALL, which is approximately 20-fold higher, as well as for myeloid leukemia of DS (ML DS), which is 150-fold higher.[32] Leukemic disease from the myeloid lineage consists of 2 entities, i.e. transient myeloproliferative disorder (TMD) and ML DS. TMD is a clonal disease that is characterized by immature megakaryoblasts in the fetal liver and peripheral blood.[35,36] In absolute numbers, ALL affects ~5 and ML DS ~8 DS children each year in the Netherlands every year (DCOG registration 2006-2011, Figure 2). The true incidence of TMD is not known, as the course of TMD is uncomplicated and asymptomatic in the vast majority of cases, a diagnosis of TMD is often missed prior to its spontaneous remission. Probably TMD occurs in

less than 10% of newborns with DS, which is based on the finding of *GATA1* mutations (see below) in 590 Guthrie cards in 4% of newborn DS children.[37] The strong predisposition for DS children to develop acute leukemias is as yet not completely understood. Remarkably, the general risk for developing cancer in DS individuals is not increased, as there is a reduced propensity for solid tumors, and even a decreased frequency of secondary malignancies after treatment for prior leukemia.[32,38] Hence, DS is not a cancer susceptibility but a leukemia susceptible syndrome.

DS-ALL patients are different from ALL patients without DS (non-DS ALL) in presenting characteristics. For instance, in DS-ALL there is a lower frequency of T cell ALL, and ALL never occurs in DS infants (<1 year).[8,39-41] Moreover, in DS ALL, favorable subtypes such as high hyperdiploidy and *ETV6-RUNX1*, as well as the unfavorable characteristics such as *MLL* rearrangements and the Philadelphia-chromosome [*BCR-ABL1*] occur less frequent.[6-9] In addition, there is a relatively large group of DS ALL patients (~40%) without known cytogenetic aberrations.[9] It is unknown whether this group comprises '*BCR-ABL1* like' patients, and leukemias with abnormalities of B-cell differentiation genes, as this has not been studied in DS ALL. ML DS is characterized by a unique acquired mutation in the globin transcription factor 1 (*GATA1*).[42-44] These mutations are disease specific and therefore can be used as a marker for MRD.[35] It is unknown whether such a unique genetic event also exists in DS ALL. Recently new molecular aberrations such as mutations of *Janus Kinase 2* (*JAK2*) and rearrangements of *Cytokine Receptor Like Factor 2* (*CRLF2*) were reported to occur in DS ALL, but later they were also identified in non-DS high-risk ALL.[45-55] Their role in DS and non-DS ALL pathogenesis is uncertain, as it is unknown whether they are true leukemia initiating events. To unravel the cytogenetic profile of DS ALL, it is needed to search with new techniques on a molecular level. Furthermore, as all published series are small, large series are needed to determine the prognostic relevance of well-established cytogenetic and novel molecular aberrations, and to study clinical relevant outcome parameters in DS ALL, thereby allowing a sufficient sample size to draw meaningful conclusions.

The outcome of DS ALL patients has been reported to be at best similar and often inferior to that of non-DS ALL patients.[6-8,39,41,56-58] This is in sharp contrast to ML DS patients, who have an excellent prognosis compared to other AML types, based on a very unique chemosensitivity profile with enhanced sensitivity to most AML drugs.[59,60] Moreover, TMD often resolves without therapy, although early death occurs in 10-20% of the affected children.[61,62] The biological basis for the reduced probability of survival in DS ALL is not fully understood. A study from the Children's Oncology Group reported that DS ALL patients treated in SR arms had an inferior outcome, while patients treated in HR arms had similar outcome as compared to non-DS ALL patients.[8] It is therefore questionable whether the National Cancer Institute classification is appropriate for risk-group stratification in DS ALL. It may also suggest that DS ALL cells are relatively resistant to chemotherapy. This is reflected in a small number of cellular cytotoxicity assays showing that DS ALL cells do not have increased sensitivity to

chemotherapy in vitro; indicating that reduction of therapy for DS-ALL patients may not be possible in contrast to ML DS.[60] However, intensification of therapy in these vulnerable patients may neither be feasible nor desirable given the increased risk of treatment-related morbidity and mortality.

Worldwide, DS ALL patients are treated according to the same protocols as their non-DS ALL counterparts, although dose reductions of Methotrexate and / or Anthracyclines are frequently applied This is different from ML DS patients, who are treated according to specific ML DS regimens with reduced treatment intensity in some countries.[63,64]

It is well known that DS ALL patients have a higher susceptibility to the toxic side effects and to treatment related mortality (TRM) compared to non-DS ALL children.[58,65,66] It is conceivable therefore that attempts to decrease morbidity and TRM by reduction of treatment intensity in individual DS ALL patients may have contributed to their inferior outcome.[67] For instance, it is well known that DS-ALL patients are more susceptible to MTX induced side effects than non-DS-ALL patients.[58,65,66,68] However, it is not known whether the enhanced susceptibility for MTX induced side effects is due to the difference in cellular sensitivity (for instance of the mucosa), but whether it also the reflects differences in pharmacokinetics between DS-ALL and non- DS-ALL patients. Moreover it is unknown whether the risk for TRM is related to a specific treatment phase or chemotherapeutic agent.

In order to guide subgroup directed therapy and the development of novel therapies, the frequency and prognostic relevance of conventional and new (cyto-) genetic aberrations needs to be established in large series of DS ALL, despite the caveat of heterogeneity in treatment over time and between different study groups. Furthermore, molecular insight into the pathogenesis of DS ALL is necessary, and this could be achieved by identifying *novel* (and drugable) molecular abnormalities that characterize DS ALL.

OUTLINE OF THIS THESIS

In this thesis we studied the clinical and biological features of DS ALL that determine prognosis (chapter 2-3). Although DS children have an increased risk of developing ALL, it remains a rare disease and almost all published series lack sufficient power to draw meaningful conclusions. Therefore, in chapter 2, we performed a large retrospective study within the international childhood ALL Ponte-di-Legno working group, with the aim to determine clinical relevant outcome parameters, the prognostic relevance of well-established (cyto-) genetic and novel molecular aberrations, and causes of treatment failure in DS ALL. Chapter 3 describes the results of a retrospective case-control study, which defined whether the well-known enhanced susceptibility for MTX-induced side effects is the result of differences in pharmacokinetics between DS-ALL and non-DS ALL patients.

In chapter 4-7 we report on the results of several genome wide techniques to identify specific molecular aberrations involved in the pathogenesis of DS ALL. In chapter 4, we searched for mutations in the kinase and pseudokinase domains of *Janus Kinase (JAK) 1-3* by direct sequencing in order to identify novel aberrations. Moreover, we analyzed the prognostic significance of *JAK2* mutations in DS ALL in our cohort, and included a meta-analysis of all published data. In chapter 5, results of the array comparative genomic hybridization and multiplex ligation-dependent probe amplification studies to determine the frequency and prognostic significance of abnormalities in B-cell development and differentiation genes in a population based DCOG DS ALL cohort are presented, which was validated with DS ALL patients from UK trials. Chapter 6 reports a study on the frequency of *BTG1* deletions in a large series of DS ALL patients and describes the prognostic significance of these abnormalities. In chapter 7 we show the results of gene-expression profiling to determine whether DS ALL can be characterized by differentially expressed genes and pathways as compared to non-DS ALL. Chapter 8 provides the general discussion of this thesis and includes future perspectives for further research. Chapter 9 contains the summary in English and the layman's summary in Dutch.

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Acute lymphoblastic leukemia in children with Down syndrome: a retrospective analysis from the Ponte di Legno study group

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ABSTRACT

Children with Down syndrome (DS) have an increased risk of B-cell precursor acute lymphoblastic leukemia (BCP-ALL). The prognostic factors and outcome of DS-ALL patients treated in contemporary protocols are uncertain. We studied 653 DS-ALL patients enrolled in 16 international trials from 1995-2004. Non-DS BCP-ALL patients from the DCOG and BFM were reference cohorts. DS-ALL patients had a higher 8-year cumulative incidence of relapse (26±2% vs. 15±1%; p<0.001) and 2-year treatment-related mortality (TRM) (7±1% vs. 2.0±<1%; p<0.0001) than non-DS patients, resulting in lower 8-year event-free survival (EFS) (64±2% vs. 81±2%; p<0.0001) and overall survival (74±2% vs. 89±1%; p<0.0001). Independent favorable prognostic factors include age<6 years (hazard ratio [HR]=0.58, p=0.002), white blood cell count (WBC) $<10x10^9$/L (HR=0.60, p=0.005) and *ETV6-RUNX1* (HR=0.14; p=0.006) for EFS, age (HR=0.48, p<0.001), *ETV6-RUNX1* (HR 0.1, p=0.016) and high hyperdiploidy (HeH) (HR 0.29, p=0.04) for relapse-free survival. TRM was the major cause of death in *ETV6-RUNX1* and HeH DS-ALLs. Thus while relapse is the main contributor to poorer survival in DS-ALL, infection-associated TRM was increased in all protocol elements, unrelated to treatment-phase or regimen. Future strategies to improve outcome in DS-ALL should include improved supportive care throughout therapy, and reduction of therapy in newly identified good-prognosis subgroups.

INTRODUCTION

Children with Down syndrome (DS) are predisposed to develop acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL),[1] which are characterized by unique biological features in comparison with those of non-DS ALL.[2-4]

Children with DS-ALL have an inferior outcome compared to non-DS patients because of both higher treatment related mortality (TRM) and higher relapse rate.[5-9] As attempts to decrease TRM by reducing treatment intensity may contribute to the increased risk of relapse in DS-ALL, it is important to determine whether the risk for TRM is related to a specific treatment phase or chemotherapeutic agent.[8-10] Small series suggest that DS-ALL patients have an increased risk of mucositis from methotrexate, myelosuppression from anthracyclines, and hyperglycemia from glucocorticoids.[10-16] Acquired leukemic cell genetic abnormalities have important prognostic significance in non-DS childhood ALL.[17] However, the impact of these abnormalities on treatment outcome in DS-ALL is unknown, as all published series lack a sufficient sample size to draw clear conclusions. Even the prognostic significance of well-known good prognostic factors in non-DS ALL such as the t(12;21)(p13;q22) [*ETV6-RUNX1*], high hyperdiploidy (HeH) and trisomies 4&10 is uncertain in DS-ALL, as well as for the unfavorable translocations including the t(9;22)(q34;q11) [*BCR-ABL1*] and t(4;11)(q21;q23) [*MLL-AF4*].[2] Of interest, these prognostic genetic features have a lower frequency in DS-ALL.[2,7,18,19]

Recently, genetic abnormalities such as *JAK2* mutations[20] and *CRLF2* rearrangements have been identified in both DS and non-DS ALL.[3,4,20-27] Activating *JAK2* R683 mutations were found in ~18% of DS-ALL patients.[20,24] Rearrangements of *CRLF2* occurred in ~60% of DS-ALL patients and in fewer than 10% of non-DS ALL patients.[3,4,23] In almost all instances *JAK2* (or rarely *JAK1*, or *IL7R*) mutations were associated with *CRLF2* gene rearrangements, suggesting a model by which *CRLF2* overexpression results in JAK-STAT activation and proliferation of the leukemic clone.[3] Thus far, *CRLF2* gene rearrangements lack prognostic relevance in DS ALL, although all series were small.[3,4,21,27]

The small size of most studies in DS-ALL patients has precluded definitive answers to the issues raised above. Hence, we undertook a large retrospective study of DS-ALL within the International ALL "Ponte di Legno" Working Group to study clinically relevant outcome parameters, the prognostic relevance of well-established and novel (cyto-) genetic aberrations in ALL, and causes of treatment failure, thereby allowing a sufficient sample size to draw meaningful conclusions, despite the caveat of heterogeneity in treatment over time and between different study groups.[28]

PATIENTS AND METHODS

Patients

Patients eligible for this study were enrolled in various national or collaborative group clinical trials between January 1, 1995 and December 31, 2004, were ≤18 years at diagnosis and were treated with curative intent. The Institutional Review Boards of each participating center approved treatment protocols according to the local law and guidelines. Participating study groups and their number of patients are mentioned in Supplementary Table S1. A predefined set of data was collected, consisting of clinical data obtained at diagnosis, treatment, and cytogenetic and molecular data (Supplementary Table S2).

DS-ALL patients were treated according to standard ALL treatment protocols, but modifications of the standard protocol did occur. None of the protocols provided specific supportive care measures for DS-ALL children. In total, 42.3% (n=276) DS-ALL patients received a reduced dose of chemotherapy. Most of these dose-reductions (79%) were planned prior to the administration of specific courses of chemotherapy and gradually increased by observed clinical toxicity. Modifications for MTX consisted of dose-reductions of high dose MTX, varying from 10-75% of the maximum dose, and intensified leucovorin rescue. DS ALL patients enrolled in EORTC 58951 protocols from September 2002 (n=7) received 0.5g/m^2 of MTX instead of 5g/m^2. In addition, patients treated on protocol POG 9405 (n=10) started with 50% of the total dose of Daunorubicin, Cytarabine, Teniposide, HDAC and Peg-asparaginase, which was successively increased or reduced depending on toxicity. Supplementary Table S3 provides an overview of the main chemotherapeutic agents of treatment protocols used by the various study groups.

Data on either *JAK2* R683 mutations and/or *CRLF2* gene rearrangements were available from a subset of patients (n=182) included in this study. There were no statistical differences between patients with and without available data. Some of these data have been previously reported.[20,24,25] However, several study groups contributed new unpublished data.

Non-DS ALL reference cohort

For comparison, population-based B-cell precursor ALL reference cohorts from the DCOG and the ALL-BFM Study Group, from exactly the same time period as the DS patients (January 1, 1995 and December 31, 2004), were added. The DCOG dataset consisted of 827 non-DS BCP-ALL patients enrolled in 3 DCOG ALL treatment protocols (ALL-8, ALL-9 and ALL-10). The BFM dataset consisted of 3618 non-DS BCP-ALL patients enrolled in 2 BFM treatment protocols (BFM-95 and BFM-2000) in Germany and Austria. Details of these protocols have been reported elsewhere, except for protocol ALL10, which is on-going.[29,30]

There were no significant differences in outcome estimates, nor in the distribution of cytogenetic subgroups, between the DCOG and BFM datasets (data not shown), nor when compared to reported data from other participating groups.[31-38] The DCOG and BFM non-DS datasets were merged for statistical analysis.

Cytogenetic analysis

Genetic abnormalities were determined by G-, Q-, or R-banded karyotyping, fluorescence in situ hybridization (FISH) or reverse-transcribed polymerase chain reaction (RT-PCR). Diagnosis of rearrangements of *ETV6-RUNX1*, *BCR-ABL1* and *MLL* were based on one or more of these techniques; diagnosis of high hyperdiploidy (HeH) was defined by modal chromosomal number ≥52 or DNA index ≥1.13 for DS-ALL patients and ≥51 chromosomes for non-DS patients. All cytogenetic data were centrally reviewed by two co-authors (N.H. and E.F.). The definition and description of clonal abnormalities followed the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN 2005).[39]

CRLF2 gene rearrangements were identified by genomic array, FISH, genomic PCR, Sanger sequencing or Multiplex Ligation-dependent Probe Amplification.

Statistical analyses

Statistical analyses were conducted using SAS software (SAS-PC, Version 9.1). The Kaplan-Meier method was used to estimate survival: complete remission rate (CR), event-free survival (EFS), overall survival (OS), relapse free survival (RFS). The survival estimates were compared using the log-rank test. The cumulative incidence of toxic death (TRM) and the cumulative incidence of relapse (CIR) were calculated by the method of Kalbfleisch and Prentice and compared with the use of Gray's test. CR was defined as less than 5% blasts in the bone marrow, with regeneration of tri-lineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere. EFS was calculated from the date of diagnosis to the date of last follow-up or to the first event, including relapse, death in CR, failure to achieve CR (considered as event on day 0) or second malignancy. Early death was defined as any death within the first 6 weeks of treatment, and was considered as an event on day 0 for statistical analysis. OS was measured from the date of diagnosis to the date of last follow-up or to the date of death from any cause. CIR included death in CR and other events as competing events.

To compare categorical variables χ^2 analyses was used and the Fisher exact test was used for small patient numbers. The non-parametric Mann-Whitney U test was applied for continuous variables. P values ≤ 0.05 were considered as statistically significant (two-tailed testing).

For multivariate analysis, the Cox regression model was used. Continuous variables were categorized according to the National Cancer Institute (NCI) risk criteria.[40] P values ≤ 0.05 were considered as statistically significant (two-tailed testing).

RESULTS

Patient characteristics

In total data of 708 DS-ALL patients were collected, of which 55 were excluded because they did not meet the inclusion criteria; i.e. the karyotype of one patient lacked constitutional trisomy 21; 39 patients were diagnosed outside the inclusion period of the study; 2 patients were not treated with curative intent; and the age of 9 patients was above 18 years at diagnosis (range 18.2 – 21.9). Furthermore, we excluded the 5 patients with T-cell ALL as this number was considered too small for meaningful statistical analysis. However, clinical and cyto-genetic characteristics of these 5 T-cell ALL patients are described in Supplementary Table S4. Hence, 653 patients with DS BCP-ALL were analyzed. DS-ALL patients were slightly older than non-DS patients at diagnosis (median 5.0 vs. 4.7 years; p=0.002) (Table 1), and DS-ALL did not occur in infants. The initial white blood-cell count (WBC) of DS-ALL patients was not different compared with non-DS (median $10.2x10^9$/L (range 0.2–459) vs. $8.9x10^9$/L (range 1.7–998), p=0.14).

Genetic data

All leukemic karyotypes, FISH and RT-PCR results underwent central review; 68% (n=444) of the DS patients had adequate genetic data (Table 1). In total, 40.3% had a cytogenetically normal (CN) karyotype (i.e. only constitutional trisomy 21) compared to 6.9% of the non-DS cases (p<0.001). Nine percent of DS patients had a HeH karyotype compared to 33% of non-DS patients (p<0.001). HeH DS patients were significantly older than HeH non-DS patients (median, 7.2 years vs. 4.2; p<0.001). Trisomies of both chromosomes 4 and 10 were found in 45% of the HeH DS-ALL patients, similar to non-DS HeH patients (42.6%; p=0.77).[18,41]

ETV6-RUNX1 fusion was found in 8.3% of the DS-ALL patients (compared with 25.8% in non-DS; p<0.001), *BCR-ABL1* fusion in 0.7% compared with 2.4% in non-DS (p=0.02) and *MLL* rearrangements in <1% compared with 1.2% in non-DS (p=0.2). The previously reported t(8;14)(q11.2;q32) translocation was found in DS-ALL patients only (2%).[2,42,43]

In total, 182 patients had available data on either *JAK* and/or *CRLF2* aberrations. *JAK2* R683 mutations were found in 21% (n=30) of the 141 DS-ALL patients with available data, of which 83% (n=25) also had a *CRLF2* gene rearrangement. In 69% (n=93) of the 134 DS-ALL patients with available data, *CRLF2* gene rearrangements were found, including 5.4% (n=6) with *IGH@-CRLF2* translocations, and 94.6% (n=87) with *P2RY8-CRLF2* fusions. DS patients with *CRLF2* gene rearrangements were younger compared to DS patients with wildtype *CRLF2* (4.1 vs. 7.7 years, p<0.001), but no difference in diagnostic WBC was observed (14.8 vs. $11.8x10^9$/L, p=0.7). This differs from non-DS patients with *CRLF2* gene rearrangements who

Table 1. Patient characteristics of DS-ALL patients and the DCOG non-DS BCP ALL reference cohort

	DS ALL	non-DS ALL	p
Number	653	4445	
Age at diagnosis (range)	5.0 (1.2-17.9)	4.7 (0.1 - 17.9)	0.002
Sex			
Male	343	2431	
Female	310	2014	0.3
Median initial WBC x 10^9/L (range)	10.5 (0.2-459)	8.8 (0.2 - 999)	0.14
Extra medullary disease			
CNS (%)	16/624* (2.5)	98/4258* (2.2)	0.69
Lymphnodes (%)	134/412* (32.5)	1471/4339* (33.1)	0.57
Hepatomegaly (%)	245/469* (52.2)	3156/4357* (71)	<0.001
Testis (%)	1/296* (<1%)	28/4317 (<1%)	0.51
Cytogenetic subgroups			
Normal karyotype	179 (40.3)	45/650* (6.9)	<0.001
BCR-ABL1 t(9;22)	3 (0.7)	93/3898* (2.4)	0.02
MLL (11q23)	2 (0.5)	36/2966* (1.2)	0.15
ETV6-RUNX1 t(12;21)	37 (8.3)	841/3264* (25.8)	<0.001
HeH[$]	40 (9)	235/708* (33)	<0.001
HeH trisomy 4 & 10	18 (4.1)	100/650* (15.4)	<0.001
HeH, other	22 (5.0)	135/708* (19.1)	<0.001
Others	183 (41.2)	225/650* (34.6)	0.03
8-year OS	74 ± 2%	89 ± 2%	<0.001
8-year EFS	64 ± 2%	81 ± 2%	<0.001
8-year CIR	26 ± 2%	15 ± 1%	0,001
2-year TRM	7 ± 1%	2 ± <1%	<0.001

DCOG, Dutch childhood oncology group; BCP, B-cell precursor; WBC, white blood cell count; CNS, central nervous system involvement at diagnosis (>5 WBC/μl; CNS-3); HeH[$] DS: 52-60 chromosomes, non-DS 51-60 chromosomes; OS, overall survival; EFS, event-free survival; TRM, treatment-related mortality; CIR, cumulative incidence of relapse, *Number of patients available for analysis.

had lower WBC (14.6 vs. 34.6x10^9/L, p=0.004), but did not differ in age (5.1 vs. 4.7 years, p=0.7) compared to wild-type patients (Supplementary Table S5).

Treatment outcome according to clinical data

The median follow up time was 6.8 years for DS-ALL and 8.4 years for non-DS survivors. The CR rate was 96.7% in DS-ALL and 99% in non-DS patients (p<0.001). Induction failures were more frequent in DS-ALL compared to non-DS (3.0% and 1.0% respectively, p<0.001). DS patients had a higher cumulative incidence of relapse (CIR, 26±2% vs. 15±1% at 8 years; p<0.0001), and treatment related mortality (TRM, 7±1% vs. 2±<1% at 2 years; p<0.0001) than non-DS patients, resulting in a lower EFS (64±2% vs. 81±2% at 8 years; p<0.0001) and OS

(74±2% vs. 89±2%; p<0.0001) (Figure 1). In total, 144 DS patients relapsed compared to 650 non-DS patients. The time-to-relapse after CR was significantly longer for DS (median 2.8 years, p25: 1.8 years, p75: 4.0 years), than for non-DS patients (median 2.4 years, p25: 1.4 years, p75: 3.5 years; p=0.007). In addition, 23 DS ALL patients relapsed after 5 years versus 33 non-DS ALL patients, p<0.001. Treatment outcome did not differ significantly between the early (1995-2000) and late treatment eras (2000-2004) for DS patients (8-year: OS 77±3% vs. 73±3%;p=0.7, CIR 26.7±3% vs. 31±6%; p=0.4).

The 379 DS-ALL children below the age of 6 years fared significantly better than the 272 older children (8-year: EFS 70±3% vs. 54±4%; p<0.0001; OS 78±2% vs. 67±3%; p=0.002, CIR 21±2% vs. 34±3%; p=<0.001, and 2-year cumulative incidence of TRM 7±1% vs. 8±2%; p=0.33). Notably, the 126 children aged 6-9 years had a relatively poor outcome (8-year: EFS 51±3%, OS 70±5%), which was due to a very high frequency of relapse (CIR 41±6%), not attributable to any known risk factor(s). Outcome declined with increasing WBC, and was best for the 319 patients with WBC <10x10^9/L due to a low risk of TRM (8-year: 4±1% vs.11±2% for WBC ≥10x10^9/L; p=0.0003) and relapse (8-year: 21±3% vs. 30±3%; p=0.03). These features thus define a favorable risk-group with age <6 years and WBC <10x10^9/L, when compared to the remaining DS patients (8-year: EFS: 78±3% vs. 58±3%, p<0.0001; OS: 87±3% vs. 68±3%, p<0.0001; CIR: 17±3% vs. 30±2%, p=0.003; 2-year TRM: 3±1% vs. 9±1%, p=0.002) (Figure 2, Table 2). These criteria predicted outcome more accurately than the classical NCI-criteria (Figure 3).[40] These features remained significant after excluding patients with *ETV6-RUNX1* rearrangements or trisomies 4&10 from the analysis. The effect of this new PdL risk stratification was consistent among the larger study groups including AIEOP, BFM, CCG, POG, and the

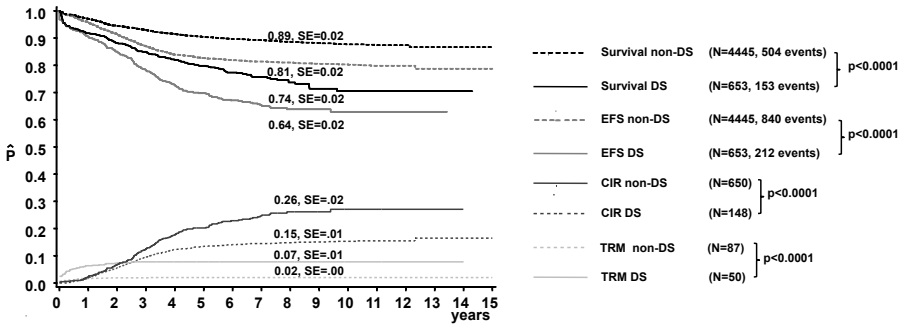

Figure 1. Treatment outcome of the Down syndrome and non Down syndrome ALL patients

The continuous lines represent the DS-ALL patients, the dotted lines represent the non-DS ALL patients. The 100%-black line represents overall survival, the 50%-black line event free survival, the 25%-black line the cumulative incidence of treatment-related mortality and the 75%- black line the cumulative incidence of relapse.

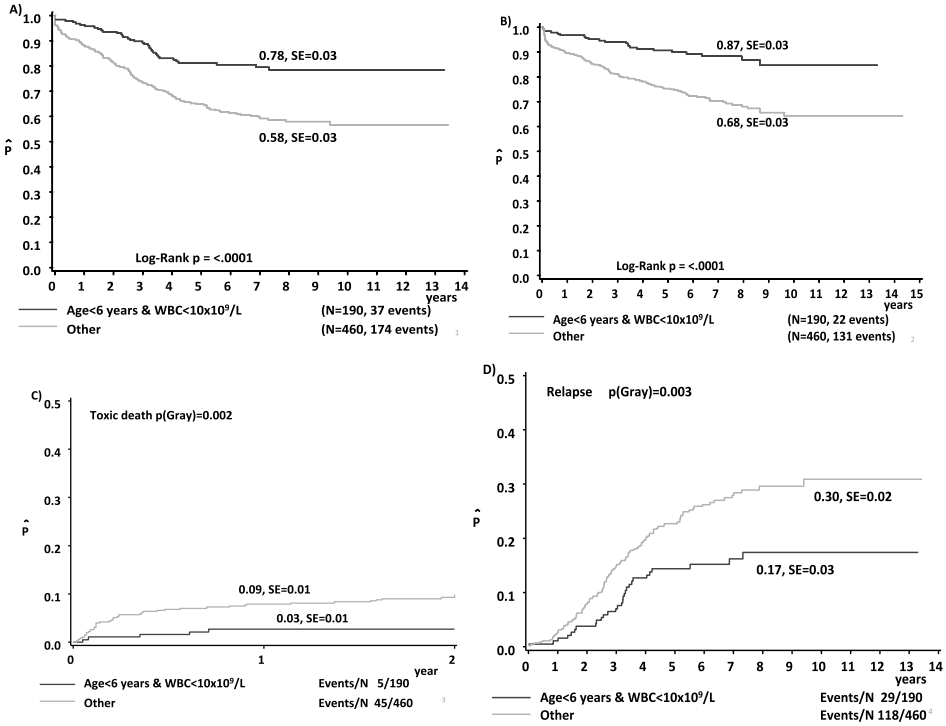

Figure 2. Treatment outcome according to age and white blood cell count in Down syndrome ALL

The overall survival (A), event-free survival (B), cumulative incidence of treatment-related mortality (C) and cumulative incidence of relapse (D) are depicted for patients with age <6 years and WBC <10x10^9/L (black line) versus all other DS-ALL patients (grey line). The numbers on the curves for overall survival and event-free survival represent results at 8 years. The numbers on the curves for treatment related mortality are 2-year results (during treatment only) and those for relapse are results at 8 years.

UK with a HR of 1.62 for high-risk patients from the UK, and 3.79 for BFM patients. Among patients with age >6 years and WBC >10x10^9/L, DS patients had a poorer outcome than non-DS patients (8-year: EFS: 58±3% vs. 78±1%, p<0.001; OS: 68±3% vs. 86±1%, p<0.001; CIR: 30±2% vs. 17±1%, p<0.001; 2-year TRM: 10±1 vs. 2±<1%, p<0.0001). The clinical characteristics of DS-ALL patients (n=246) classified as NCI low risk, but considered high-risk according to our criteria are described in Supplementary Table S6.

In total, 18 (2.8%) of the DS-ALL patients received a stem-cell transplantation, 3 in CR1 and 15 in CR2. Of these patients, 6 are alive in continuous CR, and 12 patients died (1 graft versus host disease, 1 toxic non-infectious event, 1 infection, and 9 relapsed).

Table 2. Contingency table representing outcome of Down syndrome patients by NCI risk group and PdL risk group criteria

		Ponte di Legno Risk Model		
		Low risk	High risk	
Classical NCI criteria	Low risk	N=187	N=246	
		EFS 78±3%	EFS 63±4%	NCI LR
		OS 87±3%	OS 73 ±3%	N=433
		TRM 3±1%	TRM ±%	
		CIR 17±3%	CIR ±%	
	High risk		N=218	
			EFS 57±4%	NCI HR
		N=0	OS 62±4%	N=218
			TRM 12±2	
			CIR 29±3%	
		PdL LR model	PdL HR model	Total
		N=187	N=464	N=651

EFS, event-free survival; OS, overall survival; TRM, treatment-related mortality; CIR, cumulative incidence of relapse; SR, standard risk; HR, high risk; PdL, Ponte di Legno; Classical NCI risk criteria, age 1-9 or ≥10 years at diagnosis and WBC < or ≥50x10^9/L; Ponte di Legno low risk criteria; age <6 years and WBC <10x10^9/L, Ponte di Legno high risk criteria; all other patients.

Treatment outcome according to genetic data

The 37 DS-ALL patients with *ETV6-RUNX1* had significantly better outcome than the other DS patients: 8-year EFS 95±4% vs. 63±3% (p=0.001), OS 97±3% vs. 75±2% (p=0.007), CIR 3±3% vs. 26±2% (p=0.004), and 2-year: TRM 3±3% vs. 8±1%; (p=0.2). DS ALL patients with *ETV6-RUNX1* did not differ in outcome when compared to the 841 non-DS patients with this abnormality (8-year: EFS 95%, p=0.48; OS 96%, p=0.91; CIR 7%, p=0.32; 2-year TRM 1%, p=0.19).

The 40 HeH DS-ALL patients had a significantly lower CIR than the other DS-ALL patients (8-year: 8±5% vs. 26±3%, p=0.02). However a relatively high rate of TRM (2-year: 13±5% in HeH vs. 7±1% in non-HeH DS; p=0.2) resulted in similar 8-year EFS (77±7% vs. 65±3%, p=0.28) and OS (79±6% vs. 76±2%, p=0.88). TRM in these HeH patients was not exclusively seen in one treatment strategy, but was spread across the different treatment protocols. HeH DS-ALL patients showed lower OS when compared to the 235 HeH non-DS patients due to increased TRM (8-year: OS 79±6% vs. 93±2%; p=0.009, EFS 77±7% vs. 86±2%; p=0.06, CIR 8±5% vs. 11±2%; p=0.7, 2-year: TRM 13±5% vs. 1±1%, p<0.001).

The subgroup of HeH DS-ALL patients with trisomies 4&10 (n=18) showed a trend towards better outcome, when compared to all other DS-ALL patients (8-year: EFS 88±8% vs. 65±3%, p=0.09; OS 88±8% vs. 76±2%, p=0.32; CIR 0% vs.25±2%, p=0.03; 2-year: TRM 12±8% vs.7±1%, p=0.6). No DS patients with these trisomies did relapse, and all events were due to toxicity. Their outcome was similar when compared to non-DS patients with trisomy 4&10 (8-year: EFS 90.8±3%; p=0.75, OS 92.3±4%; p=0.65, CIR 5.1±2%; p=0.34, 2-year: TRM 3.0±2%, p=0.1).

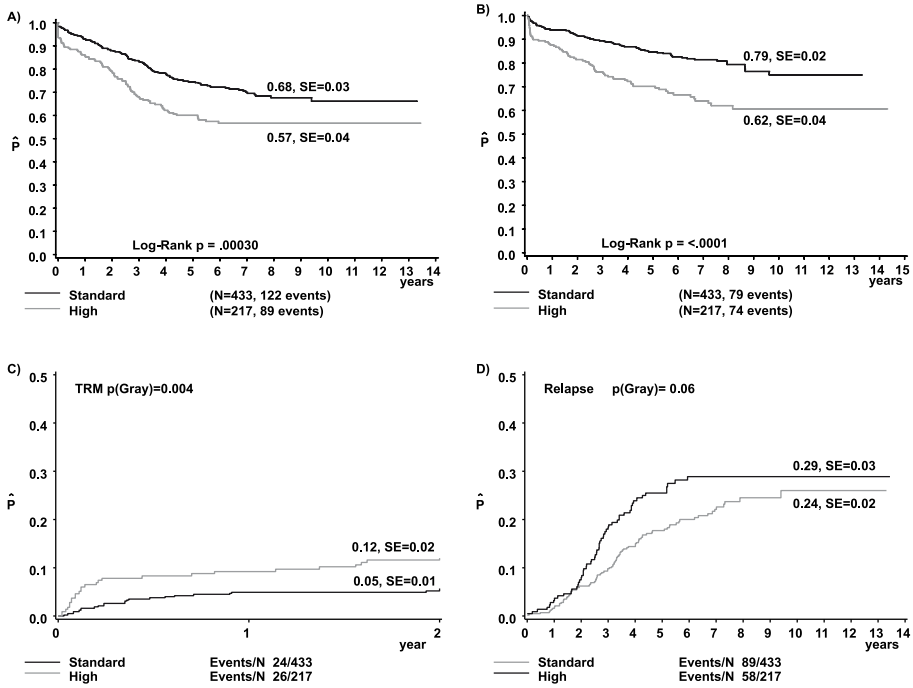

Figure 3. Treatment outcome according to standard- and high-risk National cancer Institute criteria in Down syndrome ALL patients

The overall survival (A), event-free survival (B), cumulative risk of mortality (C) and cumulative risk of relapse (D) depicted for patients with NCI standard-risk (black line) and high-risk (grey line) patients with cut off values for WBC < or ≥ $50x10^9$/L and age < or ≥10 years. The numbers on the curves for overall survival and event-free survival represent results at 8 years. The numbers on the curves for treatment-related mortality are 2-year results and those for relapse are results at 8 years.

DS ALL patients with or without *JAK2* mutations had similar treatment 8-year outcomes (EFS 57±10% vs. 69±5%, p=0.1; CIR 26±9% vs. 23±5%, p=0.48). No data were available in the reference cohort. The 93 DS ALL patients with *CRLF2* aberrations showed no significant difference in 8-year survival compared to the 41 wild-type DS ALL patients (EFS 62±6% vs. 71±8%, p=0.21; OS 73±5% vs. 83±8%, p=0.13; CIR 26±6% vs. 22±8%; p=0.44). DS ALL patients with *CRLF2* gene rearrangements did not differ in outcome from non-DS ALL patients with these aberrations (8-year: EFS 62±6% vs. 58±9%; p=0.7; OS 73±5% vs. 79±8%; p=0.6; CIR 26±6% vs. 38±9%; p=0.15). Median time to relapse for DS patients with *CRLF2* aberrations was 29 months versus 51 months in patients with wildtype *CRLF2* (p=0.11).

Treatment related mortality

In total, 7.7% of the DS-ALL patients died from other causes than relapsed/refractory disease compared to 2.3% in non-DS (p<0.001). TRM occurred at all phases of therapy, including main-

tenance (supplementary Table S7). TRM death during induction occurred in 2.8% (n=18) of the DS patients (13 infectious, 5 non-infectious deaths). In CR, 4.9% (n=32) of the DS patients died of TRM (25 infectious, 7 non-infectious). The most common cause of TRM was infection, mainly respiratory and bacterial infections. Only 0.3% (n=2) of the DS ALL patients died of second malignancies in CR1 (secondary AML and Epstein-Barr virus lymphoproliferative disease), compared to 1.3% of the non-DS patients, p<0.04. Secondary malignancies in non-DS patients included 28 AML/MDS, 5 brain tumors, 9 other tumors, and 13 other malignancies.

TRM was not significantly different between DS patients treated on the CCG/POG/UK studies (3-drug induction) and those DS patients treated on AIEOP/BFM-studies (4-drug induction): the rate of death during induction was 1.1±1% vs. 1.9±1% (p=0.7) and the 2-year cumulative rate was 7±2% vs. 8±3 % (p=0.99). The inclusion of an anthracycline in induction (4-drug induction) had no impact on TRM.

Multivariate analysis

Stepwise multivariate Cox regression analysis of EFS revealed age <6 years (HR=0.58; 95%CI= (0.41–0.81); p=0.002), WBC <10x10^9/L (HR=0.60; 95%CI= (0.42–0.86); p=0.005), and *ETV6-RUNX1* (HR=0.14; 95%CI=(0.03–0.57); p=0.006) as independent predictors for favorable outcome. They also independently predicted OS (Age HR=0.66, p=0.04; WBC<10x10^9/L HR=0.51 p=0.003; and *ETV6-RUNX1* HR=0.12, p=0.04). Relapse-free survival (RFS) was predicted by age, *ETV6-RUNX1*, and HeH (Table 3).

In non-DS ALL the classical NCI criteria are comprised by age and the initial WBC, however *ETV6-RUNX1* and trisomy 4&10 are independent predictors for favorable outcome (*ETV6-*

Table 3. Multivariate analysis of the DS-ALL dataset

Outcome	Variable	HR	95% CI	*P*-value
EFS	Age < 6 years	0.58	0.41 - 0.81	0.002
	WBC < 10 x 10^9/L	0.60	0.42 - 0.86	0.005
	TEL/AML1	0.14	0.03 - 0.57	0.006
	HeH	0.68	0.34 - 1.36	0.275
OS	Age < 6 years	0.66	0.44 - 0.99	0.044
	WBC < 10 x 10^9/L	0.51	0.33 - 0.79	0.003
	TEL/AML1	0.12	0.02 - 0.86	0.035
	HeH	1.01	0.48 - 2.11	0.983
RFS	Age < 6 years	0.48	0.32 - 0.73	0.000
	WBC < 10 x 10^9/L	0.71	0.46 - 1.08	0.105
	TEL/AML1	0.01	0.01 - 0.64	0.016
	HeH	0.29	0.09 - 0.92	0.036

HR, hazard ratio; CI, confidence interval; EFS, event free survival; OS, overall survival; RFS, relapse-free survival; WBC, white blood-cell count; HeH, high hyperdiploid (≥52 chromosomes).

RUNX1: HR=0.29; 95%CI= (0.15–0.58); p<0.001; or trisomy 4&10: HR=0.37; 95%CI= (0.17–0.79); p=0.011). NCI-criteria retained their prognostic value in a Cox model with these three variables (HR 1.96; 95%CI= (1.30-2.95), p=0.001). In addition, multivariate analysis showed that the PdL criteria are not driven by the large group of DS-ALL patients having *CRLF2* aberrations (HR=0.66; 95%CI= (0.33–1.33); p=0.25), but more likely by age and initial WBC (HR=2.16; 95%CI= (0.95–4.90); p=0.07).

DISCUSSION

Many study groups have reported the worse clinical outcome of DS-ALL, however, almost all reports lack sufficient power to answer relevant biological questions in DS-ALL, which is the reason the Ponte di Legno group undertook this retrospective review. The unprecedented size of this study cohort resolves the controversy of the frequency and clinical impact of specific (cyto-) genetic aberrations in DS ALL.[2,18] Moreover, the scale of the study enabled the identification of relatively small subgroups of DS-ALL with favorable outcomes. Analysis of 444 DS-ALL patients with known cytogenetics, demonstrated that the genetic subgroups predicting favorable outcome in non-DS ALL,[2,6,7,18,41,44] also predict favorable outcome in DS-ALL. Most significant is the discovery that *ETV6-RUNX1* conferred an excellent prognosis, and that HeH with trisomy of chromosomes 4&10 was associated with a very low CIR. Hence these patients, comprising 12% of DS-ALL, may be eligible for future treatment reduction to reduce TRM, and can be treated according to the same risk-stratified algorithms as non-DS patients in the collaborative study group protocols.

Another novel finding of this study was the identification of a clinically favorable prognostic subgroup of DS-ALL patients, characterized by age <6 years and WBC <10x10^9/L. These cut points differ from those used in the classical NCI ALL risk criteria, although the biological basis for this difference is not fully understood.[40] No genetic abnormalities were identified that could explain this difference between the classical NCI- and the herein reported criteria. Remarkably, children aged between 6 and 9 years at diagnosis, had a relatively poor outcome similar to high-risk ALL patients, which was due to a high frequency of relapse. This subgroup may be treated according to a medium or high-risk arm of future collaborative study group protocols. Unraveling the genetic background of the leukemia in this subgroup will be required in order to design more rational therapy for these patients. Noteworthy, MRD was not routinely determined during the era of this study, and it is unclear whether MRD would confirm these novel risk-groups. Since MRD was proven to be a powerful tool in non-DS ALL risk assignment,[45,46] further research is needed to validate whether a MRD based strategy is desirable in future DS ALL treatment protocols.

In general, we showed that DS-ALL patients have an inferior survival when compared to a representative non-DS ALL cohort treated in the same time period, which is in agreement

with previous smaller studies.[5,10,47] Despite a high rate of TRM, and different from what is often suggested, relapse remained the main cause of treatment failure in DS patients. Interestingly the relapses tend to occur later in DS. It is unclear if this is due to the genetic makeup of DS-ALL or to decreased immune surveillance of the residual leukemia in DS patients. It cannot be ruled out that under-reported treatment reduction of patients with DS-ALL contributes to the increased relapse risk.[48] This finding suggests that the currently accepted strategy of treatment reduction in DS-AML, which is characterized by a chemotherapy-sensitive phenotype,[49] is not applicable to DS-ALL.[47] The only exception may be DS-ALL patients with *ETV6-RUNX1* or HeH, in which TRM outweighed the risk of relapse, for whom a 3-drug induction and a limited re-induction might be adequate. Interestingly and in accordance with previous results, the incidence of secondary malignancies was significantly lower in DS patients as compared to non-DS ALL patients. This is in agreement with the reduced propensity for solid tumors in DS patients reported before.[50]

The genetic basis of the aggressive clinical behavior of DS-ALL is still unknown. A high proportion of DS-ALLs have normal karyotype (40.3% compared with 6.9% of non-DS), suggesting the presence of cytogenetically invisible molecular abnormalities. One of these abnormalities, detected in 60% of DS-ALLs is the aberrant expression of *CRLF2*, which is often associated with *JAK-STAT* mutations. In contrast to some studies showing deleterious effects of *CRLF2* alterations in non-DS high-risk ALL,[26,51] no such association was found in this study, nor in several prior smaller studies of DS-ALL.[3,4,21,27] Nevertheless, a substantial proportion of DS ALL patients carry these aberrations, thereby providing a pathway which might be targeted by inhibitors of the JAK-STAT pathway or mTOR signaling.[52]

IKZF1 mutational status was unknown in our dataset. Recently it was shown that this gene was frequently deleted in DS-ALL patients (in ~35%), and was found to be an independent predictor for dismal outcome.[27] Of note, the median age of patients with *IKZF1* aberrations in the DS-ALL study was significantly higher compared to wildtype patients (8.2 vs. 4.3 years), which could be an important genetic factor underlying the biological basis for the age cut-off point of 6 years reported here as clinically significant.

Previous studies reported increased TRM in children with DS-ALL[9], also in relapse protocols.[53] The large size of our cohort enabled the observation that the increased TRM is present throughout treatment, with about half of the deaths occurring during maintenance therapy. While doses of myelosuppressive chemotherapy are typically adjusted during maintenance therapy, to maintain an adequate neutrophil count, this phase of treatment may nevertheless lead to B-cell depletion and hypo-gammaglobulinemia, and hence to a higher infection rate in already immune-compromised DS patients.[54,55] To reduce TRM, we suggest improving supportive care throughout the treatment period with aggressive treatment of infections, and studies analyzing the potential benefit of anti-bacterial and anti-fungal prophylaxis, and/or immunoglobulin substitution. Patients should be leucocyte depleted as non-DS patients during maintenance in order to prevent relapse, but with prompt interruptions for aplasia

and with more intensive surveillance than non-DS children. In conclusion, this large international study demonstrated that the poorer survival seen in DS-ALL is mainly due to a higher relapse rate, and less so to TRM. Therefore, treatment reduction is not warranted, except for the 12% of patients with HeH or *ETV6-RUNX1* in which toxicity is the major cause of mortality. As TRM occurs throughout therapy and is not associated with a specific chemotherapy regimen, better surveillance and improved supportive care measures throughout therapy need to be evaluated. As a result of this study an initiative is underway to develop an international treatment protocol for children with DS-ALL.

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Supplementary Table S1. Participating study groups

Study group	Number of included patients
North American Children's Oncology Group including the Children's Cancer Group and the Pediatric Oncology Group studies	202
German Berlin-Frankfurt-Münster Study Group	109
UK Medical Research Council	66
Italian Associazione Italiana di Ematologia ed Oncologia Pediatrica	63
Scandinavian Nordic Society for Pediatric Hematology and Oncology	43
Belgian European Organization for Research and Treatment of Cancer	31
Dutch Childhood Oncology Group	30
North American Dana Faber Cancer Institute	27
Japan Association of Childhood Leukemia Study	18
German Cooperative study-group for childhood acute lymphoblastic leukemia	16
St. Jude Children's Research Hospital	13
Tokyo Children's Cancer Study Group	10
Taiwan Pediatric Oncology Group	9
Polish Paediatric Leukaemia and Lymphoma Study Group	8
Israel National Study group for Childhood ALL	8

Supplementary Table S2. Collected variables in the dataset

Collected variables in the dataset	
Patient ID	Name of protocol
Study group	Use of Anthracylines in induction: yes/no
Lab ID cytogenetic lab	Steroids in induction: Prednisone or Dexamethasone
Date of birth	L' asparaginase in induction: yes or no
Gender	Dose of Methotrexate: gram/m^2
Age at diagnosis	Therapy reduction: yes/no, agent, dose
Date of diagnosis	Day 6-9 BM evaluated
Hepatomegaly	Day 6-9 BM evaluated
Splenomegaly	Day 6-9 PB evaluated
Lymphnodes	Day 6-9 PB evaluated
Mediastinal involvement	Day 12-16 BM evaluated
Testicular infiltration	Day 12-16 BM evaluated
Hemoglobine	Start date and name of therapy block 1
Platelets	Start date and name of therapy block 2
White bloodcell count	Start date and name of therapy block 3
CNS involvement	Start date and name of therapy block 4
Percentage blasts in PB by morphology	Start date and name of therapy block 5
Percentage blasts in PB by immunology	Start date and name of therapy block 6
BM aspirate: percentage blasts morphologically	Start date and name of therapy block 7
BM aspirate: percentage blasts immunological	Start date and name of therapy block 8

Supplementary Table S2. (Continued)

Collected variables in the dataset	
Trephine % blasts morphologically	Irradiation and dose
Trephine % blasts immunology	Therapy Comments
Immunophenotype determined on PB or BM	Stemcell transplantation, type and date
Immunophenotype	Date of CR
Date karyotype	Date of relapse
Karyotype on PB or BM	Date and type of secondary malignancy
Karyotype (ISCN 1995 nomenclature)	Date and status at last follow up
Number of metaphases	Date of death
Ploidy	Cause of death
FISH for *ETV6-RUNX1*, *BCR-ABL*, *MLL-AF4*, and any other FISH performed.	General comments
Percentage of positive cells in FISH	
Number of assessed nuclei in FISH	
PCR for *ETV6-RUNX1*, *BCR-ABL*, *MLL-AF4*, and any other PCR performed.	
CRLF2 gene rearrangement	
JAK 1-3 mutation	
DNA index	
If available percentage of positive cells of CD33, CD 117, TdT, HLA-DR, CD19, CD10, CD20, CD22, CD79a, CD1, TCR a/b, TCR g/d, CD13, CD33, cylgM, CD2, CD3m, CD3cy, CD4, CD5, CD7, CD8	

CNS, central nervous system; PB, peripheral blood; BM, bone marrow; CD, cluster of differentiation; CR, complete remission.

Supplementary Table S3. Details of the various treatment protocols used in this study

Study group	Protocol	Methotrexate (gram/m^2)	Anthracycline	Induction steroids	L'asparaginase
AIEOP	AIEOP ALL 95 SR	1[1]	No	Prednisone	Yes
AIEOP	AIEOP ALL 95 MR	1[1]	Yes	Prednisone	Yes
AIEOP	AIEOP ALL 95 HR	1[1]	Yes	Prednisone	Yes
AIEOP	AIEOP ALL 2000	1[1]	Yes	Prednisone	Yes
AIEOP	AIEOP ALL 2000 (SR1)	1[1]	Yes	Prednisone	Yes
AIEOP	AIEOP ALL 2000 (SR2)	1[1]	Yes	Prednisone	Yes
AIEOP	AIEOP ALL 2000 (MR1)	1[1]	Yes	Prednisone	Yes
AIEOP	AIEOP ALL 2000 (MR2)	1[1]	Yes	Prednisone	Yes
AIEOP	AIEOP ALL 2000 (DXM)	1[1]	Yes	Dexamethasone	Yes
AIEOP	AIEOP ALL 2000 (DXM-SR1)	1[1]	Yes	Dexamethasone	Yes
AIEOP	AIEOP ALL 2000 (PDN-MR1)	1[1]	Yes	Prednisone	Yes
AIEOP	AIEOP ALL 2000 (DXM-MR2)	1[1]	Yes	Dexamethasone	Yes
AIEOP	AIEOP ALL 2000 (PDN-MR2)	1[1]	Yes	Prednisone	Yes

Supplementary Table S3. (Continued)

Study group	Protocol	Methotrexate (gram/m^2)	Anthracycline	Induction steroids	L'asparaginase
BFM-A	ALL-BFM 95 SR	5	Yes	Prednisone	Yes
BFM-A	ALL-BFM 95 MR	5	Yes	Prednisone	Yes
BFM-A	ALL-BFM 2000 SR	5	Yes	Prednisone	Yes
BFM-A	ALL-BFM 2000 MR	5	Yes	Prednisone	Yes
BFM-G	ALL-BFM 95 SR	5	Yes	Prednisone	Yes
BFM-G	ALL-BFM 95 MR	5	Yes	Prednisone	Yes
BFM-G	ALL-BFM 2000 SR	5.0[2]	Yes	Prednisone	Yes
BFM-G	ALL-BFM 2000 MR	5.0[2]	Yes	Prednisone	Yes
BFM-G	ALL-BFM 2000 HR	5.0[2]	Yes	Prednisone	Yes
CCG	1952	No HD MTX	No	No	No
CCG	1961	No HD MTX	Yes	No	No
CCG	1991	No HD MTX	No	No	No
POG	9201	0.5[3]	No	No	No
POG	9405	1[4]	No	No	No
POG	9406	1[4]	Yes	No	No
POG	9605	0.5[3]	No	No	No
POG	9904	0.5[3]	No	No	No
POG	9905	0.5[3]	No	No	No
POG	9906	0.5[3]	Yes	No	No
DCOG	ALL8	5	Yes	Prednisone	Yes
DCOG	ALL9 NHR	3	No	Dexamethasone	Yes
DCOG	ALL9 HR	5	Yes	Dexamethasone	Yes
DCOG	ALL10	5	Yes	Prednisone	Yes
DFCI	95001 (SR)	4	Yes	Prednisone	Yes
DFCI	95001 (HR)	4	Yes	Prednisone	Yes
DFCI	00001 (SR)	4	Yes	Prednisone	Yes
DFCI	00001 (HR)	4	Yes	Prednisone	Yes
EORTC	EORTC CLG 58881	5	Yes	Prednisone	Yes
EORTC	EORTC CLG 58951	5[5]	Yes	Randomized trial	Yes
Israel	93-01(ALL -BFM 90 mod)	5	Yes	Prednisone	Yes
Israel	98-01(ALL-BFM 95 SR)	5	Yes	Prednisone	Yes
Israel	98-02(ALL-BFM 95 MR)	5	Yes	Prednisone	Yes
Israel	03-02-00(IC-BFM 2002)	5	Yes	Prednisone	Yes

[1] First high dose MTX course is 1g/m^2, if tolerated 2nd course 2g/m^2; [2] First high dose MTX course 0.5g/m^2, if tolerated 2nd course 2g/m^2, if tolerated following course 5g/m^2; [3] First course of MTX 0.5g/m^2, if tolerated 2nd course 1g/m^2. If not tolerated, continue to reduce each dose by 25% until tolerated; [4] DS patients not eligible for randomization to 2.5 mg/m^2 MTX; [5] From September 2002, DS ALL patients were only enrolled in VLR and AR1 risk groups, and received 0.5g.m^2 MTX; [6] Children <4 years of age receive 8 g/m^2, and those older received 6g/m^2. [7]High dose MTX is limited to 0.5g/m^2 for all course, leucovorin rescue from T=30 and vigorous hydration; [8] High dose MTX is limited to 0.5g/m^2, normal dose is 2.5 g/m^2 for SR and 5.0 g/m^2 for HR patients.

Supplementary Table S4. Patient characteristics and outcome parameters of the 5 excluded T cell DS-ALL patients

ID no.	Age, y	Sex	WBC, x10^9/L	CNS	Lymphnodes	Hepatomegaly	Testis	Karyotype	CR	Relapse (RFS)	Dead (OS)
1	13,6	M	55,9	No	No	No	No	47,XY,+21c	Yes	No	No (101)
2	9,1	F	231,6	No	No	No	NK	47,XX,+21c	Yes	No	No (78)
3	2,2	M	50,2	No	No	No	No	47,XY,t(1;16)(p34),t(3;6)(q24;p12),del(4)(q34),der(7)t(1;7)(q32;q36),+21c[18]	Yes	Yes (13.2)	Yes (18.5)
4	11,2	M	0,6	No	Yes	Yes	No	NK	NA	NK	No (60)
5	3,5	M	5,5	No	NK	Yes	NK	47,XY,+21c[10]	Yes	No	No (154)

WBC, white blood cell count x 10^9/L; CNS, central nervous system; CR, complete remission; RFS, relapse free survival in months; OS, overall survival in months; M, male; F, female; NK, not known

Supplementary Table S5. *JAK2* and *CRLF2* subgroup analysis

	JAK2/*CRLF2* cohort (n=182)	Residual cohort (n=471)	P-value
Age at diagnosis (range)	5.1 (1.2-17.2)	5.0 (1.2-17.9)	0,6
Sex			
Male (%)	98 (54)	245 (52)	
Female (%)	84 (46)	226 (48)	0,68
Median initial WBC x 10^9/L (range)	11.7 (1.0-322)	9.5 (0.2-459)	0,15
Extra medullary disease			
CNS (%)	8/176* (4.5)	8/440* (1.8)	0.06
Molecular aberrations			
JAK1	2/141* (1.4)		
JAK2 R683	30/141* (21.3)		
IGH@-CRLF2	6/134* (4.5)		
P2RY8-CRLF2	87/134* (69.4)		
8-year OS	73 ± 5%	83 ± 8%	0,13
8-year EFS	62 ± 6%	71 ± 8%	0,21
8-year CIR	26 ± 6%	22 ± 8%	0,44

*Number of patients available for analysis.

Supplementary Table S6. Patient characteristics of NCI standard risk DS ALL patients considered high risk by Ponte di Legno DS ALL risk group criteria

	High risk modified model
Number	246
Age at diagnosis (range)	5.2 (1.2 - 9.9)
Sex	
Male	136
Female	110
Median initial WBC x 10^9/L (range)	16.4 (0.5 - 48.8)

Supplementary Table S6. (Continued)

	High risk modified model
Extra medullary disease	
CNS (%)	6/234* (2.6)
Lymph nodes (%)	64/159* (40.3)
Hepatomegaly (%)	100/171* (58.5)
Testis (%)	1/117* (<1)
Survival	
10-year OS	73±3%
10-year EFS	63±4%
10-year TRM	7±2%
2-year CIR	30±4%
Median time to relapse in years (range)	3.2 (4.4 months - 9.3 years)
Cytogenetic subgroups	171*
Normal karyotype (%)	71 (41.5)
MLL (%)	1 (<1)
ETV6-RUNX1 (%)	11 (6.4)
HeH (%)	18 (10.5)
Other (%)	70 (40.9)

WBC, white blood cell count; CNS, central nervous system; OS, overall survival; EFS, event-free survival; TRM, treatment-related mortality; CIR, cumulative incidence of relapse; HeH, high hyperploidy (≥52 chromosomes); *Number of patients available for analysis.

Supplementary Table S7. Specified cause of death for treatment related mortality

Patient ID	Cause of death	Treatment phase	Study group
1	Infection	Induction	DFCI
2	Infection	Induction	AIEOP
3	Infection	Induction	UKCLWP
4	Suspected infection, unknown organism	Induction	NOPHO
5	Suspected infection, unknown organism	Induction	NOPHO
6	Infection and Epidermolysis Bullosa	Induction	UKCLWP
7	Gram positive infection: Streptococcus alpha	Induction	CCG
8	Pseudomonas aeruginosa	Induction	NOPHO
9	Pseudomonal aeruginosa septicaemia	Induction	TPOG
10	Serratia marcescens in blood and urine	Induction	CCG
11	Para-influenza pneumonitis	Induction	UKCLWP
12	Adenovirus during leukopenia	Induction	COALL
13	Candida Albicans pneumonia	Induction	SJCRH
14	Chemotherapy related toxicity	Induction	NOPHO
15	Chemotherapy related toxicity	Induction	BFM-Austria
16	Central nervous system toxicity	Induction	CCG

Supplementary Table S7. (Continued)

Patient ID	Cause of death	Treatment phase	Study group
17	Coagulation disturbances (thrombosis in oment to intestinal ischemia)	Induction	NOPHO
18	Hemorrhage	Induction	UKCLWP
19	Pseudomonal septicemia	Delayed intensification	UKCLWP
20	Gram negative septicemia, aspiration and septic shock	Delayed intensification	UKCLWP
21	Rhinovirus	Delayed intensification	UKCLWP
22	Respiratory syncytial virus	Delayed intensification	CCG
23	Sepsis	Interim maintenance	BFM-Germany
24	Cardiopulmonal decompensation	Interim maintenance	BFM-Germany
25	Infection during aplasia	Consolidation	BFM-Germany
26	Infection and neutropenia	Consolidation	UKCLWP
27	Hemorrhage	Consolidation	UKCLWP
28	Atypical pneumonia during aplasia	Consolidation	BFM-Germany
29	Candida septicemia	Intensified consolidation	POG
30	Infection	Maintenance	DFCI
31	Fulminant sepsis, multi organ failure	Maintenance	BFM-Germany
32	Detection of adenovirus in stool and serum, acute liver failure	Maintenance	BFM-Germany
33	Infection, pneumonia	Maintenance	BFM-Germany
34	Sepsis, aplasia	Maintenance	BFM-Germany
35	Septic shock	Maintenance	UKCLWP
36	Septic shock	Maintenance	TPOG
37	Septic shock, Acute Respiratory Distress Syndrome	Maintenance	DCOG
38	Pseudomonal septicaemia	Maintenance	UKCLWP
39	Pneumonia, septicaemia, acute renal failure	Maintenance	UKCLWP
40	Infection and lympho proliferative disease (EBV associated)	Maintenance	POG
41	Bacterial pneumonia	Maintenance	UKCLWP
42	Bacillus meningitis	Maintenance	CCG
43	Aspergillus pneumonia	Maintenance	UKCLWP
44	RSV pneumonitis	Maintenance	UKCLWP
45	Influenza	Maintenance	UKCLWP
46	Cardiopulmonary failure secondary to overwhelming sepsis, presumed viral etiology	Maintenance	POG
47	Cardiopulmonal circulatory failure during conditioning for SCT	Maintenance	BFM-Germany
48	Chemotherapy related toxicity	Maintenance	CCG
49	Renal insuffiency	Maintenance	EORTC-CLG
50	Hemorrhage	Maintenance	UKCLWP

3

Methotrexate induced side effects are not due to differences in pharmacokinetics in children with Down syndrome and acute lymphoblastic leukemia

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ABSTRACT

Children with Down Syndrome (DS) have an increased risk of developing acute lymphoblastic leukemia (ALL). They have a poor tolerance for methotrexate (MTX). This is assumed to be caused by a higher cellular sensitivity of the tissues in DS children. However, whether differences in pharmacokinetics (PK) play a role is unknown. We compared MTX induced toxicity and pharmacokinetics in a retrospective case-control study between DS-ALL and non-DS ALL patients. Population PK-models were fitted to data from all individuals simultaneously, using non-linear mixed effect modeling. 468 MTX courses (1-5 g/m^2) were given to 44 DS and 87 non-DS ALL patients. Grade 3-4 gastro-intestinal toxicity was significantly more frequent in DS versus non-DS children (25.5% vs. 3.9%; p=0.001). Moreover, the occurrence of grade 3-4 gastro-intestinal toxicity was not related to plasma MTX area under the curve (AUC). MTX-clearance was 5% lower in DS-ALL patients (p= 0.001), however this small difference is probably clinically not relevant, because no significant differences in MTX plasma levels were detected at T=24 and at T=48 hours. No major safety concerns were observed when using intermediate dosages of MTX (1-3 gr/m^2) in DS-ALL children in this study, and hence this may be a safe dose for DS-ALL children.

INTRODUCTION

Down syndrome (DS) is one of the most common congenital chromosome abnormalities, with a prevalence of 16 per 10.000 live births in the Netherlands.[1] Children with DS have an increased risk of developing both acute myeloid, as well as acute lymphoblastic leukemia (DS-ALL).[2]

DS-ALL patients differ in presenting characteristics from ALL patients without DS (non-DS ALL). For instance, in DS-ALL a lower frequency of T-cell ALL[2-5] and CD-10 negative ALL (pro-B cell ALL) is found.[4] Moreover, there are differences in the distribution of genetic abnormalities, with lower frequencies of unfavorable characteristics such as MLL-AF4 and the Philadelphia-chromosome, as well as lower frequencies of favorable characteristics such as high hyperdiploidy and TEL-AML1 in DS-ALL cases.[2,3,6]

Several studies indicate a poorer outcome for children with DS-ALL compared to non-DS ALL patients.[2,3,7] Whitlock et al. reported that children with DS-ALL treated according to the NCI (National Cancer Institute) standard-risk arm in Children's Oncology Group (COG) protocols, had a worse outcome when compared with children with non-DS ALL.[2] In contrast, DS-ALL patients stratified in the NCI high-risk arm showed no significant differences in outcome compared to high-risk non-DS ALL. This suggests that DS-ALL cells are relatively resistant to chemotherapy, and that intensification of therapy for DS-ALL patients may be warranted.[2] Moreover, this study may suggest that the NCI-classification is not appropriate for risk-group stratification in DS ALL. Preliminary results of the ALL-BFM 2000 study showed no significant differences in minimal residual disease levels in the first 3 months of treatment between DS and non-DS ALL children, nor in relapse risk (6.1% in DS versus 11.4% in non-DS patients).[8,9] Of interest, the risk of serious adverse events was significantly higher in DS-ALL patients (23.4%) vs. non-DS ALL (6%) patients, as well as the cumulative incidence of treatment related deaths (9% vs. 2%).[8,9] These data suggest that treatment intensification in DS ALL patients need to be carefully balanced against enhanced toxicity and potential excess in treatment related mortality.

One of the key agents used in ALL treatment is methotrexate (MTX). MTX inhibits dihydrofolate reductase, and leads to inhibition of DNA-synthesis. MTX-polyglutamylation increases the intracellular retention of MTX, which is an important parameter of MTX efficacy.[10-13] MTX is associated with side effects, especially mucositis, liver-toxicity and myelo-suppression. Patients can be rescued from excessive toxicity with leucovorin, which is routinely administered following the infusion of higher dosages of MTX. It is well-known that patients with Down syndrome are more susceptible to MTX-induced side-effects than non-DS ALL patients,[14,15] which is due to the higher cellular sensitivity of the tissues affected by MTX, such as the mucosa and bone-marrow. This vulnerability often results in MTX dose reductions. It is not known whether the differences in toxicity between DS and non-DS children also reflect differences in MTX-pharmacokinetics. The only available study from Garré et al., showed that

MTX plasma concentrations 42 hours after the start of infusion were significantly higher in 5 DS-ALL patients compared to 3 non-DS ALL patients.[16]

We performed a retrospective case-control study of 44 children with DS-ALL and 87 non-DS ALL controls, enrolled on Dutch Childhood Oncology Group (DCOG) studies. The aim of the study was to identify differences in MTX pharmacokinetics between DS and non-DS children, and whether this was related to side effects.

PATIENTS AND METHODS

Patients

We identified all DS-ALL patients enrolled in 3 consecutive DCOG treatment protocols, i.e. study DCOG ALL-8, -9 and -10, conducted between November 1991 and December 2006. The children were enrolled in the 8 participating University Hospitals in the Netherlands. Only children who were treated according to the protocol and in complete remission (CR) after induction therapy were included. In addition, for each DS ALL case, we selected 2 non-DS ALL controls that were matched for treatment protocol, sex and body surface area (BSA).

Treatment protocols and MTX-administration

From 1991 until January 1997, children with newly diagnosed ALL were enrolled in the BFM-based treatment protocol DCOG-ALL-8.[17] Patients were stratified in 3 risk groups; standard (SRG), medium (MRG) and high risk (HRG). SRG and MRG patients received HD-MTX courses (5 g/m^2/course), given every 2 weeks for a total of 4 courses; combined with intrathecal triple therapy (ITT) consisting of MTX/DAF/ARA-C, and oral 6-mercaptopurine (6-MP; 25 mg/m2/day) given once daily for 8 weeks. MRG patients were randomized to receive this bock with either oral low-dose 6-MP oral, or high-dose 6-MP intravenously (1300 mg/m^2, directly following the MTX infusions) every two weeks. HRG patients received HD-MTX (5 g/m^2/course) in 2 of the 3 high-risk blocks. Three doses of leucovorin rescue (SRG: 15 mg/m^2; MRG and HRG patients: first dose 30 mg/m^2; subsequent dosages 15 mg/m^2) were given every 6 hours, starting 36 hours after the start of the MTX infusion for SRG patients, and at 42 hours for MRG and HRG patients.

The DCOG-ALL-9 protocol (1997 – 2004) stratified children into 2 risk groups; non-high risk (NHR) and high risk (HR). NHR patients received 3 HD-MTX courses (2 g/m^2/course) given once weekly and HR patients received 4 HD-MTX courses (3 g/m^2/course), given every 2 weeks. HD-MTX courses were combined with ITT at the start of every MTX infusion. Children treated according to the SR group did not receive 6-MP; the HR group received oral 6-MP (50 mg/m^2,

once daily) for 8 weeks. Leucovorin rescue therapy (15 mg/m^2) was initiated at 36 hours after start of the infusion, and was administered every 6 hours for 3 doses.[18]

From November 2004 onwards, children with ALL were treated according to the DCOG-ALL-10 protocol, which is ongoing. Patients were stratified in three risk groups; standard (SR), medium (MR) and high risk (HR). SR and MR patients received HD-MTX courses (5 g/m^2/course), given every 2 weeks for a total of 4 courses. HD-MTX courses were combined with ITT and oral 6-MP (25 mg/m^2, once daily) for 8 weeks. Leucovorin rescue (15 mg/m^2) was given every 6 hours starting at 42 hours after the start of the MTX infusion, for a minimum of 3 doses. HR patients received 3 HR blocks with HD-MTX (5g/m^2/course) after which patients who were eligible received stem cell transplantation, or 3 subsequent HR courses when patients did not have a suitable donor. Leucovorin rescue therapy (15 mg/m^2) was initiated at 42 hours after start of the infusion and was given every 6 hours.

All protocols used similar supportive care guidelines for administration of high dose MTX, including hyperhydration (2.5-3.0 L/m^2/day), and urine alkalinization (using sodium bicarbonate infusion, aiming at producing urine with a pH between 7 and 8). In case of MTX plasma levels ≥ 0.4 µmol/L at time point (T) T=48 hours after the start of the MTX infusion, hyperhydration, alkalinization and leucovorin rescue were continued for at least another 24 hours. The required MTX plasma level to discontinue these measures was ≤ 0.25 µmol/L at T=72 hours or later. No specific guidelines for DS patients regarding MTX administration were provided in any of these protocols. The MTX and Leucovorin dosages are specified in detail in Table 1.

MTX toxicity and plasma levels

The data were extracted from patient files, and included the number of MTX courses, the dose of MTX that was prescribed, the MTX plasma levels, the leucovorin rescue that was given, the hyperhydration and urine alkalinization procedures, as well as the side-effects during and after the MTX infusion until the next block of chemotherapy. Toxicity data were graded according to the Common Toxicity Criteria for Adverse Events version 3.0 (CTCAE). MTX plasma levels 48 hours after the start of the MTX infusion were collected, as well as additional time-points in case of high levels, or in case the hospital also routinely determined plasma levels at other time-points. Other items that were tabulated included co-medication, delays in starting subsequent therapy elements, creatinine and liver function tests. In a few cases the exact time of MTX plasma level determination was missing, and then the assumption was made that the physicians followed the treatment protocol, and that samples were taken at the prescribed time-points.

Table 1. Methotrexate and standard leucovorin rescue dosages in the 3 Dutch Childhood Oncology Group ALL treatment protocols

Protocol	MTX	6-MP	Leucovorin rescue
DCOG ALL 8			
SRG	4 x 2 gram/m^2 every 14 days	25mg/m^2/day, orally for 56 days	15 mg/m^2, every 6 hrs from T=36 for 3 dosages
MRG	4 x 5 gram/m^2 every 14 days	25mg/m^2/day, orally for 56 days, or 1300mg^2 iv every 14 days	30 mg/m^2 at T=42; followed by 15 mg/m^2 at T=48 and T=54
HRG	2 x 5 gram/m^2 every 21 days	100 mg/m^2 for 5 days	30 mg/m^2 at T=42; followed by 15 mg/m^2 at T=48 and T=54
DCOG ALL 9			
NHR	3 x 2 gram/m^2 every 7 days	NA	15 mg/m^2, every 6 hours from T=36 for 3 dosages
HR	4 x 3 gram/m^2 every 14 days	50mg/m^2/day for 56 days	15 mg/m^2, every 6 hours from T=36 for 3 dosages
DCOG ALL 10			
SR	4 x 5 gram/m^2 every 14 days	25mg/m^2/day for 56 days	15 mg/m^2, every 6 hours from T=42 for 3 dosages
MR	4 x 5 gram/m^2 every 14 days	25mg/m^2/day for 56 days	15 mg/m^2, every 6 hours from T=42 for 3 dosages
HR	5 gram/m^2 every 50 days, for 3-6 courses	25mg/m^2/day for 14 days	15 mg/m^2, every 6 hours from T=42 for 3 dosages

In some protocols 6-mercaptopurine (6-MP) was added during this treatment block. SRG: standard risk group; MRG: medium risk group; HRG: high risk group; NHR: non high risk; HR: high risk; SR: standard risk; MR: medium risk; T: time-point after start of MTX-infusion; m^2: meter square; DCOG: Dutch Childhood Oncology Group; MTX: methotrexate. NA: not applicable, since none of the ALL9-NHR patients did receive 6-MP.

Pharmacokinetic analysis

The pharmacokinetic model was fitted to the data from all individuals simultaneously, using non-linear mixed effect modeling (NONMEM).[19] The population parameters, intra- and inter-patient and residual variances were estimated using the NONMEM software program (double precision; version VI, level 1.0). The first-order conditional estimate method was used throughout the analysis.

MTX pharmacokinetics was described according to a two-compartmental model with a first order elimination from the central compartment. The following parameters were estimated: the volume of distribution of the central compartment (V1), clearance from the central compartment (CL), volume of distribution of the peripheral compartment (V2) and inter-compartmental clearance (Q). In the structural model pharmacokinetic parameter values were standardized for a body weight of 70 kg using an allometric model.[20] For instance CL and V1 were standardized as to CLpop = CLstd • (WT / 70)$^{0.75}$ and V1pop = V1std • (WT / 70), where CLpop and V1pop are typical population parameter values in individuals with a certain weight (WT) and CLstd and V1std are the standard values for patients with a weight of

70kg. Inter-and intra-patient variability of the pharmacokinetic parameters was estimated using an exponential error model. For instance, inter- and intra-individual variability in CL was estimated using: CLi = CLpop x exp (ηi + κi) where CLi represents the clearance of individual i, and η and κ are the respective inter- and intra-patient random effects with a mean of zero and variance ω2. The covariance between inter-patient variability was estimated as well. For a NONMEM model, the residual variance corresponds to the difference between the observed concentration (Cobs) and predicted concentration (Cpred). The latter is predicted on basis of individual parameters (e.g. CLi, V1i, etc.). Residual variance was modelled with a combined additive and proportional error model: Cobs, i = ε1 + Cpred, i (1 + ε2), where ε1 and ε2 are independent random variables with zero mean and common variances of σ2. The adequacy of the developed model was evaluated by examination of the precision of the parameter estimates, the values of random-effect variances and various diagnostic plots.[20-23] In order to explain the pharmacokinetic variability between and within the patients, relationships were investigated between pharmacokinetic parameters and various patient characteristics. Covariates were introduced in a multiplicative way. Categorical variables, like for example Down syndrome, were modelled as: CLi = CLpop x θ^{DOWN}, where CLpop is the population value for MTX-clearance in non-Down patients (exponent DOWN=0) and θ is the fractional change in clearance in Down-patients (DOWN = 1). Continuous variables, like creatinine clearance (CRCL), were modelled centred around the median value in the population: CLi = CLpop * $(CRCL/142)^{\theta}$, where CLpop is the MTX-clearance in individuals with a creatinine clearance of 142 ml/min and θ is an exponential. The objective function value (OFV) was used for comparison of the models. Discrimination between hierarchical models was based on the OFV using the log-likelihood ratio test. A value of p=0.05, representing a decrease in OFV of 3.8 units was considered statistically significant (df=1).[19]

Individual pharmacokinetic parameters were generated by Bayesian analysis. On basis of these parameters, individual plasma concentration-time profiles were generated for assessment of the area under the plasma concentration versus time curve (AUC), and the plasma concentration at 48 hours after the start of the MTX infusion.

Statistics

For statistical comparisons the Statistical Package for the Social Sciences (SPSS) Analysis system (v.15.0, SPSS Inc., Chicago, IL, USA) was used. To analyze differences between DS-ALL patients and non-DS ALL patients, non-parametric matched paired analysis was applied. For toxicity parameters with binary values, the non-parametric Cochran test for k related samples was used. Analysis were 2-tailed at the significance level of $p<0.05$.

RESULTS

Patient characteristics

In total 47 DS-ALL patients, enrolled in protocols DCOG ALL 8, 9 and 10, were identified in the DCOG database. Three patients died during induction therapy, and could therefore not be evaluated. For the remaining 44 DS-ALL patients (25 boys, 19 girls), 87 matched non-DS-ALL controls (50 boys, 37 girls) were selected. One patient with DS was matched to 1 instead of 2 non-DS ALL patients, because no other appropriate control could be identified.

Patient characteristics are shown in table 2. All DS-ALL patients had B-cell-precursor ALL, and 7/87 (8%) non-DS ALL patients had T-cell ALL. DS-ALL patients were slightly older compared to non-DS-ALL patients (3.4 vs. 5.4 years respectively; p=0.02), which was the result of matching on BSA. There was a difference in median presenting white blood cell count (WBC) between DS and non-DS children ($8.8x10^9$/l. vs. $26.9x10^9$/l. respectively; p= 0.005). Significant co-morbidity was present in 5 DS-ALL patients, including a complex cor vitium (n=3; surgically corrected before diagnoses of ALL in all of them), hypothyroidism (n=1), and diabetes mellitus (n=1). However, all patients were in a clinically good condition before they were diagnosed with DS-ALL, and all children were treated with curative intent.

MTX treatment

In total, 468 HD-MTX courses were administered to 44 DS children (n = 152 courses) and 87 non-DS children (n = 366 courses). Dose reductions were applied in 26 of 152 (17.1%) MTX courses, in 9 out of 44 (20.5%) patients, whereas none of the non-DS ALL patients received a dose-reduction. Three DS-ALL patients received one course less than required per protocol, and 1 non-DS ALL patient received 3 instead of 4 courses because of severe MTX induced side effects (p= 0.68). Dose-reduction was electively initiated from the 1st course onwards, in anticipation of possible greater toxicity in 18/26 courses in 5 DS children. In 8 of 26 courses in 4 DS patients, dose-reductions were applied from the 2nd or subsequent courses onwards because of documented excessive toxicity in earlier HD-MTX courses. Dose-reductions occurred in protocol ALL-9 at the 2 gr/m^2 MTX dose (2 courses in 1 patient), and in protocol ALL-10 at the 5 gr/m^2 MTX dose (6 courses in 3 patients). Of interest, the number of DS patients requiring dose-reductions in the 2nd or subsequent courses due to excessive toxicity in earlier courses was therefore 1/27 (3.7%) patients when treated at the 2-3 gr/m2 dose-level, and 3/12 (25%) patients when treated at the 5 gr/m2 dose level (p=0.046).

Table 2. Patient characteristics of Down syndrome ALL patients and their non-Down syndrome ALL matched controls

Parameter	DS-ALL patients	Non DS-ALL patients	p-value
N	44	87	
Matching parameters			
Sex, n and (%)			
Male	25 (56.8)	50 (57.5)	
Female	19 (43.2)	37 (42.5)	
BSA, median (range), m^2	0.70 (0.3-1.6)	0.74 (0.5-1.6)	
Treatment protocol.			
ALL 8, n and (%)	10 (22.7)	20 (23)	
ALL 9, n and (%)	24 (54.5)	48 (55.2)	
ALL 10, no and (%)	10 (22.7)	19 (21.8)	
Patient characteristics			
Age, median (range), years	5.4 (2.0-17.1)	3.6 (1.3-14.7)	0.03
Initial WBC, median x 10^9/L	8.8 (1.2-460)	27.0 (0.8-684.0)	0.005
Immunophenotype			
Pro B-ALL, n and (%)	0	4 (4.6)	
BCP-ALL, n and (%)	48 (100)	76 (87.4)	
T-ALL, n and (%)	0	7 (8)	
DNA index			
<1,16	29/32 (90.6%)	45/87 (51.7%)	0.524
>= 1,16	3/32 (9.3%)	8/87 (9.2)	
unknown	12	34 (39.1)	
Cytogenetic abnormalities			
t(9;22), n and (%)	2/44 (4.5)	1/87 (1.1)	0.261
t(12;21), n and (%)	4/44 (9.1)	6/87 (6.9)	0.732
MTX Courses			
Number of MTX courses (n)[1]	152	316	
Administered at 2 gr/m^2	89	126 (39.8)	
Administered at 3 gr/m^2	15	71 (22.5)	
Administered at 5 gr/m^2	39	119 (37.6)	
Administered other	9		
Courses not received, n.	3	1	0.68
Reduced dosage, n and (%)			
All courses, n and (%)	26 (17.1)	0 (0)	0.001
Dose reduction below the prescribed of 2 gr/m^2	2/74	0/126	
Dose reduction below the prescribed of 3 gr/m^2	0/15	0/71	
Dose reduction below the prescribed of 5 gr/m^2	24/63	0/119	

[1] This is the actual dose that was given to patients, which sometimes differs from the prescribed dose in the protocol. *Dose reductions were either given empirically before the 1st course of MTX, or dosages were adapted based on experiences toxicity in the 1st course. WBC: white blood cell count; BSA: body surface area; BCP-ALL: B-cell precursor acute lymphoblastic leukemia.

Toxicity of high dose MTX courses

We first evaluated the frequency of grade 3/4 toxicities after the first HD-MTX course only (after excluding the 5 DS-ALL patients with initial dose reductions in anticipation of greater toxicity), as toxicity in later courses was influenced by dose-reductions and cumulative toxicity. DS patients experienced a significantly higher frequency of grade 3-4 gastrointestinal toxicity when compared with non-DS ALL patients (DS 13/38 patients (34.2%) vs. non DS 3/76 patients (3.9%); p= 0.001), as shown in Table 3.

Next, we compared the cumulative frequencies of grade 3-4 toxicities; now only including the MTX-courses 2, 3 and 4 in all patients. Despite dose-reductions, children with Down syndrome still had an increased risk of cumulative grade 3-4 gastrointestinal toxicity compared with non-DS ALL patients (27/102 patients (26.5%) vs. 8/204 patients (3.9%), respectively; p=0.001).

DS patients did not experience enhanced hematological toxicity. Moreover, no difference in hematological toxicity was found between DS-ALL patients who received 6-mercaptopurine (6-MP) during MTX therapy, compared with those DS ALL patients who did not receive 6-MP

Table 3. Frequency of grade 3/4 toxicities in DS- and non-DS-ALL patients after high dose Methotrexate therapy blocks

Side-effects	DS	Non-DS	P-value
First course HD-MTX*			
Number of MTX courses	39	87	
Anemia	0/5	0/10	
Leukopenia	0/5	3/10	0.71
Neutropenia	0/4	4/7	0.37
Thrombocytopenia	0/4	0/8	
Neurological toxicity	1/38	1/76	0.60
Gastro-intestinal toxicity (mucositis)	13/38	3/76	0.001
Cumulative toxicity: courses 2-4			
Number of MTX courses	108	229	
Anemia	2/43	1/86	0.36
Leukopenia	10/43	9/86	0.06
Neutropenia	8/24	11/48	0.36
Thrombocytopenia	5/43	4/86	0.33
Liver toxicity (transaminases)	1/15	0/30	0.36
Neurological toxicity	1/102	1/204	0.60
Gastro-intestinal toxicity (mucositis)	27/102	8/204	0.001

Toxicity was graded according to the Common Toxicity Criteria for Adverse Events version 3.0. Not all toxicities could be evaluated in all subjects, as many centers did not routinely check blood values in between courses. Number of grade 3-4 toxicity divided by number of measurements for the specific parameter. *Patients with dose reduction in anticipation of greater toxicity were excluded from the analysis. HD-MTX: high dose methotrexate.

treatment (p=0.58). The same lack of difference in hematological toxicity was observed when comparing the non-DS ALL controls that received 6-MP versus those that did not (p= 0.74).

Neurological toxicity (grade1-4) was reported in 3 DS (in 5 courses) and in 3 non-DS patients (in 4 courses). Grade 4 MTX encephalopathy, consisting of either seizures, unconsciousness, and/or transient hemi-paresis, occurred in 1 DS (in 2 courses) and in 2 non-DS patients (in 1 course per patient).

MTX pharmacokinetics

Figure 1 shows the observed MTX plasma concentrations and the diagnostic plots of the developed population pharmacokinetic model in DS and non-DS ALL patients. The population pharmacokinetic parameters are given in Table 4. Predicted concentrations are evenly distributed around the line of unity, indicating the 'goodness of fit' of the model. For each patient the individual estimates of CL, Q, V1 and V2 were obtained by Bayesian analysis. On basis of these parameters, individual plasma concentration-time profiles were calculated.

The two-compartment pharmacokinetic model adequately described the data, and the parameters were generally well estimated as indicated by their standard errors. Allometric normalization of clearances for weight reduced the inter-patient variability from 45% to 31%. Both inter- and intra-patient variability in clearance was moderate with respective values of 31% and 15%. Covariate analysis revealed that MTX-clearance was 5% lower in DS-ALL patients compared to non-DS ALL patients (p=0.001). Median (range) posthoc values for clearance were 4.7 (2.4 – 11.9) L/hr and 4.9 (1.3 – 10.4) L/hr in DS ALL and non-DS ALL patients, respectively; standardized values were 12.3 (7.3 – 18.9) L/hr/70kg and 13.0 (4.6 – 25.2) L/hr/kg. No relationship was found between the pharmacokinetic parameters and the treatment center or treatment protocol, MTX dose, hyper hydration (L/m^2), number of leucovorin dosages, creatinine clearance, age, WBC, bilirubin or ASAT levels.

The 5% difference in MTX-clearance between DS-ALL and non-DS ALL is small, which is further demonstrated by the fact that no significant differences were detected in the plasma concentration at T=24 and T=48 hours after the start of the MTX infusions. At T=24, the median MTX-level in DS-ALL patients was 38.74 µmol/L (range 0.38-133.11 µmol/L; 25^{th} and 75^{th} percentiles: 19.7–66.3 µmol/L) versus 36.49 µmol/L (range 7.62-261.49 µmol/L; 25^{th} and 75^{th} percentiles: 22.8-63.5 µmol/L; p=0.51) in non-DS patients. At T=48 hours, the median MTX level in DS-ALL patients was 0.28 µmol/L (range 0.04-9.57 µmol/L; 25^{th} and 75^{th} percentiles: 0. 15-0.51 µmol/L), versus 0.27 µmol/L (range 0.06-14.63 µmol/L; 25^{th} and 75^{th} percentiles 0.17-0.41; p=0.41) in non-DS patients. After stratification for the various dosages of MTX that were administered to the patients (either 2, 3 or 5 $gram/m^2$/course), again MTX plasma levels did not differ significantly between DS and non-DS ALL children.

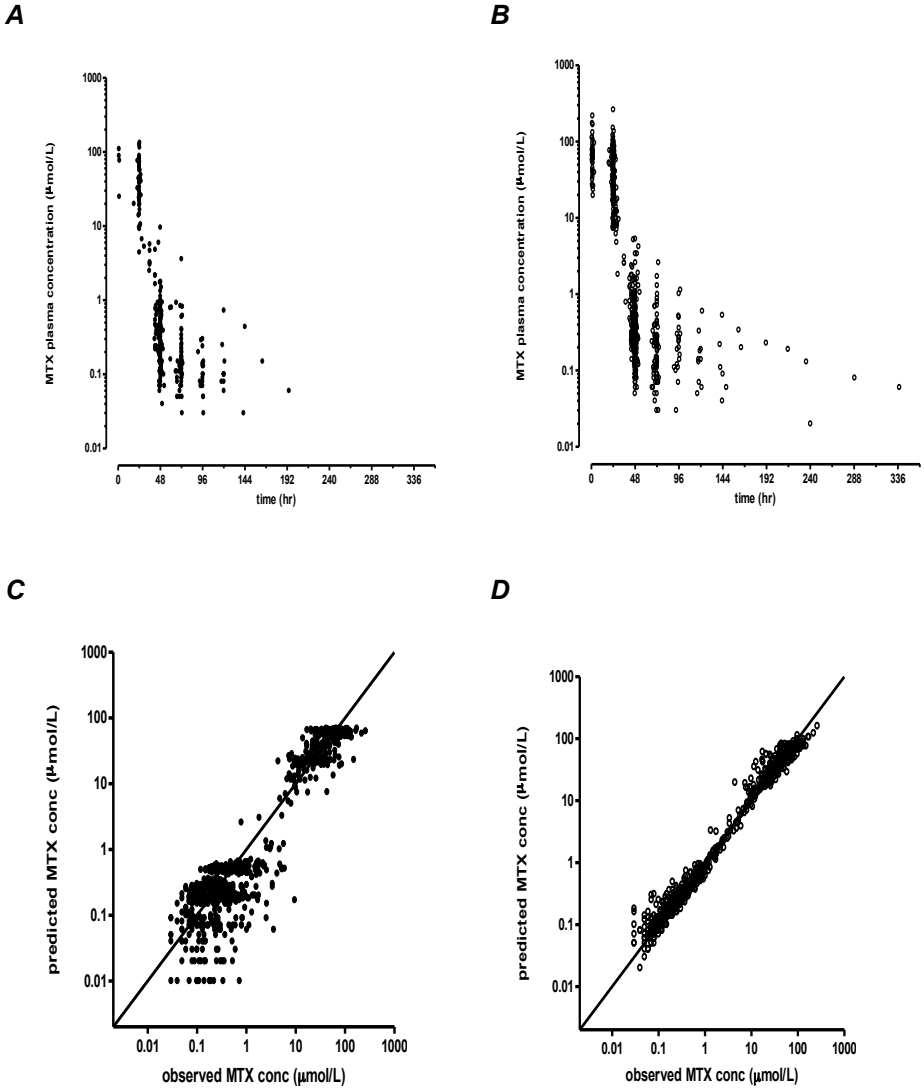

Figure 1. Methotrexate plasma levels and diagnostic plots of the population pharmacokinetic model

Methotrexate (MTX) plasma levels at various time-points following the start of a 24-hour MTX-infusion in DS (graph A, n=152 courses in 44 patients) and non-DS (graph B, n=316 courses in 87 patients) ALL**.** Each dot represents a plasma level in a patient measured at a given time-point. Plasma concentrations at T=48 hours were available from all patients, whereas levels at T=1 hour and T=24 hour were only determined in some of the hospitals. In case of T=48 MTX levels > 0.4 µmol/L monitoring of MTX levels was continued. C) Model predicted MTX concentrations calculated by the NONMEM two-compartment model versus observed concentrations for all patients. The points are evenly distributed around the line of unity indicating the goodness of fit of the model. Deviations from the line are caused by intra- and inter-patient variability and residual variability. D) Individually (Bayesian) predicted MTX concentrations versus observed concentrations for all patients. All dots are close to the line of unity indicating limited residual variability.

Table 4. Population pharmacokinetic parameters for Methotrexate in children with ALL

	Estimate	SE (%)
Population parameter		
V1 (L/70kg	46	12
CL (L/hr/70kg)	13	8
θ^{Down}	0.95	5
θ^{Gender}	0.87	9
V2 (L/hr/kg)	10	19
Q (L/hr/kg)	0.3	31
Inter-patient variability		
V1 (%)	38	45
CL (%)	31	34
V2 (%)	74	38
Q(%)	57	51
Correlation		
V1 – CL	0.9	
V1 - V2	0.86	
CL - V2	0.63	
V1 – Q	0.72	
CL – Q	0.73	
V2 – Q	0.81	
Intra-patient variability		
V1 (%)	37	54
CL (%)	15	65
Residual variability		
Additive (µmol/L)	0.02	22
Proportion (%)	35	31

V1 and V2, central and peripheral volume of distribution, respectively; CL, clearance in male non-DS-ALL patients; θ^{DOWN}, fractional change in clearance in DS-ALL patients; θ^{GENDER}, fractional change in clearance in DS-ALL patients; Q, inter compartmental clearance; SE: standard error of the estimate.

In 36.4% of the MTX courses in DS-ALL patients, the MTX plasma levels were ≥0.4 µmol/L at T=48 hours, which is the cut-off value used in DCOG centers for additional leucovorin rescue, compared to 27.7% of the MTX courses in non Down syndrome patients (p=0.14).

No correlation was found between the MTX area under the curve (AUC, range 276-2603 µmol/L*hr) of the 1st course of MTX and grade 3-4 toxicity in the DS ALL patients, although the number of patients in the 5 gr/m^2 group was limited (Figure 2). We also did not observe a clear correlation when all subsequent courses were included. Grade 3-4 toxicity occurred both at low and high AUC, and was even seen at the lowest AUC of 276 µmol/L*hr in one DS-ALL patient.

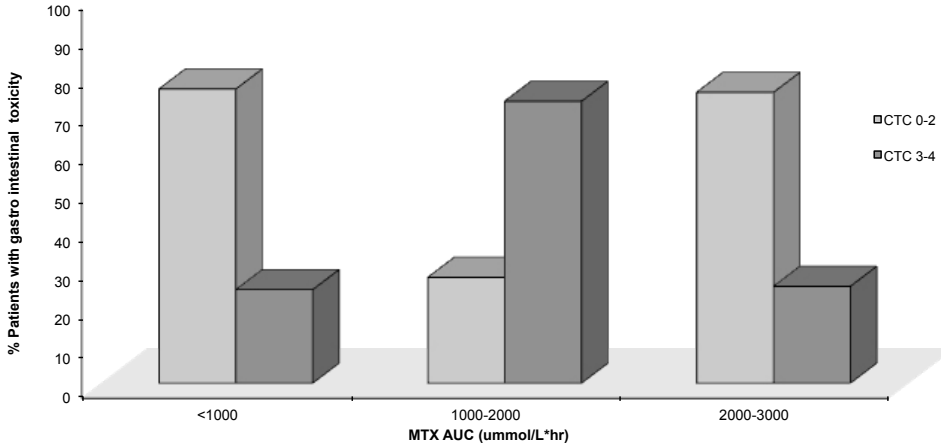

Figure 2. Correlation of Methotrexate area under the curve versus gastrointestinal toxicity
Grade 1-4 gastro intestinal toxicities in the first Methotrexate (MTX) course versus the MTX area under the curve in Down syndrome patients only. Number of patients per subgroup: <1000 μmmol/L*h: n=29, 1000-2000 μmmol/L*h: n=11, 2000-3000 μmmol/L*h: n=4. ALL, acute lymphoblastic leukemia, CTC grade 0-2 light grey, CTC grade 3-4 dark grey.

DISCUSSION

Given the well-known reduced tolerance for MTX in children with DS, we performed a retrospective case-control study to define whether the enhanced susceptibility for MTX-induced side-effects is not only be due to the well-known difference in cellular sensitivity (for instance of the mucosa), but whether this is also the result of differences in pharmacokinetics between DS-ALL and non-DS ALL patients.[2,16,24]

In our study, indeed a significantly higher proportion of children with DS experienced MTX-induced gastrointestinal toxicity compared with the non-DS controls, which is consistent with other reports.[2,16,24,25] Dose reductions were applied both in anticipation of possible toxicity, and because of apparent excessive toxicity, and were restricted to DS patients only. However, due to excessive toxicity both DS ALL (n=3) as well as 1 non-DS ALL patient, each received one course less than required per protocol.

A two-compartment pharmacokinetic model was constructed to characterize the pharmacokinetics of MTX. The MTX-clearance we observed in this study was in agreement with other studies.[26-29] For instance, Relling et al. reported a mean MTX-clearance of 99.9 ml/min/m^2 (~0.149 L/hr/kg), in 134 children enrolled on the St. Jude Total Therapy study XII for newly diagnosed ALL.[26] We found that the MTX-clearance was 5% lower in DS-ALL when compared to non-DS ALL patients. This is only a marginal difference, and probably not clinically relevant, which is reflected by the fact that MTX plasma concentrations in DS-ALL versus non-DS ALL patients did not differ at T=24 nor at T=48 hours after the start of the infusion. Altogether, we did not observe major differences in MTX pharmacokinetics between DS- and non-DS

children, which would explain the enhanced rate of side effects in DS children. The only other study regarding MTX pharmacokinetics in DS-ALL found significantly higher plasma concentrations for DS ALL patients, but numbers were small (5 DS-ALL and 3 non-DS ALL patients).[16]

We could not relate clinical severe toxicity to the MTX AUC, and toxicity was not restricted to DS-patients with higher plasma levels only. This suggest that the enhanced frequency of gastrointestinal side effects in the DS patients must have been related to pharmacodynamic differences of the gastrointestinal mucosa between DS and non-DS children. Several differences in MTX pharmacodynamics between DS and non-DS children have been reported in the literature. For instance, patients with DS have decreased folate levels when compared to control patients without DS, which may result in enhanced polyglutamylation and MTX-induced cell-kill.[16,30] Another plausible explanation for the observed MTX toxicity in DS patients could be a gene dosage effect for enzymes found on chromosome 21.[30,31] In particular, the reduced folate carrier gene (RFC), which is responsible for MTX transport over the cell-membrane, is localized on chromosome 21q22 [15,32]. However, at higher concentrations passive diffusion of MTX across the cell-membrane may also occur.[33,34] This may explain why, in an earlier study, we could not demonstrate higher sensitivity of DS-ALL cells to MTX, compared to non-DS ALL cells.[32]

Furthermore, polymorphisms in genes linked to the pharmacodynamics of MTX, such as folate-metabolism related genes, could give rise to enhanced toxicity, as has been shown in previous reports by us and others.[35-38] Children harboring polymorphisms exhibited significantly more GI toxicity. More knowledge on folate related polymorphisms might contribute to further individualization of MTX treatment in ALL and specifically for DS-ALL patients.

It remains a challenge to advise clinicians on the right dose of MTX to use in DS patients. Even in non-DS ALL patients, different protocols use different dosages, and there seems to be no consensus when it comes to the best dose of MTX to be used. In this retrospective study, a significantly higher number of DS-patients was given a dose-reduction in subsequent courses when treated with higher dosages (5 gram/m2/course) of MTX, compared with intermediate MTX-dosages (1–3 g/m^2/course). In absence of major safety concerns, it seems safe to start with intermediate dosages of MTX, followed by careful monitoring. The fear of enhanced toxicity, however, needs to be balanced against efficacy, as DS ALL cells are not more sensitive to chemotherapy when compared to non-DS ALL cells, which is different from the situation in myeloid leukemia of Down syndrome, which is characterized by hypersensitivity to chemotherapy.[32,39-41]

In summary, we did not find evidence for differences in MTX pharmacokinetics between DS-ALL and non-DS ALL patients, which might have explained the higher rate of grade 3-4 gastrointestinal toxicity and the greater need for MTX dose-reductions in DS-ALL patients due to excessive toxicity in earlier courses. Hence, these differences are most likely explained by differential pharmacodynamic effects in the tissues/organs of MTX between DS and non-DS children. Based on the clinical experience in this retrospective study, no major safety

concerns were observed when using intermediate dosages of MTX (1-3 gr/m^2) in DS-ALL children, and hence this might be a safe dose to consider in future studies.

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4

Frequency and prognostic implications of *JAK 1-3* aberrations in Down syndrome acute lymphoblastic and myeloid leukemias

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ABSTRACT

Children with Down Syndrome (DS) have an increased risk of developing acute lymphoblastic leukemia (DS ALL) and acute myeloid leukemia (ML-DS). ML-DS can be preceded by transient leukemia (TL). As most studies focus only on known mutations, the true frequency of *JAK* mutations in acute leukemias in Down syndrome may be underestimated. We performed mutational analysis of the whole kinase and pseudokinase domains of *JAK 1-3* by direct sequencing of 6 TL, 14 ML-DS and 35 DS ALL samples and related this to outcome. *JAK1* mutations were found in 1 ML-DS patient and 1 DS ALL patient. One TL patient and 1 ML-DS patient harbored a *JAK3* mutation. Six DS ALL patients had a *JAK2* mutation and their 10-year event free survival (EFS) was 100% vs. 75 +/- 9 % in wildtype patients (p=0.27); the 10-year overall survival (OS) was 100% vs. 86 +/- 7% (*p*=0.3) and the cumulative incidence of relapse (CIR) was 0% vs. 21 +/- 9% (*p*= 0.32). Moreover, a large meta-analysis did not show any differences in survival of *JAK2* mutants compared to wildtype patients. In summary, *JAK* mutations are rare in DS-leukemias, except for *JAK2* mutations in DS ALL, which have no prognostic value.

INTRODUCTION

Children with Down Syndrome (DS) have an increased risk of developing leukemia, including both acute myeloid (ML-DS) and acute lymphoblastic leukemia (DS ALL).[1] These leukemias differ in clinical characteristics and biology from leukemias in non-DS children. ML-DS is characterized by a low diagnostic white blood cell count (WBC), young age, FAB M7 morphology, excellent clinical outcome with survival rates of >90%, and a high sensitivity to chemotherapy in vivo and in-vitro.[2,3] Reduced intensity treatment in ML-DS does not lead to an increase in relapse rates, however, due to a decrease in treatment related mortality, it does result in improved overall survival.[4,5] ML-DS is often preceded by transient leukemia in newborns (TL), which in most cases resolves spontaneously. Approximately 20% of TL-patients subsequently develop ML-DS.[6,7] Both the TL and ML-DS blasts are characterized by mutations in the *GATA-1* gene, a hematopoietic transcription factor, which result in a truncated protein GATA1s.[8] Because these mutations occur both in TL and ML-DS, additional genetic abnormalities are needed in the progression from TL to ML-DS.

The prognosis of DS ALL patients is at best similar and often inferior to that of non-DS ALL patients.[9,10] This is in agreement with findings from cellular cytotoxicity assays that showed that. DS ALL cells do not have increased sensitivity to chemotherapy in vitro.[3] Consequently, in DS ALL dose-reduction should only be considered in case of unacceptable toxicity arising during treatment. Favorable prognostic factors in non-DS ALL such as high hyperdiploidy and *ETV6-RUNX1* gene-rearrangements are less frequently found in DS ALL, as well as unfavorable factors such as Philadelphia-chromosome or *MLL*-rearrangements.[11,12]

Janus kinases (*JAK)* belong to a family of intracellular non-receptor protein tyrosine kinases that transduce cytokine-mediated signals via the STAT family of transcription factors. *JAK* plays an important role in regulating the processes of cell proliferation, differentiation and apoptosis in response to growth factors. The *JAK2* V617F mutation is well-known in myeloproliferative disorders (MPD), and result in the impaired ability of the pseudokinase domain to negatively regulate the kinase domain.[13] The expression of *JAK2* V617F in mouse models leads to the development of a disease with a similar phenotype to polycythemia vera, with eventual progression to myelofibrosis, underscoring the role of this mutation in the pathogenesis of myeloproliferative disorders.[14,15] We and others have previously described activating mutations in *JAK2* and *JAK3* in TL and ML-DS.[16,17] Mutations within the pseudokinase domain of *JAK2* in DS ALL patients have also recently been reported. These activating *JAK2* R683 mutations occur at a different site than the V617F mutation, but both these mutations are localized in the pseudokinase domain and both have the same functional consequence, i.e. constitutive kinase activity.[18-20]

As most studies only focus on known mutations in *JAK*, and hence may underestimate mutational frequency, we performed mutational analysis of the whole kinase and pseudokinase domains of *JAK 1-3* by direct sequencing.[16,17,19-22] Moreover, we analyzed the prognostic

significance of *JAK2* mutations in DS ALL in our cohort. As all cohorts of the prognostic impact of *JAK2* mutations in DS ALL are small, we collected all clinical data from these series,[18,19] and performed a meta-analysis, assuming that since most *JAK* mutations have been described to be activating mutations,[18] they will result in increased proliferation and hence in increased relapse rates.

DESIGN AND METHODS

Patient samples

We screened 6 TL, 15 ML-DS (TL and ML-DS samples were unpaired) and 35 DS ALL samples taken at initial diagnosis. TL and ML-DS patients were diagnosed between 1994 and 2007. DS ALL patients were diagnosed between 1992 and 2008 and were treated according to subsequent DCOG treatment protocols ALL 8, 9 and 10. Details of these treatment protocols have been reported elsewhere, except for protocol ALL10, which is currently ongoing.[23,24] Clinical and cell-biological data, including cytogenetic data, were available for all mutated cases. Samples were provided by the Dutch Childhood Oncology Group, the AML-'Berlin-Frankfurt-Munster' Study Group, and the Nordic Society for Pediatric Hematology and Oncology. All study groups performed central review of the diagnosis, classification and clinical follow-up of the patients. All investigations had been approved by the Institutional Review Board, and informed consent was obtained according to local law and regulations.

Low-density mononuclear cell populations of bone marrow or peripheral blood were isolated after density gradient centrifugation of the sample using Ficoll Isopaque. All resulting samples contained 80% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)–stained cytospins. Genomic DNA was extracted using standard methods.

Mutation analysis

For mutational analyses, all exons encoding the kinase and pseudokinase domains of *JAK1* (exon 12-25), *JAK2* (exon 12-25) and *JAK3* (exon 12-24) were PCR amplified. All ML-DS and TL samples were screened for *GATA-1* mutations in exon 2 and 3. Purified PCR products of *JAK 1-3* and *GATA-1* were bi-directionally sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA). The sequence data were assembled and analyzed for mutations using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark).

Statistics

For statistical comparisons the Statistical Package for the Social Sciences (SPSS) Analysis system (v.15.0, SPSS Inc., Chicago, IL, USA) was used. P-values less than or equal to 0.05 were considered statistically significant (two-tailed testing).

To evaluate outcome, the following parameters were used: complete remission rate (CR), event-free survival (EFS), overall survival (OS) and cumulative incidence of relapse (CIR). CR was defined as less than 5% blasts in the bone marrow, with regeneration of tri-lineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere. Patients who did not achieve CR were considered as treatment failure on day 0. OS was measured from the date of diagnosis to the date of last follow-up or date of death from any cause. EFS was calculated from the date of diagnosis to the date of last follow-up or to the first event, including relapse, death in CR, and failure to achieve CR. The Kaplan-Meier method was used to estimate the 10-year probabilities of OS (OS), and EFS (EFS), and survival estimates were compared using the log-rank test. CIR (with treatment related death as competing event) was constructed by the method of Kalbfleisch and Prentice and compared by the Gray test. Statistical analysis was only performed when at least 5 patients were available in a given subgroup.

For the meta-analysis we collected the original outcome data from all participating study groups and evaluated OS and EFS.

RESULTS

Patient characteristics

All 35 DS ALL patients were classified as B-cell precursor ALL and enrolled in consecutive DCOG treatment protocols (DCOG ALL 8, 9 and 10). The median age of DS ALL patients was 4.5 years (range 2.0-17.1 years), the median WBC was 8.7x10^9/l (range 1.2-390x10^9/l), and 51% of the patients were male. The median age of the ML-DS patients was 2.0 years (range 0.7-2.4 years), the median WBC was 8,0 x10^9/l (range 2.6-168 x10^9/l) and 36% were male. The TL patients had a median age of 1.5 days (range 1-6 days) with a median WBC of 172.8 x10^9/l (range 35-410 x10^9/l) and 67% were male.

The median follow up time for survivors with DS ALL was 5.2 years (range 1.1 – 15.4 years) and for survivors with ML-DS 1.9 years (range 0-16.4 years). The patient characteristics of the TL, ML-DS and DS ALL patients as well as *JAK* 1-3 mutations are described in detail Table 1 and Table 2.

Table 1. Clinical and genetic characteristics of the included transient and myeloid leukemia of Down syndrome

ID	Disease	Sex	Age	WBC	Karyotype	*JAK*	*GATA 1 mutation*
1	TL	Male	1 day	64	NA	JAK3 A573V	itd 105 bpt
2	TL	Female	2 days	193	47,XX,+21c	WT	del 2 bp
3	TL	Male	1 day	35	NA	WT	ins 14 bp
4	TL	Female	2 days	173	NA	WT	del 1 bp, ins 2 bp
5	TL	Male	6 days	NA	NA	WT	del 2 bp
6	TL	Male	1 day	410	NA	WT	ins 14 bp
7	ML-DS	Female	0.7	4	NA	JAK3 A573V	del 4 bp
8	ML-DS	Female	2.2	7	45-46,XX,der(1)t(1;6)(q31;q?),ins(4;1)(q12;q25q44),-6,-7,der(7)t(6;7)(p21;p22),der(7)t(7;8)(q2?2;q2?3),der(11)t(7;11)(p14;p15),+21c,der(22)t(1;22)(q25;p11)[cp13]/47,XX,+21c[28]	JAK 1 D625R	ins 7 bp
9	ML-DS	Male	1.3	6	47,XY,+21c 2/47,idem,t(4;15),(q?21;q?21),del(7)(q?31q?33)	WT	ins 16 bp
10	ML-DS	Male	2.0	26	NA	WT	del 2 bp
11	ML-DS	Female	2.2	9	NA	WT	del 2 bp
12	ML-DS	Female	2.0	168	NA	WT	pointmutation
13	ML-DS	Female	2.0	6	NA	WT	ins 1 bp
14	ML-DS	Male	2.3	12	NA	WT	ins 14 bp
15	ML-DS	Female	0.9	19	NA	WT	del 5 bp
16	ML-DS	Male	1.8	7	47,XY,+21c [3]	WT	ins 4 bp
17	ML-DS	Male	1.4	6	48,X,ins(Y;5)(q11;?),der(3)t(3;6)(q2?8;?) or ins(3;6)(q2?8;?),-5,del(6)(q1?4q2?4),+21c,+21,+mar [24]	WT	del 3 bp, ins 5 bp
18	ML-DS	Female	2.3	49	47,XX,der(9)inv(9)(p24;q2?1)del(9)(q2q3) [14]	WT	del 3 bp
19	ML-DS	Female	1.9	40	47,XX,der(1)t(1;1)(p36;q21),t(5;6)(p15;p23),+21c [23]	WT	del 6 bp
20	ML-DS	Female	2.4	3	47,XX,r(7)(p22q22),+21c,.ish r(7)(WCP7+,D7Z1+,D7S486-,164D18-,3K23-)	WT	pointmutation

WBC= white blood cell count (*109/l); WT = wildtype; NA = not available. Age in years (except for TL). Karyotype nomenclature according to ISCN 1995.

Table 2. Clinical and genetic characteristics of the included Down syndrome acute lymphoblastic leukemia patients

ID	IPT	Sex	Age	WBC	Karyotype	*JAK*	*GATA 1*
21	C-ALL	Male	3.1	7	48,XY,+X,+21c[19]	JAK-2 R683 insertion GGCCCCATC	WT
22	C-ALL	Female	2.7	2	50,XX,+X,+4,+17,+21c[5]/47,XX,21c[5]	JAK-2 R683	WT

Table 2. (Continued)

ID	IPT	Sex	Age	WBC	Karyotype	*JAK*	*GATA 1*
23	C-ALL	Male	2.2	18	47,XY,+21c[20]	JAK-2 R683	WT
24	PRE-B	Male	3.8	3	47,XY,+21c[22]	JAK-2 R683	WT
25	C-ALL	Male	3.3	4	47,XY,+21c	JAK-2 R683	WT
26	PRE-B	Female	6.2	5	46,XX,der(14;21)(q10;q10)c,+21c[27]	JAK-2 R683	WT
27	C-ALL	Female	3.4	17	47,XX,+21c[20]	JAK-1 V651M	WT
28	C-ALL	Male	9.2	30	48,XY,+X,t(8;14)(q12;q32),+21c/47,XY,+21c	WT	WT
29	PRE-B	Female	4.9	4	47,XX,+21c[30]	WT	WT
30	PRE-B	Female	2.6	18	48,XX,+X,add(18)(q22),+21c[2]/47,XX,+21c[30]	WT	WT
31	C-ALL	Male	13.5	1	56,XY,+X,+4,+10,+14,+14,+17,+18,+18,+21c,+mar[13]/47,XY,+21c[9]	WT	WT
32	C-ALL	Male	15.4	4	47,XY,t(1;3)(q32;q26),t(2;12)(q23;q13),t(7;8)(q31;q12),del(13)(q14q32),+21c[17]/40-46,idem[4]	WT	WT
33	PRE-B	Female	4.0	48	NA	WT	WT
34	PRE-B	Female	2.6	9	57,XX,+5,+6,+17,+18,+21c,+5mar[cp23]/47,XX,+21c[9]	WT	WT
35	C-ALL	Male	4.2	41	46,XY,t(8;9)(q24;p13),del(12)(p13.1),?dic(12;13)(p11.2;p10),+21c[20].ish del(12)(TEL-,AML1-,CEP12+),?dic(12;13)(TEL-,AML1-,CEP12+)	WT	WT
36	PRE-B	Female	2.8	33	47,XX,+21c	WT	WT
37	PRE-B	Male	3.9	78	47,XY,+21c	WT	WT
38	PRE-B	Male	5.6	5	47,XX,+21c	WT	WT
39	C-ALL	Female	7.9	13	47,XX,+21c[21]	WT	WT
40	C-ALL	Male	6.8	199	47,XY,der(9)del(9)(p13p2?2)t(9;22)(q34;q11),+21c,der(22)t(9;22)[15]/47,idem,add(21)(q22)[7]/47,XY,+21c[1]	WT	WT
41	C-ALL	Male	3.7	17	48,XY,+21c,+mar[4]/48,XY,+X,+21c[3]/47,XY+21c[13]	WT	WT
42	C-ALL	Male	17.1	20	47,XY,+21c[20]	WT	WT
43	C-ALL	Female	7.0	33	47,XX,del(12)(p11p13),+21c[18]/47,XX,+21c[2]	WT	WT
44	C-ALL	Male	2.0	112	47,XY,+21c	WT	WT
45	C-ALL	Female	8.4	4	58~59,XX,+4[3],+6,+10[2],+11,+?14,+18[2],+21,+21c,+1~5mar,inc[cp4]/47,XX,+21c[5]	WT	WT
46	PRO-B	Male	13.3	7	46,XY,-13,+21c[17]/47,XY,+21c[3]	WT	WT
47	C-ALL	Female	13.8	2	49,XX,+X,+5,+21c	WT	WT
48	C-ALL	Male	5.5	5	47,XY,+21c	WT	WT
49	C-ALL	Female	8.1	390	47,XX,-2,-8,+21c,+mar1,+mar2[8]/46,idem,-X/47,XX,+21c[3]	WT	WT
50	PRE-B	Female	5.4	4	47,XX,add(17)(q2?5),+21c[4]/47,XX,+21c[28]	WT	WT
51	PRE-B	Male	4.9	11	47,XY,+21c[32]	WT	WT
52	C-ALL	Female	3.7	9	47,XX,+X,-13,i(17)(q10),der(19)t(13;19)(q1?3;q1?2),+21c[15]/47,idem,del(12)(q1?4q2?1)[7]/52,idem,+3,+10,+14,+21,+21[2]/47,XX,+21c[29]	WT	WT
53	C-ALL	Male	2.3	6	47,XY,+21c	WT	WT
54	PRE-B	Female	4.5	6	47,XX,+21c[32]	WT	WT
55	C-ALL	Female	4.5	5	47 XX +21c [5]	WT	WT

WBC=white blood cell count (*10^9/l) ; WT = wildtype; IPT= immunophenotype; NA = not available. Age in years. Karyotype nomenclature according to ISCN 1995

Mutation analysis

GATA1 mutations were identified in all ML-DS and TL cases (details are given in Table 1). Mutations in *JAK1* were found in 1 (7%) ML-DS patient (D625R), and in 1 (3%) DS ALL patient (V651M). These were both missense mutations leading to substitution of a single amino acid which are predicted to result in an altered protein. No events occurred in either of the patients with a follow-up of 2.4 and 3.1 years, respectively. See Figure 1 for the localization of the mutations in *JAK* 1-3.

JAK2 mutations were not identified in any of the TL and ML-DS patients. However, *JAK2* R683 activating mutations were found in 6/35 (17%) of the DS ALL patients. In 5 patients a substitution of nucleotides A à G was found which resulted in a substitution of arginine with glycine and 1 patient had an insertion of 9 base pairs (GGCCCCATC) immediately upstream of R683. In DS ALL, cases with *JAK2* mutations were significantly younger than the wildtype patients, with a median age of 3.2 year versus 4.9 years (p= 0.044). There were no significant differences in other characteristics such as WBC, sex, and cytogenetics between the *JAK2* mutated and the wildtype DS ALL patients.

One TL-patient (17%) and 1 ML-DS patient (7%) harbored the *JAK3* A573V-mutation. Both patients are in continuous complete remission (CCR) with a follow up of 1.4 and 1.9 years, respectively.

Survival analysis

None of the 6 *JAK2* mutated DS ALL patients experienced an event, whereas 5 of the 29 patients with wildtype *JAK2* relapsed, and one died of treatment-related toxicity. Using

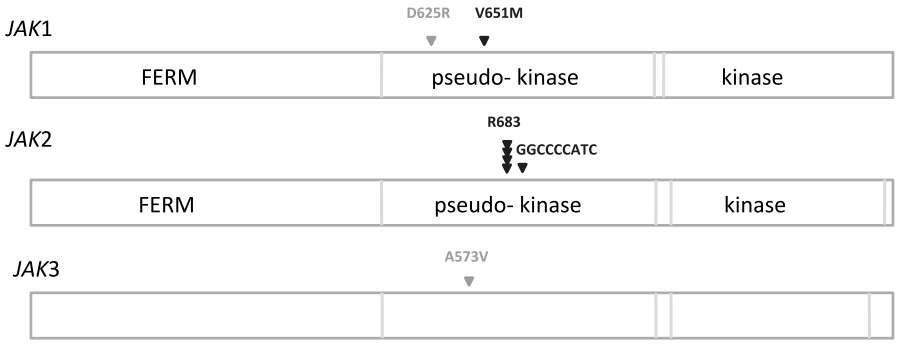

Figure 1. Localization of mutations in *JAK 1-3*

Schematic overview of the structure of *JAK 1-3* with the kinase, pseudokinase and FERM (4.1-ezrin-radixin-moesin) domain in which the location of the mutations we identified is indicated. Mutations in Down syndrome ALL are depicted in black, mutations in myeloid leukemia of Down syndrome and transient myeloproliferative leukemia in grey.

Kaplan-Meier analysis (see graphs in Figure 2), no significant differences in clinical outcome were detected: the 10-year EFS for *JAK2* mutated versus *JAK2* wildtype patients was 100% vs. 75 +/- 9%; (p=0.27), the 10 year OS 100% vs. 86 +/- 7% (p=0.3). The cumulative incidence of relapse at 10 years after diagnosis in *JAK2* wildtype patients was 21 +/- 9% versus 0% in the *JAK2* mutated group; (p(Gray)= 0.32).

Meta-analysis

To assess the impact of JAK2 mutations on the survival in a larger cohort of patients, we performed a meta-analysis of the data of 3 studies taken together pooling our data with those of Bercovich et al, and Gaikwad et al.[17-18] This analysis did not show a statistical significant difference for the *JAK2* wildtype versus the *JAK2* mutated patients. Six year EFS was 71 +/- 5% vs. 74 +/- 10%; P= 0.63 and OS was 76.0 +/- 4% vs. 89 +/- 6%; P= 0.30 (Figure 3A and 3B).

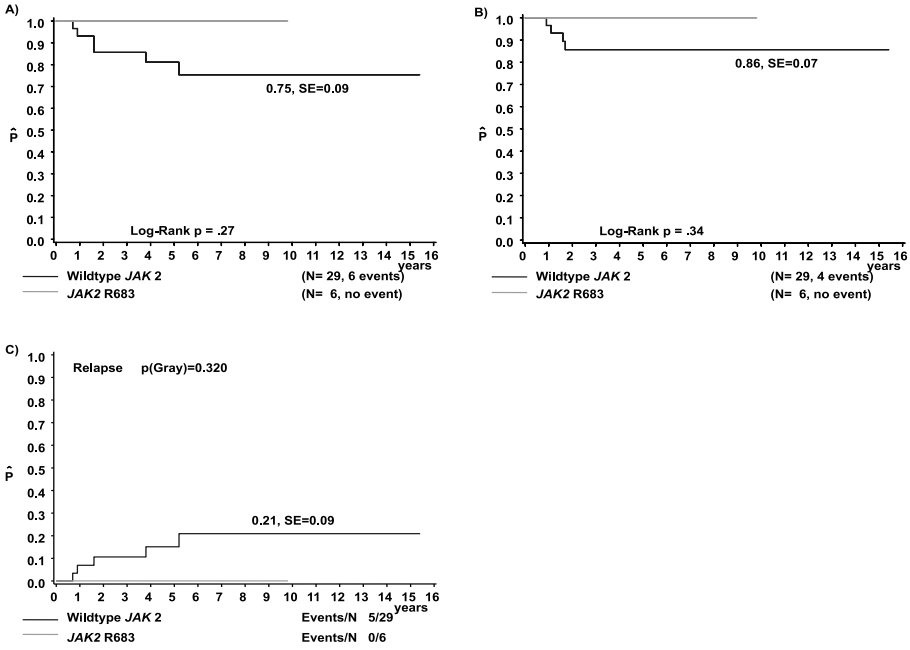

Figure 2. Ten years survival parameters of *JAK2* R683 mutations in DS ALL patients

The event-free survival, 100% versus 75±9%; p= 0.27 (A), overall survival, 100% versus 86±7%; p= 0.30 (B), and cumulative incidence of relapse, 0% versus 21±9%; p= 0.32 (C) are depicted for patients with *JAK2* R683 mutations (grey line) and wildtype *JAK2* (black line).

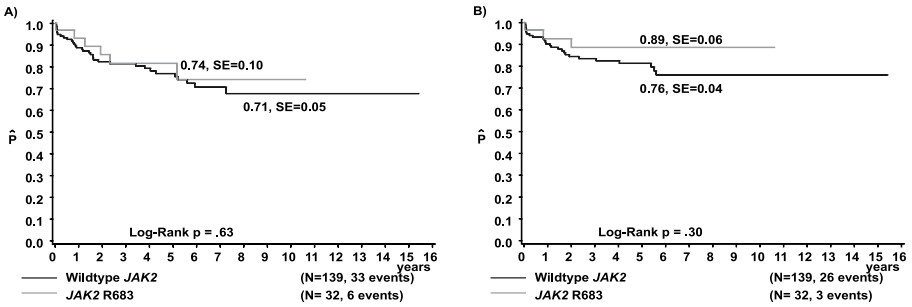

Figure 3. Survival parameters of *JAK2* mutant and wildtype patients; meta-analysis of three datasets

The event-free survival, 74±10% versus 71±5%; p=0.63 (A), and overall survival, 89±6% versus 76±4%; p=0.30 (B) are depicted for patients with *JAK2* R683 mutations (grey line) and wildtype *JAK2* (black line). DS ALL patients were diagnosed between 1992 and 2008.

DISCUSSION

Our study focused on the frequency of mutations in all exons encoding the kinase and pseudokinase domains of *JAK 1-3* in DS leukemia patients. We did not detect a higher mutational frequency than previously described,[13-16] suggesting that there are clear mutational hotspots, which can be screened for, in clinical studies. However, given the number of patients we have screened we may still have missed rare mutations that occur in a relatively low frequency in any of these leukemias.

Both *JAK1* mutations (one in ML-DS and one in DS ALL), which we found, were localized in the same region of the pseudokinase domain, but not identical to the activating mutations in *JAK1* described in non DS ALL patients.[25] *JAK1* mutations in non-DS cases are mainly found in T-cell ALL (27.3 %),[26] and in low frequencies in adult ALL T-ALL and B cell precursor ALL (3.4%)[27] and AML (2.1%).[28] These mutations are very heterogeneous in the sense that they are dispersed over several *JAK1* domains, and differ in their ability to transform hematopoietic cells and to activate downstream signaling pathways such as the STAT, PI3K and MAPK cascades.[26-28] T-cell ALL is exceedingly rare in DS patients, with frequencies varying between 0% and 7.8 % in several larger series,[9,29,30] versus approximately 15% in non-DS ALL. No patients with DS and T-cell ALL were included in this study.

Recently, Bercovich and others reported activating *JAK2* R683 mutations in 18% of DS ALL patients.[18-20] This mutation was thought to be unique for DS ALL, in a similar fashion as *GATA-1* mutations are uniquely found in ML-DS. However, the same *JAK2* mutations were also reported in non DS B-cell precursor ALL patients with a high risk for relapse, which implies that this mutation is not specific for DS ALL.[25] In our series, the typical *JAK2* R683 mutations were found in 17% of the DS ALL patients, which is in line with the frequency described by others.[18-20] One patient had an insertion of 9 base pairs immediately upstream of R683. This specific mutation has not been described before, but two different insertions of multiple base

pairs at this position have been reported, and were considered to have an effect analogous to R683 mutations because of their location.[18]

In contrast to the non DS B-cell precursor high-risk ALL patients with *JAK2* mutations who were found to have a high relapse rate,[25] none of the *JAK2* mutated DS ALL patients in our cohort experienced an event. The observed better outcome for *JAK2* mutated patients was, however, not statistically significant. So far, only two other studies report on the clinical relevance of *JAK2* mutations in DS-ALL and both studies are in agreement with our results reported here. Bercovich et al. reported a 5-year EFS of 73% in *JAK2* wildtype patients (n=62) versus 78% in *JAK2* mutated patients (n= 16), which was also not statistically significant.[17] In addition, Gaikwad et al. described a 5-year EFS in *JAK2* wildtype patients (n=43) of 76.3 % versus 87.5% in *JAK2* mutated patients (n=10), again a statistically non-significant difference.[19] A combined analysis of our data plus the 2 studies just mentioned above showed no statistically significant difference in survival between *JAK2* mutated and *JAK2* wildtype patients. Since this meta-analysis includes 32 mutated cases and 139 wildtype cases, it provides substantially greater certainty that there is no survival advantage for DS ALL patients with a *JAK2* mutation.

JAK mutations play a role in activation of the JAK-STAT pathway, resulting in a proliferation advantage for leukemic cells,[18] which led to our initial hypothesis that they would be associated with poor clinical outcome. One possible explanation for the observed good clinical outcome may be that this increased proliferation could contribute to enhanced sensitivity to chemotherapy. However, this is contradictory with the outcome of *JAK2* mutants in high-risk ALL, in which patients with a *JAK2* mutation have a high risk for relapse.[25] This may be due to currently unknown differences in step-wise leukemogenesis or cooperating genetic events between DS and non-DS *JAK2* mutated ALL.

Both one TL patient and one ML-DS patient harbored a *JAK3* A573V-mutation. This activating mutation has previously been described in ML-DS patients and the megakaryoblastic cell line CMY.[21] It has been suggested that *JAK3* mutations may be associated with a more aggressive form of ML-DS.[16] However, our 2 patients with a *JAK3* mutation are in continuous complete remission (CCR) with a follow up of 1.4 and 1.9 years, respectively.

Of interest, *JAK* mutated cases may be sensitive to JAK-inhibitors.[25] This might be of benefit, since it is well-known that DS patients have an increased risk for chemotherapy related morbidity and mortality. In myeloproliferative disorders, several JAK-inhibitors are already used.[31] Remarkably, patients with and without the *JAK2* V617F mutation may benefit to the same extent which is due to the fact that the current inhibitors do not differentially inhibit mutated and wildtype *JAK2*, because of the location of the mutation outside the ATP-binding pocket of the enzyme.[10] Treatment results in a decrease in organomegaly in responding patients.[31,32] The major side-effect of *JAK2* inhibitors is myelosuppression, which is due to suppression of wildtype *JAK2* that is required for normal hematopoiesis,[13] and which may render it difficult to combine *JAK* inhibitors with chemotherapy. Given that *JAK2* mutated DS ALL cases do not

have worse outcome it is difficult to predict whether the cells are addicted to the constitutive activity of *JAK2*, and hence whether inhibiting this will provide a benefit to these patients. We therefore feel that studies with *JAK2* inhibitors in DS ALL patients require further pre-clinical evidence of potential benefit before implementation.

Unfortunately, due to limited availability of our samples, we were not able to show activation of the JAK-STAT pathway at the protein level in the Down syndrome patients, and hence we may have missed patients that have JAK-STAT activation due to other mechanisms than mutations in *JAK*. Hence, further research is needed to identify potential JAK-STAT activation and its causes in patients without JAK-mutations. For instance, it is known that *MPL* (myeloproliferative leukemia virus oncogene) mutations may also activate the JAK-STAT pathway.[13] In an earlier study in 8 TL and ML-DS patients these *MPL* mutations were not found.[22]

In conclusion, *JAK1* and *JAK3* mutations are rare in DS-leukemias (although there seem to be mutational hotspots) whereas *JAK2* mutations occur relatively frequently in approximately 17% DS ALL cases. Of interest, none of the DS ALL cases with a *JAK2* in our cohort mutation relapsed so far, and a meta-analysis confirmed the lack of prognostic significance for *JAK2* mutated DS ALL patients.

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Outcome in children with Down syndrome and Acute Lymphoblastic Leukemia: role of *IKZF1* deletions and *CRLF2* aberrations

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ABSTRACT

Children with Down syndrome (DS) have an increased risk of developing acute lymphoblastic leukemia (ALL) and have a low frequency of established genetic aberrations. We aimed to determine which genetic abnormalities are involved in DS ALL. We studied the frequency and prognostic value of deletions in B-cell development genes and aberrations of *JAK2* and *CRLF2* using array-CGH, and MLPA in a population-based cohort of 34 DCOG DS ALL samples. A population-based cohort of 88 DS samples from UK trials was used to validate survival estimates for *IKZF1* and *CRLF2* abnormalities. In total, 50% of DS ALL patients had ≥1 deletion in the B-cell development genes: *PAX5* (12%), *VPREB1* (18%), and *IKZF1* (35%). *JAK2* was mutated in 15% of patients, genomic *CRLF2* rearrangements in 62%. Outcome was significantly worse in patients with *IKZF1* deletions (6-year EFS 45±16% vs. 95±4%; p=0.002), which was confirmed in the validation cohort (6-year EFS 21±12% vs. 58±11%; p=0.002). This *IKZF1* deletion was a strong independent predictor for outcome (Hazard-Ratio EFS 3.05; p=0.001). Neither *CRLF2*, nor *JAK2* were predictors for worse prognosis. If confirmed in prospective series, *IKZF1* deletions may be used for risk-group stratification in DS ALL.

INTRODUCTION

Children with Down syndrome (DS) have an increased risk of developing acute myeloid (AML) and acute lymphoblastic leukemia (ALL).[1] While AML in children with DS is characterized by unique acquired mutations in the transcription factor *GATA1* (globin transcription factor 1),[2-4] there is no evidence as yet for such a unique genetic event in DS ALL.

The established cytogenetic abnormalities, such as high hyperdiploidy (51-65 chromosomes), *ETV6-RUNX1*, *BCR-ABL1*, *MLL*-rearrangements and *TCF3-PBX1*, occur in about two-third of non-DS B-cell precursor (BCP) ALL patients. However, these abnormalities are found only in approximately 20% of DS children with ALL.[5,6] Recently, genomic abnormalities of Cytokine Receptor Like Factor 2 (*CRLF2*) have been described in approximately 60% of DS ALL patients.[7] These rearrangements include a) translocations of *CRLF2* with the Immunoglobulin heavy chain locus (*IGH@*) at chromosome 14q32 and b) interstitial deletions in the pseudo-autosomal region 1 (PAR1) resulting in *P2RY8-CRLF2* fusion.[7-9] Functionally, both aberrations result in overexpression of *CRLF2* and activation of the JAK-STAT pathway.[7-9] Activating mutations in amino acid R683 of *Janus Kinase 2* (*JAK2*) occur in around 20% of DS ALL cases, which were initially thought to be unique to DS ALL.[6,10-12] Mutations of *JAK2* and rearrangements of *CRLF2* are found in <10% and up to 15% of *high-risk* non DS ALL patients, respectively.[8,12-16]

Deletions in B-cell development and differentiation genes, including *IKZF1*, *TCF3*, *EBF1*, *PAX5* and *VPREB1*, have recently been described in DS and non-DS ALL.[17-20] The prognostic significance of abnormalities of these genes in DS ALL is currently unknown. Therefore, we analyzed a population-based cohort of DS ALL samples for deletions in these genes using genomic profiling and related these to clinical outcome. The survival parameters were validated using another population-based cohort of DS ALL patients from UK trials for abnormalities involving *IKZF1* and *CRLF2*.

MATERIALS AND METHODS

Patients and patient samples

We identified all DS ALL patients (n=58) enrolled in 3 consecutive Dutch Childhood Oncology Group (DCOG) ALL treatment protocols (DCOG ALL8, ALL9 and ALL10). Details of DCOG ALL8 and ALL9 have been reported elsewhere, and ALL10 is currently ongoing (Supplementary figure 1).[21,22] In addition, we identified all DS ALL patients (n=114) enrolled in the UK National Cancer Research Institute (NCRI) Childhood Cancer and Leukaemia Group (CCLG) treatments protocols ALL97, ALL97/99 and ALL2003, which were used as a validation cohort.[23,24] Both, DCOG and UK treatment protocols were approved by the Institutional Review Board of the participating centers according to local law and guidelines, and informed consent was ob-

tained from all patients, their parents or guardians. Data on clinical characteristics, biological features and outcome were extracted from national databases.

Vials with frozen cells were available from 34/58 DCOG patients. After thawing, contaminating non-leukemic cells were eliminated as previously described.[25] Blast percentages were assessed morphologically on May-Grünwald-Giemsa–stained cytospin slides. All patient samples contained ≥ 80% leukemic blasts (median 95.5%, range 83% – 99%). Genomic DNA and total cellular RNA were extracted from leukemic cells using Trizol reagent (Gibco BRL, Life Technologies, Breda, the Netherlands), as previously described.[25] DNA quality was tested using gel electrophoresis, and DNA quality and quantity were measured on the Nanodrop 1000 spectrophotometer (Isogen, De Meern, the Netherlands). Analyses for UK patients (n=88) were performed on pre-treatment samples using standard methodologies and were reported using established nomenclature and definitions.[26,27]

Cytogenetics and Fluorescence in situ Hybridization

Diagnostic samples were routinely analyzed using standard cytogenetic procedures by the reference cytogenetic laboratories in the University Hospitals participating in the DCOG and UK trials. Ploidy status was determined from the karyotypes; if these data were missing array-CGH results were used. Hypodiploidy was defined as <47 chromosomes, diploidy was defined as 47 chromosomes, low hyperdiploidy as ≥48 ≤50 chromosomes, and high hyperdiploidy as ≥ 51 chromosomes. All DCOG and UK samples were further analyzed for the presence of the *ETV6-RUNX1* fusion using fluorescence in-situ hybridization (FISH) with the dual-color translocation probe set: LSI ETV6 (TEL)/RUNX1(AML1)ES (Vysis).

Genomic *CRLF2* aberrations in the DCOG and UK cohorts, and *IKZF1* deletions in the UK cohort were detected using FISH as previously described.[8,16] Briefly, we used a break-apart FISH probe to identify *CRLF2* involvement. A break-apart probe to *P2RY8* was designed to map the centromeric breakpoint of the deletion in patients with loss of the centromeric signal indicating deletion within the pseudo autosomal region centromeric of *CRLF2*. Combined results from these two break-apart probes indicated the presence of the *P2RY8-CRLF2* fusion. The involvement of *IGH@* was determined by interphase FISH using the LSI *IGH* Dual Color Break-Apart Rearrangement Probe (Abbott Diagnostics). Results were recorded using a Zeiss Axioscop fluorescence microscope (Zeiss) fitted with a 100_/1.30 oil objective, CCD camera and digital imaging software: ISIS software version 5.1.9 (Metasystems, Germany) and Cytovision Version 4.5 (Leica Microsystems, UK).

Array-Comparative Genomic Hybridization

To identify copy number changes, particularly in the B-cell development and differentiation genes: *PAX5*, *VPREB1*, *TCF3*, *EBF1* and *IKZF1*, we performed 105-K oligonucleotide array-CGH

on genomic DNA on the DCOG cohort as previously described.[17,28] Genomic losses and gains were identified as a minimum of 3 consecutive probes deviating beyond the threshold of -0.8 for single copy loss and -1.8 for bi-allelic loss (log ratio). Deletions were defined as a loss ≥0.5 million bases, whereas focal deletions were defined as losses < 0.5 million bases.[17]

To further specify deletions of *IKZF1* in the DCOG cohort, genomic DNA was PCR amplified to detect Isoform 6 of the *IKZF1* gene with primers previously described.[19]

Mutation analysis

To analyze mutations in the coding exons of *IKZF1* (exon 2-8) and in the *JAK2* R683, PCR amplified DNA was purified and bi-directionally sequenced as previously described.[8,29,30]

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was used in both DCOG and UK cases for detection and validation of aberrations in B-cell development and differentiation genes found by array-CGH and to further define *IKZF1* deletions into different splice variants. MLPA analysis was performed using the SALSA MLPA kit P335-A3 ALL-IKZF1 which contains probes for selected B-cell development and differentiation genes, and the SALSA MLPA kit P202-A1 IKZF1, which contains an increased density of probes for *IKZF1* (MRC Holland). The full list and location of the MLPA probes can be downloaded from the MRC Holland website (http://www.mrc-holland.com). The data were normalized, by dividing the peak area of each probe by the mean peak area of the control probes. Peak heights below 0.7 (0.75 for MRC UKALL samples) and above 1.3 times the control peak height were considered abnormal, with those below 0.7 representing deletions, and those above 1.3 representing duplications.[31,32]

Gene-expression profiling and Quantitative real-time PCR

Biotinylated cRNA of DCOG subjects was synthesized, hybridized and processed to Affymetrix U133 plus 2.0 GeneChips (Affymetrix, Santa Clara, CA USA) as previously described.[17,31] Data was acquired using Expresso (BioConductor package Affymetrix) and probe-set intensities were normalized using the variance-stabilization normalization (VSN) BioConductor package in the statistical data analysis environment R, version 2.7.0. VSN RMA normalized data was used to test for statistical differences in the expression of *CRLF2* (probe set 208303_s_at). We performed Quantitative real-time PCR (RT-qPCR)[33] with primers as previously described and used SYBRgreen (Finnzymes) for expression analysis.[7] The average cycle threshold (Ct) value was used to calculate mRNA expression levels of *CRLF2* relative to the expression level of the reference gene '*ribosomal protein S20*' (*RPS20*) by use of the comparative cycle time (_Ct) method.[34]

Statistics

For statistical comparisons the Statistical Package for the Social Sciences (SPSS) Analysis system (v.15.0, SPSS Inc., Chicago, IL, USA) was used. To compare categorical variables we used χ^2 analyses, for continuous variables the Mann-Whitney U test and Spearman Rho (ρ) was used to calculate correlation coefficients. P-values ≤ 0.05 were considered statistically significant (two-tailed testing).

To evaluate outcome, statistical analyses were conducted using SAS software (SAS-PC, Version 9.1). Complete remission (CR) was defined as less than 5% blast in the bone marrow, with regeneration of tri-lineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere. Event free survival (EFS) was calculated from the date of diagnosis to the date of last follow-up or to the first event, including relapse, death in CR, and failure to achieve CR (considered as event on day 0). Early death was defined as any death within the first 6 weeks of treatment, and was considered as an event on day 0. Overall survival (OS) was measured from the date of diagnosis to the date of last follow-up or to the date of death from any cause. The Kaplan-Meier method was used to estimate survival rates, and survival estimates were compared using the log-rank test. The cumulative incidence of relapse (CIR), with other events and death in CR as competing events was constructed by the method of Kalbfleisch and Prentice and compared using the Gray test.

For multivariate analysis, a stepwise Cox proportional-hazard regression model was used. Data from both the DCOG and UK validation cohorts were merged for this purpose. Continuous variables known to be of prognostic value in ALL were categorized according to the National Cancer Institute (NCI) risk criteria [35], and with the cut-offs previously described in DS ALL and BFM-95.[36,37]

RESULTS

Patient samples and characteristics

A total of 34/58 (59%) DCOG and 88/114 (77%) UK DS ALL patients were enrolled in this study. All 122 patients were classified as B-cell precursor ALL and treated with curative intent. The clinical and presenting characteristics of the tested cohort and the remaining DS ALL samples (residual cohort), for both DCOG and UK, were compared to test for selection bias, and no differences were detected (Supplementary Table S1 and S2). Furthermore, we tested for differences between the DCOG and the UK sample cohorts (Table 1). Patients from UK trials had a lower 6-year survival estimate, which was due to a higher rate of death and relapse in early treatment protocols.[38] In addition, WBC was slightly higher in UK patients then in DCOG patients (18.8 vs. 8.8 $x10^9$/L; p=0.07).

Table 1. Down syndrome ALL patient characteristics of the DCOG cohort compared with the UK Cohort

	DCOG sample cohort	UK sample cohort	p
Number	34	88	
Age at diagnosis (year)	4.9	4.8	0.85
Gender			0.29
Male	20	58	
Female	14	26	
Median initial WBC (x 10^9/L)	8.8	18.8	0.07
Immune phenotype			
Pro-B	2	NK	
C-ALL	21	NK	
Pre-B	11	NK	
Treatment protocol			
ALL 8	8	NA	
ALL 9	17	NA	
ALL 10	9	NA	
EFS	76.2 ± 8.2%	58 ± 6%	0.05
OS	85.1 ± 6.2%	63 ± 6%	0.01
CIR	18 ± 8%	16 ± 5%	0.8
Cytogenetic aberrations			
ETV6-RUNX1	2 (1.5)	15/85* (18)	0.15
HeH	4 (11.8)	4/73* (5)	0.26
IKZF1	12 (35)	23/85* (27)	0.37
CRLF2	21/33* (62)	43/84* (51)	0.07
JAK 2	5 (15)	8/20* (40)	0.05

DCOG, Dutch childhood oncology group; UK, united kingdom; NK, not known; NA, not applicable; WBC, white blood cell count, HeH, high hyperdiploidy (>52 chromosomes); ALL, acute lymphoblastic leukemia; * Not all genomic aberrations were analysed in all patients.

Cytogenetics and Fluorescence in situ Hybridization

Cytogenetic data were available for 31/34 DCOG patients (3 were based on array-CGH) and 88 UK patients (Supplementary Table S3A and B, respectively). A normal karyotype was found in 14 (45%) DCOG and 34 UK patients based on the full analysis of least 10 cells (there was failure to achieve a successful karyotype in 6 UK patients); 1 DCOG patient had a *BCR-ABL1* fusion, while 1 DCOG and 2 UK patients had t(8;14)(q11;q32).[5] The gain of an X chromosome was observed in 6 DCOG and 18 UK patients, which were identified as additional derived X chromosomes.[5] The remaining patients (n=15, 48%) had random cytogenetic aberrations. The *ETV6-RUNX1* fusion was found in 2 (6%) DCOG patients and 15/85 (18%) UK patients.

Ploidy status of DCOG patients was determined from karyotypes (n=31) and from array-CGH (n=3). Four (12%) patients had a high hyperdiploid karyotype (HeH) (≥52 chromosomes), including trisomy 10 (n=4), trisomy 4 or 18 (n=3), and tetrasomy 21 (including the constitu-

tional gain) (n=2). A similar proportion of UK patients (4/73, 5.5%) had a HeH karyotype; and included trisomy of 4 (n=5), 10 (n=4) and 18 (n=4) and tetrasomy 21 (n=5).

In 21/33 (64%) DCOG DS ALL patients, *CRLF2* was aberrant, including *IGH@-CRLF2* (n=5) and *P2RY8-CRLF2* (n=16). One patient could not be evaluated due to the poor quality of the material. In the UK cohort 43/84 (52%) DS ALL patients had lesions of *CRLF2*, with 6/84 (7%) *IGH@-CRLF2* translocations and 37/84 (44%) *P2RY8-CRLF2* fusions. Four patients were not tested for *IGH@-CRLF2*, but were negative for *P2RY8-CRLF2*.

Array-Comparative Genomic Hybridization

In total, 17/34 (50%) DCOG cases had ≥1 deletion of B-cell development and differentiation genes. Affected genes included the transcription factors: *IKZF1* (n=12, 35%), *VPREB1* (n=6, 18%) and *PAX5* (n=4, 12%). Eight patients had focal deletions within *IKZF1*, 2 patients had a focal deletion of *IKZF1* including the 3' flanking region, and 2 patients had a deletion of the entire gene, as the whole chromosome 7p arm was deleted (Figure 1). In 6 of the patients with focal deletions within *IKZF1*, exon 3 through 6 was deleted, which was validated by PCR. This deletion results in the expression of isoform 6, a dominant-negative form of *IKZF1*.[18] No deletions were found in *EBF1* or *TCF3*. Deletions in the *PAX5* gene were always part of larger deleted regions, whereas deletions of the other genes were mainly focal. Aberrations of *IKZF1* were not mutually exclusive: i.e. in 4 cases an *IKZF1* deletion was found in combination with a *PAX5* or *VPREB1* deletion. Overlap between *IKZF1*, *CRLF2* and *JAK2* aberrations are depicted in figure 2.

Mutation analysis

No additional mutations were found after sequencing the coding exons (2-8) of *IKZF1* in DCOG DS ALL patients. Synonymous polymorphisms were found at P334 in 6 patients (17.6%) and at N392 in 2 (5.9%) patients. *JAK2* R683 activating mutations were found in 5/34 (15%) of the DCOG DS ALL patients as previously reported.[30] The incidence of *JAK2* R683 mutations was higher in the UK cohort (8/20, 40%) as previously reported.[16]

Multiplex Ligation-dependent Probe Amplification

The SALSA MLPA P202-A1 IKZF1 kit, with the majority of probes targeting *IKZF1*, confirmed that 12/34 (35%) of the DCOG DS ALL patients had deletions of *IKZF1* which were identical to those detected by array-CGH.

Using the SALSA MLPA P335-A3 ALL-IKZF1 kit, the same results were achieved for *IKZF1* deletions as with the P202 MLPA kit. In addition, in 4 patients (12%), all probes for *PAX5* were deleted, while no focal deletions of *PAX5* were seen. No aberrations of *EBF1* (0/34) were

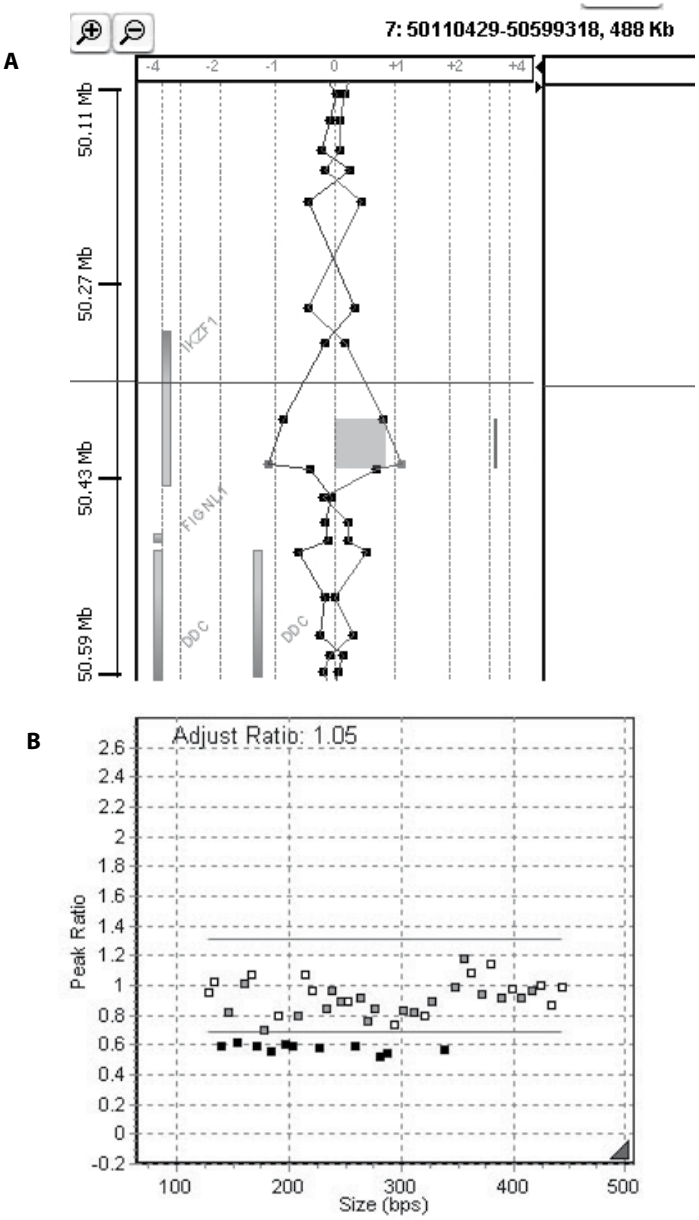


Figure 1. *IKZF1* aberration detected by Array-CGH and MLPA

A) The figure shows a chromosome 7 oligonucleotide Array Comparative Genomic Hybridization (CGH) plot of the log-ratio of patient DNA/control DNA ratios (grey tracing) versus the dye-swap experiment (black racing) from a patient with an *IKZF1* deletion. The figure zooms in on the deleted area and presents the genes located in this area.

B) Multiplex Ligation-dependent Probe Amplification (MLPA) plot of a patient with a Isoform6 deletion of *IKZF1*. Each square indicates a different probe. The white squares are control probes, grey squares represent normal probes, and the black squares are deleted probes. All probes located in exon 3 through 6 are deleted, resulting in Isoform 6 of *IKZF1*.

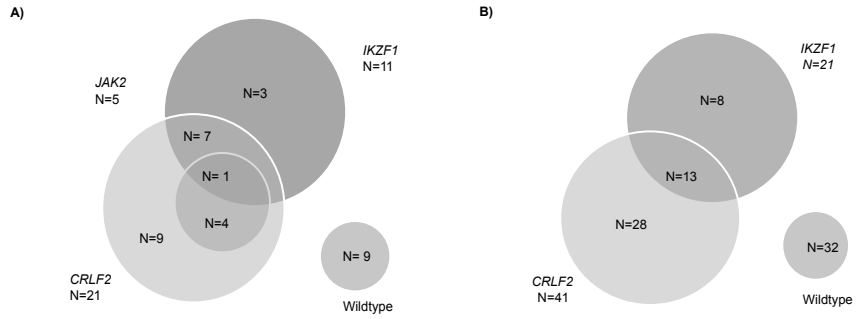


Figure 2. Venn-diagram showing the overlap between aberrations

A) DCOG cohort. One patient was excluded from the figure since the *CRLF2* status was not determined. Four patients were excluded from the figure of the UK cohort (B), as *IKZF1* or *CRLF2* were not known for these cases.

detected. In the UK cohort, *IKZF1* was found to be aberrant in 23/85 (27%) patients, of which 5 (29%) resulted in *IKZF1* Isoform 6, 5 (29%) had whole gene deletions and 7 (41%) had different deletion profiles. Interestingly, patients with *IKZF1* deletions are significantly older then patients with wildtype *IKZF1* (8.2 vs. 4.3; p=0.03) (Table 2).

Gene-expression profiling and real-time PCR

Of 23 DCOG DS ALL patients, RNA was available for gene-expression profiling and RT-PCR. The median variance-stabilization normalized (VSN) expression value of patients with *P2RY8-CRLF2* (n=11; median 9.4) was significantly higher than in patients with wildtype *CRLF2* (n=8; median 7.8); p 0.001), and was also higher as compared to patients with *IGH@-CRLF2* (n=4; median 8.4); p=0.001) (Figure 3A), which was confirmed by RT-PCR (correlation coefficient of $\rho = 0.7$, p=0.0002) (Figure 3B). The median relative mRNA expression of *CRLF2* in patients with *P2RY8-CRLF2* was 6.5 fold higher (median 6.5) than in patients with *IGH@-CRLF2* (median: 0.001) and wildtype *CRLF2* (median: 0.001); p<0.001.

Table 2. Characteristics of patients with aberrant and wildtype *IKZF1*

	IKZF1 deletions	Wildtype *IKZF1*	p
Number	35	84	
Median age at diagnosis (year)	8.2	4.3	0.03
Gender			0.49
Male	21	56	
Female	14	28	
Median initial WBC (10^9/L)	17.5	14.9	0.07

WBC, white blood cell count

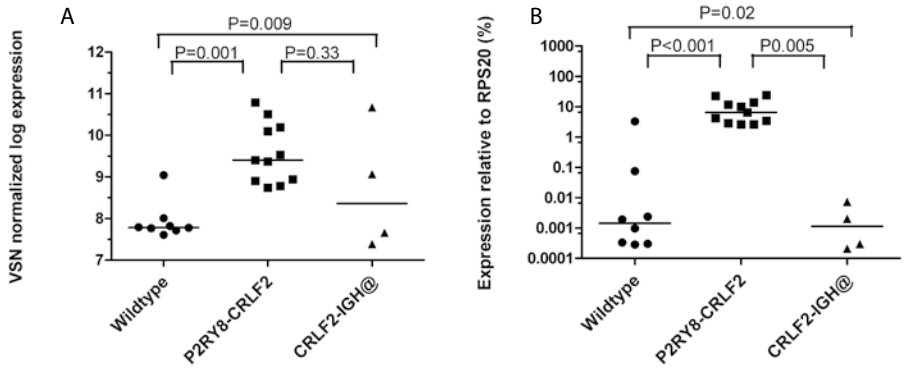


Figure 3. *CRLF2* expression as determined by gene expression profiling and RT-qPCR

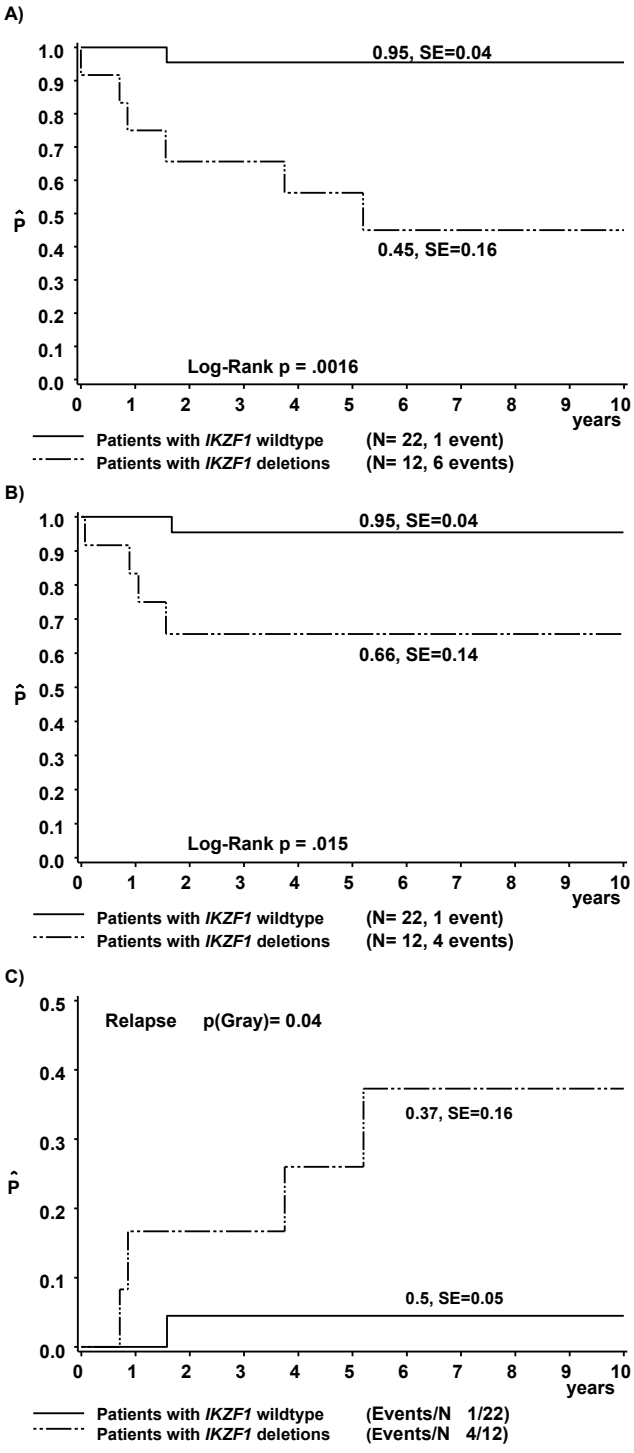
Graphs showing the expression of *CRLF2*. Bars represent the median expression in each group. Differences in *CRLF2* expression are shown between patients with wildtype *CRLF2*, *IGH@-CRLF2* translocation and *P2RY8-CRLF2*. **A)** Expression of probe 208303_s_at representing the *CRLF2* gene after log transformation. Significant differences in median expression are shown between patients with *CRLF2* wildtype (n=8; 7.8) and patients with *P2RY8-CRLF2* (n=15; 9.4), p=0.001, between *P2RY8-CRLF2* and *IGH@-CRLF2* translocation (n=4; 8.4, p=0.33), and between wildtype and all patients with CRLF2 lesions, p=0.009. **B)** Cumulative mRNA expression levels of *CRLF2* relative to RPS20 (%). Significant differences are observed between patients *CRLF2* wildtype (n=8; 0.001) and patients with *P2RY8-CRLF2* (n=15; 6.5), p<0.001, between *P2RY8-CRLF2* and *IGH@-CRLF2* translocation (n=4; 0.001), and between wildtype and all patients with CRLF2 lesions, p=0.02.

Univariate survival analysis

The median follow up time for survivors in the DCOG sample cohort was 5.7 years (range 1.2–15.4 years), the median FU time for UK survivors was 4.6 years (range 0.4-12.0 years). The 6-year EFS was 76±8.2%, the 6-year OS 85±6.2% and the CIR was 18±8% in DCOG DS ALL patients. UK DS ALL patients had a 6-year EFS of 58±6%, a 6 year OS of 63±6.1% and a CIR of 16±5%.

DCOG DS ALL patients with a WBC ≥20x10^9/L at diagnosis (n= 11) had a significantly worse outcome than patients with lower WBC (n=23) (6-year EFS 38±16.1% vs. 96±4.3%; p<0.001, 6-year OS 60±15.5% vs. 96±4.3%; p=0.005), and this was confirmed in the UK cohort (6-year EFS 43±8.7% vs. 70±7.4%; p=0.01, 6-year OS 50±8.6% vs. 70±8.3%; p=0.03). There was no significant difference in outcome for patients below or above 6 years of age at diagnosis in both DCOG and UK patients.

Patients with an *IKZF1* deletion (n=12) had a significantly worse outcome than patients without an *IKZF1* deletion. In fact, with the exception of a single patient, all 7 events occurred in patients with an *IKZF1* deletion. The 6 year EFS was 45±16% for *IKZF1* deleted vs.95±4% for *IKZF1* wild-type patients; p=0.002, the OS 66±14% vs. 95±4%; p=0.02 and CIR 37±16% vs. 5±5 %; p(Gray)=0.044. The poor outcome for *IKZF1* deleted patients was confirmed in the UK cohort (EFS 21±12% vs. 58±11%; p=0.002, OS 15±12% vs. 71±9%; p=0.02, and CIR 37±15% vs. 18±10 %; p(Gray)=0.06) (Figure 4). Also when we combined the DCOG and the UK cohort (EFS 31±10% vs. 75±7%; p<0.001, OS 40±10% vs. 82±6%; p=0.0003, and CIR 37±11% vs. 11±6


A)
1.0
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0.45, SE=0.16
Log-Rank p = .0016
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Patients with IKZF1 wildtype (N= 22, 1 event)
Patients with IKZF1 deletions (N= 12, 6 events)
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Log-Rank p = .015
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Patients with IKZF1 wildtype (N= 22, 1 event)
Patients with IKZF1 deletions (N= 12, 4 events)
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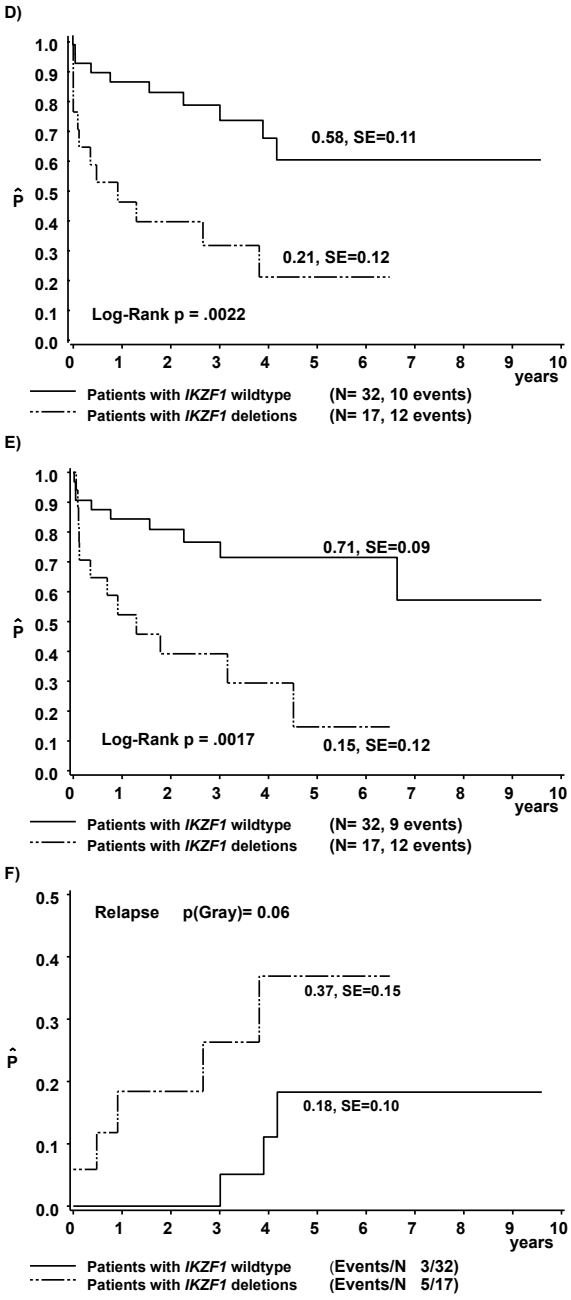

Figure 4. Kaplan-Meier estimates of DS ALL patients with and without *IKZF1* aberrations

Six year event free survival (EFS) in DCOG DS ALL patients for *IKZF1* is depicted in (A) and EFS for the UK dataset is depicted in (D), the overall survival (OS) of DCOG DS ALL patients is shown in (B), the OS for the UK dataset depicted in (E), and the cumulative incidence of relapse (CIR) of DCOG DS ALL patients is shown in (C), the CIR for the UK cohort is depicted in (F). Patients with *IKZF1* deletions have a significantly worse outcome in terms of OS, EFS, and CIR compared with *IKZF1* wildtype patients.

%; p(Gray)=0.007) (Supplementary figure S2) the poor outcome of *IKZF1* deleted cases was evident.

There was no statistically significant difference in clinical outcome between patients with or without genomic *CRLF2* aberrations, neither in the DCOG cohort (EFS 75±10% vs. 79±14% in *CRLF2* wildtype patients; p=0.6, OS 81±9% vs. 92±8%; p=0.42, CIR 15±8% vs. 21±15%; p(Gray)=0.9, supplementary figure S3) nor in the UK cohort (EFS 50±9% vs. 67±8%; p=0.26, OS 58±9% vs. 69±8%; p=0.27 and CIR 22±8% vs. 11±5%; p(Gray =0.32, supplementary figure S4).

The OS for DCOG patients with a *PAX5* deletion was significantly lower compared to those with wildtype *PAX5* due to the poor salvage rate at relapse (OS: 50±25% vs. 90±6%; p=0.03). No statistically significant differences in outcome were found in DCOG patients with and without a deletion of *VPREB1* (OS 67± 9% vs. 89±6%; p=0.12, CIR 17±17% vs. 18±9%; p(Gray) =0.85), nor for *JAK2* R683 (data not shown).

Multivariate survival analysis

A stepwise multivariate Cox regression model was performed on the total dataset (DCOG and UK) for enough statistical power and included WBC at diagnosis (≥20 x 10^9/L), sample cohort, mutational status of *IKZF1* and genomic *CRLF2* aberrations were variables included in the multivariate model.

Deletions of *IKZF1* appeared to be the strongest independent risk factor for EFS, OS and relapse-free survival (RFS) with a hazard ratio (HR) for EFS of 3.05 (95% CI 1.55 – 6.02; p=0.001), for OS of 2.82 (95% CI 1.40 – 5.70; p=0.003) and for RFS of 7.83 (95% CI 2.39 – 25.63; p=0.001). The cohort (UK v DCOG) was also an independent risk factor for EFS and OS, but not for RFS (EFS: HR 2.37, 95% CI 1.01 – 5.54; p=0.046, OS: HR 3.26, 95% CI 1.23 – 8.65; p = 0.02 (Table 3). Genomic *CRLF2* aberrations were not a predictor for prognosis.

DISCUSSION

When assessing overall outcome, children with DS-ALL have fared less well than their non-DS counterparts in most clinical trials.[39-43] There is a need to understand which genetic abnormalities contribute to the development of ALL in DS children, and which abnormalities predict for poor outcome. This study has focused on the frequency and prognostic value of aberrations involving B-cell development and differentiation genes in DS ALL patients, especially *IKZF1* deletions, based on recent findings in non-DS high-risk ALL, and the recent detection of patients with a *BCR-ABL1*-like gene expression signature by Den Boer et al from our department, with a high-risk of recurrent disease.[17,18]

Table 3. Results of multivariate analysis for overall survival, event-free survival and relapse free survival for the combined DCOG and UK cohorts

Outcome	Variable	HR	95% CI	*P*-value
EFS	*IKZF1*	3.05	1.55 - 6.02	0.001
	Collaborative Group	2.37	1.01 - 5.54	0.05
	Genomic *CRLF2*	1.69	0.83 - 3.44	0.15
	WBC ≥ $20x10^9$/L	2.41	1.20 - 4.85	0.01
OS	*IKZF1*	2,82	1.40 - 5.70	0.004
	Collaborative Group	3.26	1.23 - 8.65	0.02
	Genomic *CRLF2*	1.86	0.87 - 3.94	0.11
	WBC ≥ $20x10^9$/L	2.01	0.98 - 4.13	0.06
RFS	*IKZF1*	7.83	2.39 - 25.63	0.001
	Collaborative Group	1.38	0.45 - 4.27	0.58
	Genomic *CRLF2*	1.14	0.39 - 3.36	0.81
	WBC ≥ $20x10^9$/L	2.87	0.95 - 8.67	0.06

DCOD, Dutch childhood oncology group; UK, united kingdom; HR, hazard ration; CI, confidence interval, WBC, white blood cell count; EFS, event free survival; OS, overall survival; RFS: relapse free survival

Aberrations in genes encoding for B-cell development and differentiation, are common in non-DS B-cell precursor ALL. The frequency varies between 67% in high-risk non-DS ALL and 82% in non-DS patients with a *BCR-ABL1*-like gene expression signature.[17,18] We found that deletions in these genes, including *PAX5*, *VPREB1*, *TCF3*, *EBF1* and *IKZF1*, occurred in approximately 50% of an unselected cohort of DS ALL, which also included low risk patients. The incidence of *PAX5* deletions reported in this study is rather low (12%) when compared to non-DS ALL patients (21% - 29%) [19,29,44], but similar to a group of non-DS ALL patients with genetically unclassified disease (B-other), as previously reported by Den Boer et al.[17] None of the DS ALL patients had deletions in *EBF1*, which is in correspondence to the low frequency reported in non-DS ALL.[17,19,29]

The frequency of *IKZF1* deletions in the unselected DCOG (35%) and UK cohorts (26%) is comparable to the 29% found in high-risk non DS ALL patients [18] and to the 39% in a group of high-risk non-DS ALL patients with a '*BCR-ABL1* like' gene-expression signature.[17] Interestingly, 38% of the DCOG DS ALL have a gene-expression signature similar to those of *BCR-ABL1*-like non-DS ALL as identified in our previous study by a 110 probe-set based classifier. Within this DS-*BCR-ABL1*-like group, 40% has a deletion of *IKZF1* similar to the frequency of *IKZF1* deletions observed in non-DS *BCR-ABL1*-like cases.[17] Taken together, this suggests that a relatively large proportion of DS ALL patients have a genetic profile with characteristics of high-risk B-cell precursor ALL, and thus need to be treated accordingly, although this should be balanced against the increased risk of treatment related mortality in DS children. Given that the option for intensification of chemotherapy in DS subjects are limited, it is key to unravel the underlying biology of *IKZF1* aberrant leukemias, in order to develop les toxic

(targeted) therapy options for these patients. If confirmed in future studies or by other collaborative study groups, screening for evidence of high-risk genetic abnormalities such as *IKZF1* in DS ALL may lead to improved risk-group stratification. The future DCOG ALL11 treatment protocol will already use *IKZF1* deletions to assist in this and to tailor anthracycline exposure.

Recently, Mullighan et al. reported that alterations of *IKZF1* were associated with *JAK2* R683 mutations in 87% of high-risk non-DS ALL patients.[12] These patients had a 4-year CIR of 77%, from which the authors concluded that this defined a very poor risk subgroup.[12] This is different in DS ALL, as only 1/34 DCOG patients had a combined *IKZF1* and *JAK2* aberrations and the clinical course was uneventful during 7 years of follow up. Moreover, none of the 5 *JAK2* R683 mutated DS ALL patients without *IKZF1* aberrations experienced an event (data not shown). This implies a difference in the pattern of genetic changes leading to leukemogenesis, between non-DS and DS ALL patients, which is so far not very well understood.

Aberrations of *CRLF2* are recently described genetic abnormalities in DS ALL, [8,9,13] occurring at a high frequency of ~51% in this study. Some authors have used gene expression profiling to screen for *CRLF2* overexpression, however this may occur independently from genomic aberrations in the *CRLF2* gene.[13] Loudin et al, identified with supervised clustering a gene expression signature associated with high *CRLF2* expression.[20] After supervised clustering of our DCOG patients, genomic *CRLF2* positive samples clustered separately from wildtype *CRLF2* samples. However, only 4 genes with a false discovery rate <10% were detected (data not shown), which implies that *CRLF2* is not a strong signature. Furthermore, we found that DCOG DS ALL patients who carried the *P2RY8-CRLF2* aberration had a significantly higher expression of *CRLF2* compared to patients with a translocation of *IGH@-CRLF2* or wildtype *CRLF2*, which is different from what is found in other studies, although this might be due to small numbers in our study.[8,14] Moreover, we did not identify the *CRLF2* expression level, or genomic *CRLF2* aberrations to be a significant risk factor for inferior treatment outcome, which is in agreement with the interim report of the Ponte di Legno on a large cohort of DS ALL patients [37] and several other DS and non-DS ALL studies, [7,9,16,37], but different from some other studies. [13,15]

In agreement with our findings, Harvey et al. previously demonstrated in high-risk non-DS ALL patients that only *IKZF1* deletions, but not *CRLF2* aberrations were independently associated with relapse.[14] Furthermore, genomic aberrations of *CRLF2* were strongly associated with *JAK2* mutations.[7,8,14] Indeed, in our DS ALL cohort, all patients (n=5) with a *JAK2* R683 mutation harbored *P2RY8-CRLF2*, although, none of these patients experienced an event. Recently Herztberg et al hypothesized that overexpression of *CRLF2* is the first event in leukemogenesis of DS ALL, followed by mutations such as *JAK2* and *CRLF2* [6-8,15]. However, *IKZF1* deletions, which disrupt normal lymphoid development, are not yet included in this model. Further research is needed to identify which of the aberrations is the primary event, and how they cooperate in the pathogenesis of DS ALL.

Histone gene deletions have been recently reported by Loudin et al. to be more common in DS ALL compared to non-DS ALL (22% vs. 3.1%) patients, albeit without prognostic significance.[20] In our DCOG cohort, only 1 (3%) DS ALL patient had a large deletion at the histone-cluster at 6p22, while other patients showed only single probe deletions by array-CGH, which we consider to be below the threshold for true copy number changes. We did not search for point mutations, thus the involvement of the histone cluster may be underestimated in this study. In conclusion we could not confirm a high frequency of histon deletions in DS ALL patients, but more sensitive techniques are needed.

In this study, MRD data was only available of a limited number of patients, and patients were treated on different protocols. Unfortunately we therefore could not include this parameter in the multivariate analysis.

In conclusion, this study demonstrates that *IKZF1* is a strong and independent predictor for poor clinical outcome in DS ALL, which was confirmed in an independent validation cohort of UK DS ALL patients. These data suggest that there are similarities between DS and non-DS ALL in relation to the underlying genetic background. A relatively large proportion of DS ALL patients has high-risk genetic characteristics and thus need to be treated accordingly, in balance with TRM. If confirmed in larger and prospective series, *IKZF1* abnormalities may be integrated into risk stratification of DS ALL patients for treatment.

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Supplementary Table S1: Characteristics of the Dutch childhood oncology group sample cohort and residual cohort

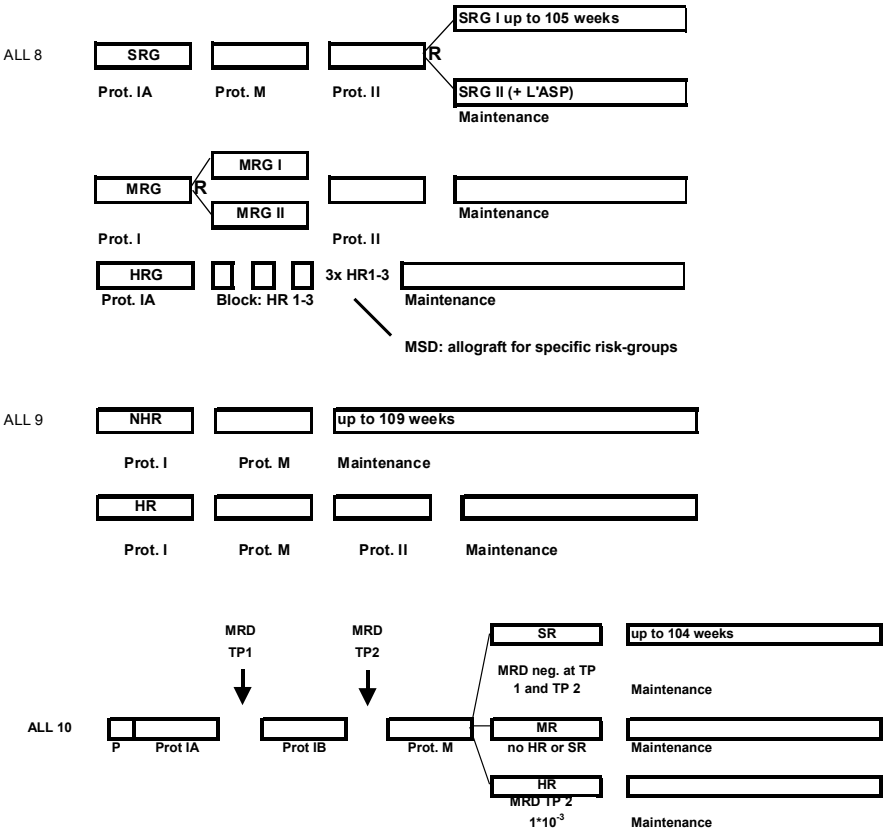
	Sample cohort	Residual cohort	p-value
Number	34	24	
Age at diagnosis (year)	4,9	5.3	0,99
Sex			
Male	20	11	
Female	14	13	0.33
Median initial WBC (x 10^9/L)	8.8	7.7	0.26
Immune phenotype			
Pro-B	2	8	
C-ALL	21	16	
Pre-B	11	0	0.48
Treatment protocol			
ALL 8	8	4	
ALL 9	17	7	
ALL 10	9	13	0,1
EFS	76% ± 8%	65% ± 11%	0,18
OS	85% ± 6%	72% ± 10%	0,16
CIR	18 ± 8%	14 ± 10%	0,6

ALL, Acute lymphoblastic leukemia; Pro, Early B-Lymphocyte Precursor Cells; C, common; Pre, precursor; WBC, white blood cell count; EFS, event free survival; OS, overall survival; CIR, cumulative incidence of relapse.

Supplementary Table S2: Characteristics of United Kingdom sample cohort and residual cohort

	Sample cohort	Residual cohort	p-value
Number	88	26	
Age at diagnosis (year)	4.9	4.9	0.89
Sex			0.22
Male	59	14	
Female	29	12	
Median initial WBC (x 10^9/L)	16.1	13.9	0.78
EFS	78.8 ± 6.1	95 ± 4.9	0,41
OS	61 ± 6%	65.3 ± 11.8%	0,48
CIR	15.7 ± 4.6%	4.2 ± 4.1%	0,4

WBC, white blood cell; EFS, event free survival; OS, overall survival; CIR, cumulative incidence of relapse



Supplementary Figure S1. Schematic overview of the different treatment blocks of the DCOG ALL-8, ALL 9 and ALL 10 treatment protocols

Prot: protocol; SRG: standard risk group; MRG: medium risk group; HRG: high risk group; NHR: non high risk; HR: high risk; SR: standard risk; MR: medium risk; L-ASP: L-asparaginase; MRD: minimal residual disease; TP: time-point.

Supplementary Table S3A. Clinical and genetic characteristics of Dutch childhood oncology group DS ALL patients

							FISH			PCR and sequencing	PCR Isoform 6	Array-CGH and MLPA				
ID	**Sex**	**Age**	**WBC**	**IPT**	**Karyotype**	**Ploidy**	***ETV6-RUNX1***	***BCR/ABL***	***CRLF2***	***JAK2* R683**	***IKZF1***	***IKZF1***	***TCF3***	***PAX5***	***VPREB1***	***EBF1***
1	Male	9.2	29.7	C-ALL	48,XY,+X,t(8;14)(q12;q32),+21c/47,XY,+21c	3	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
2	Female	4.9	4	PRE-B	47,XX,+21c[30]	2	WT	WT	TL	WT	WT	WT	WT	WT	WT	WT
3	Female	2.6	18.4	PRE-B	48,XX,+X,add(18)(q22),+21c[2]/47,XX,+21c[30]	3	WT	WT	R	WT	WT	WT	WT	WT	WT	WT
4	Male	13.5	1.2	C-ALL	56,XY,+X,+4,+10,+14,+14,+17,+18,+18,+21c,+mar[13]/47,XY,+21c[9]	4	WT	WT	WT	WT	WT	WT	WT	WT	R	WT
5	Male	3.5	22.3	C-ALL	47,XX,+21c	2	WT	WT	R	WT	WT	R	WT	WT	R	WT
6	Male	15.4	4.3	C-ALL	47,XY,t(1;3)(q32;q26),t(2;12)(q23;q13),t(7;8)(q31;q12),del(13)(q14q32),+21c[17]	2	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
7	Female	2.6	8.7	PRE-B	57,XX,+5,+6,+17,+18,+21c,+5mar[cp23]/47,XX,+21c[9]	4	WT	WT	R	WT	WT	WT	WT	WT	R	WT
8	Male	4.2	41.2	C-ALL	46,XY,t(8;9)(q24;p13),del(12)(p13.1),dic(12;13)(p11.2;p10),+21c[20].ish del(12)(TEL-,AML1-,CEP12+),dic(12;13)(TEL-,AML1-,CEP12+)	1	WT	WT	WT	WT	WT	WT	WT	R	WT	WT
9	Female	2.8	32.6	PRE-B	47,XX,+21c	2	WT	WT	TL	WT	WT	R	WT	WT	WT	WT
10	Male	3.1	7.3	C-ALL	48,XY,+X,+21c[19]	3	WT	WT	R	R	WT	WT	WT	WT	WT	WT
11	Male	3.9	77.9	PRE-B	47,XY,+21c	2	WT	WT	R	WT	R	R	WT	R	WT	WT
12	Male	5.6	5	PRE-B	47,XX,+21c	2	WT	WT	R	WT	WT	WT	WT	R	WT	WT
13	Female	7.9	12.9	C-ALL	47,XX,+21c[21]	2	WT	WT	WT	WT	WT	R	WT	WT	WT	WT

Supplementary Table S3A. (Continued)

							FISH		PCR and sequencing	PCR Isoform 6		Array-CGH and MLPA			
14	Male	6.8	199	C-ALL	47,XY,der(9)del(9)(p13p2?2) t(9;22)(q34;q11),+21c,der(22) t(9;22)[15]/47,idem,add(21)(q22)[7]/47,XY,+21c[1]	2	WT	R	WT	WT	R	R	WT	R	R	WT
15	Male	3.7	16.5	C-ALL	48,XY,+21c,+mar[4]/48,XY,+X,+21c[3]/47,XY+21c[13]	3	WT	WT	R	WT	WT	WT	WT	WT	WT	WT
16	Male	17.1	20.3	C-ALL	47,XY,+21c[20]	2	WT	WT	WT	WT	R	R	WT	WT	R	WT
17	Female	7.0	33	C-ALL	47,XX,del(12)(p11p13),+21c[18]/47,XX,+21c[2]	2	WT	WT	R	WT	WT	R	WT	WT	WT	WT
18	Male	2.0	112	C-ALL	47,XY,+21c	2	WT	WT	R	WT	WT	WT	WT	WT	WT	WT
19	Female	8.4	4.1	C-ALL	58~59,XX,+4[3],+6,+10[2],+11,+?14,+18[2],+21,+21c,+1~5mar,inc[cp4]/47,XX,+21c[5]	4	WT	WT	NE	WT	WT	R	WT	WT	WT	WT
20	Male	13.3	6.6	PRO-B	46,XY,-13,+21c[17]/47,XY,+21c[3]	1	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
21	Male	2.2	17.5	C-ALL	47,XY,+21c[20]	2	WT	WT	R	R	WT	WT	WT	WT	R	WT
22	Male	3.8	2.9	PRE-B	47,XY,+21c[22]	2	WT	WT	R	R	WT	WT	WT	WT	R	WT
23	Female	13.8	1.5	C-ALL	49,XX,+X,+5,+21c*	3*	WT	WT	WT	WT	WT	WT	WT	WT	R	amplification
24	Male	5.5	4.5	C-ALL	47,XY,+21c*	2*	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
25	Female	8.1	390	C-ALL	47,XX,-2,-8,+21c,+mar1,+mar2[8]/46,idem,-X/47,XX,+21c[3]	2	WT	WT	TL	WT	R	R	WT	WT	R	WT
26	Female	5.4	3.6	PRE-B	47,XX,add(17)(q2?5),+21c[4]/47,XX,+21c[28]	2	WT	WT	R	WT	WT	WT	WT	WT	WT	WT
27	Female	3.4	16.7	C-ALL	47,XX,+21c[20]	2	WT	WT	TL	WT	WT	R	WT	WT	WT	WT
28	Male	4.9	11.3	PRE-B	47,XY,+21c[32]	2	R	WT	WT	WT	WT	WT	WT	WT	R	WT

Supplementary Table S3A. (Continued)

								FISH		PCRand sequencing	PCR Isoform 6		Array-CGH and MLPA			
29	Female	3.7	8.9	C-ALL	47,XX,+X,-13,i(17)(q10),der(19)t(13;19)(q1?3;q1?2),+21c[15]/47,idem,del(12)(q1?4q2?1)[7]/52,idem,+3,+10,+14,+21,+21[2]/47,XX,+21c[29]	4	WT	WT	R	WT	WT	WT	WT	WT	WT	WT
30	Male	3.3	3.6	C-ALL	47,XY,+21c	2	WT	WT	R	R	WT	WT	WT	WT	WT	WT
31	Male	2.3	6.4	C-ALL	47,XY,+21c*	2*	R	WT	TL	WT	WT	WT	WT	WT	WT	WT
32	Female	4.5	5.7	PRE-B	47,XX,+21c[32]	2	WT	WT	R	WT	R	R	WT	WT	WT	WT
33	Male	7.7	4.2	PRO-B	47,XX,?der(9),del(13)(q12q31),del(16)(q23),add(20)(q11),+21c[26]	2	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
34	Female	6.2	4.5	PRE-B	46,XX,der(14;21)(q10;q10)c,+21c[27]	1	WT	WT	R	R	R	R	WT	WT	WT	WT
											6 (17.6%)	12 (35.3%)				
Total number of aberrations							2 (5.9%)	1 (2.9%)	21 (61.8%)	5 (14.7%)	12 (35.3%)		0	4 (11.7%)	10 (29.45)	0

Karyotype nomenclature according to ISCN 2009. DS ALL, Down syndrome acute lymphoblastic leukemia; WBC, white blood cell count; IPT, immune phenotype; BCP, B cell precursor; Ploidy: 1=hypodiploid (<47 (46 + 21c), 2=diploid (47 (46 + 21c), 3=low hyperdiploid (48-50), 4=hyperdiploid (≥51 (50 + 21c), * based on Array-CGH; WT, wildtype; R, rearrangement; TL, translocation.

Supplementary Table S3B. Clinical and genetic characteristics of United Kingdom DS ALL patients

							FISH			PCR and sequencing	MLPA	
ID	**Sex**	**Age**	**WBC**	**IPT**	**Karyotype**	**HeH**	***ETV6-RUNX1***	***BCR/ABL***	***CRLF2***	***JAK2* R683**	***IKZF1* subgroup**	***IKZF1***
1	Female	16.92	21.7	C- or Pre-B	48,XX,+21c,+21[4]/47,XX,+21c[19]	No	WT	WT	TL	NK	WT	WT
2	Male	5.01	2.8	C- or Pre-B	47,XY,t(8;14)(q11;q32),+21c[5]/47,XY,+21c[23]	No	WT	WT	WT	NK	IK6	R
3	Male	7.27	42.8	C- or Pre-B	47,XY,+21c[12]	NK	WT	WT	WT	R	IK6	R
4	Male	2.62	6.9	C- or Pre-B	46,XY,der(14;21)(q10;q10)c,+21c[18]	No	WT	WT	R	NK	WT	WT
5	Male	2.46	1.9	C- or Pre-B	48,XY,+X,+21c[2]/47,XY,+21c[8]	No	WT	WT	R	NK	WT	WT
6	Male	9.08	39.5	C- or Pre-B	48,XY,+X,+21c[3]/46,XY,-4,-7,+21c,+mar[6]/47,XY,+21c[3]	No	NK	WT	R	R	WT	WT
7	Male	5.47	53.1	C- or Pre-B	47,XY,del(9)(p1?3p2?),+21c[10]/53,idem,+X,+?4,+?8,+9,+18,+22[2]	No	WT	WT	R	NK	Other	R
8	Male	5.78	5.2	C- or Pre-B	47,XY,+21c[20]	No	R	WT	WT	NK	WT	WT
9	Male	9.41	2.5	C- or Pre-B	47,XY,?r(6)(::p?25q2?1::),-10,add(12)(p1?3),add(18)(q23),+21c,+r	No	R	WT	WT	NK	WT	WT
10	Female	2.97	82.8	C- or Pre-B	Fail	NK	R	WT	WT	R	WT	WT
11	Female	3.67	9.5	C- or Pre-B	48,XX,+X,+21c[7]/47,XX,+21c[6]	No	WT	WT	R	NK	WT	WT
12	Male	3.74	102.2	C- or Pre-B	48,XY,+X,t(7;17)(p11;q25),+21c[11]/47,XY,+21c[9]	No	WT	WT	R	NK	NK	R
13	Female	1.81	20.1	C- or Pre-B	47,XX,+21c[20]	No	R	WT	WT	WT	WT	WT
14	Female	16.33	22	C- or Pre-B	47,XX,+21c[10]	No	WT	WT	TL	NK	Whole Gene	R
15	Female	14.16	9.2	C- or Pre-B	47,XX,+21c[10]	NK	WT	WT	R neg, TL NK	NK	Whole Gene	R

Supplementary Table S3B. (Continued)

									FISH		PCR and sequencing	MLPA	
16	Female	2.84	58	C- or Pre-B	48,XX,+X,+21c[10]/47,XX,+21c[10]	No	WT	WT	R	NK	WT	WT
17	Male	10.65	5.2	C- or Pre-B	46,X,-Y,del(6)(q?13q?21),inv(9)(p24q21),del(11)(q2?1q23),+21c[5]/47,XY,+21c[13]	No	WT	WT	WT	NK	Whole Gene	R
18	Male	2.88	32.4	C- or Pre-B	47,XY,+21c[18]/46,XY[2]	No	WT	WT	R	R	NK	NK
19	Male	6.12	47.7	C- or Pre-B	47,XY,del(9)(p22p24),+21c[20]	No	NK	WT	R	NK	IK6	R
20	Male	2.04	29	C- or Pre-B	47,XY,+21c[30]	No	WT	WT	WT	R	WT	WT
21	Male	4.68	9.7	C- or Pre-B	47,XY,+9,der(9;16)(q10;p10),+21c[12]	No	WT	WT	R	NK	WT	WT
22	Female	1.34	111	C- or Pre-B	49,XX,+6,+19,+21c[10]	No	WT	WT	WT	NK	WT	WT
23	Female	5.59	19	C- or Pre-B	48,XX,+X,+21c[7]/47,XX,+21c[3]	No	WT	WT	R	WT	WT	WT
24	Male	5.13	2.2	C- or Pre-B	47,XY,+21c[22]	No	WT	WT	R	NK	WT	WT
25	Male	3.03	47.2	C- or Pre-B	47,XY,+21c[10]	NK	WT	WT	R	NK	WT	WT
26	Female	3.59	10.2	C- or Pre-B	47,XX,del(12)(p1?),+21c[2]/47,XX,+21c[20]	No	R	WT	WT	R	NK	NK
27	Male	4.24	6.4	C- or Pre-B	48,Y,t(X;14)(p22;q32),+der(X)t(X;14),del(15)(q11.2q15),+21c[2]/47,XY+21c[18]	No	NK	WT	TL	NK	Other	R
28	Female	3.57	3.8	C- or Pre-B	47,XX,+21c[20]	No	WT	WT	WT	NK	WT	WT
29	Male	3.58	19.4	C- or Pre-B	47,XY,der(11)t(X;11)(q?2;q2),+21c[4]/47,idem,del(6)(q)[4]/ 47,XY,+21c[2]	No	WT	WT	R	WT	NK	NK
30	Male	8.45	89.1	BCP	47,XY,+21c	No	WT	WT	R	NK	Other	R
31	Male	10.70	4	BCP	47,XY,+21c[30]	No	WT	WT	WT	NK	WT	WT

Supplementary Table S3B. (Continued)

								FISH		PCR and sequencing	MLPA	
32	Male	4.08	1.9	BCP	47,XY,+21c[12]	NK	WT	WT	WT	NK	WT	WT
33	Male	13.45	1.2	BCP	47,XY,+21c[20]	No	R	WT	WT	NK	WT	WT
34	Female	7.21	11.8	BCP	48,XX,+21c,+mar[cp3]/47,XX,+21c[8]	No	WT	WT	WT	NK	WT	WT
35	Female	10.78	14	BCP	46,XX,dic(7;12)(p1;p1),+21c,add(21)(p1)[10]	No	WT	WT	WT	NK	Whole gene	R
36	Female	12.76	24.9	BCP	Fail	NK	WT	WT	WT	NK	WT	WT
37	Male	3.20	85	BCP	47,XY,del(12)(p?11.1),+21c[5]/47,XY,+21c[10]	No	WT	WT	R	WT	WT	WT
38	Male	3.90	48.8	BCP	47,XY,+21c[20]	No	WT	WT	R	WT	Whole gene	R
39	Female	2.38	8.4	BCP	47,XX,+21c[13]	NK	WT	WT	R	NK	WT	WT
40	Male	8.18	4.5	BCP	51-52,XY,+X,-4,+5,+8,+9,-10,t(14;17)(q32;q21),+der(14)t(14;17),+19,+20,+21c,+22,inc[cp4]/47,XY,+21c[79]	HeH	WT	WT	WT	NK	IK6	R
41	Male	8.38	1.9	BCP	52,XY,+X,+2,+4,+9,+21c,der(21;21)(q10;q10)c,+der(21;21)(q10;q10)[8]/46,XY,+21c,der(21;21)(q10;q10)c[2]	HeH	WT	WT	WT	NK	WT	WT
42	Female	3.28	102.3	BCP	47,XX,t(4;4)(q10;q10),+21c[3]/47,XX,+21c[15]	No	R	WT	WT	NK	WT	WT
43	Male	11.49	40.2	BCP	48,XY,+X,+21c[10]	NK	WT	WT	WT	WT	WT	WT
44	Male	2.70	65	BCP	47,XY,+21c[20]	No	WT	WT	R	NK	WT	WT
45	Male	15.76	6.5	BCP	47,XY,+21c[20]	No	R	WT	WT	NK	WT	WT

Supplementary Table S3B. (Continued)

							FISH		PCR and sequencing	MLPA	
46	Male	9.66	3.7	BCP	47,XY,del(6)(q2?1q2?5),+21c[9]/47,XY,+21c[3]	No	WT	WT	WT	NK	WT	WT
47	Male	2.36	17.3	BCP	Fail	NK	WT	WT	R	NK	WT	WT
48	Male	4.35	25.2	BCP	48,XY,+X,del(1)(q32),+21c[6]/48,XY,+X,add(1)(q32),+21c[3]/ 47,XY,+21c[1]	No	WT	WT	R	NK	WT	WT
49	Male	8.57	10.6	BCP	48,XY,t(2;12)(p1;q1),-13,-20,+21c,+21,+3-4mar,inc[cp7]	No	WT	WT	WT	NK	WT	WT
50	Male	10.94	151	BCP	47,XY,add(1)(q2?),+21c,inc[3]/47,XY,+21c[2]	No	WT	WT	WT	NK	Other	R
51	Male	13.57	32.9	BCP	47,XY,+21c[20]	No	WT	WT	WT	NK	WT	WT
52	Male	3.63	24.8	BCP	47,XY,+21c[14]	NK	WT	WT	R	WT	WT	WT
53	Male	2.49	12.6	BCP	47,XY,+21c[20]	No	WT	WT	R	WT	IK6	R
54	Female	3.18	3.9	BCP	47,XX,add(19)(p13.3),+21c[8]/47,XX,+21c[7]	No	WT	WT	R	NK	WT	WT
55	Male	3.70	26	BCP	53~55,XY,+X,+4,+9,+10,+18,+21c,+21,+2~3mar[cp12]	HeH	WT	WT	WT	NK	WT	WT
56	Male	3.62	1.7	Null	46,XY,add(6)(q1),-13,+21c[12]/47,XY,+21c[8]	No	WT	WT	WT	R	WT	WT
57	Female	3.80	103	BCP	47,XX,+21c[2]	NK	WT	WT	R	WT	WT	WT
58	Male	8.31	400	BCP	47,XY,-2,?add(14)(q32),+21c,+mar[5]	No	WT	WT	R	NK	IK6	R
59	Female	8.14	11.5	BCP	47,XX,+21c[20]	No	WT	WT	R	NK	WT	WT

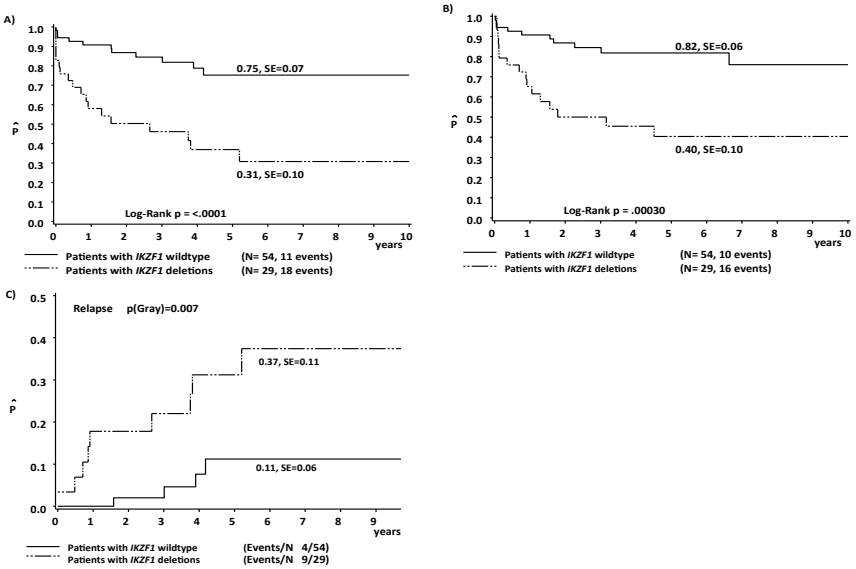
Supplementary Table S3B. (Continued)

								FISH		PCR and sequencing	MLPA	
60	Male	4.94	9.7	Null	47,XY,-13,+21,+21c[7]/47,XY,t(9;13)(q2?2;q3?3),-13,+21c[3]/47,XY,+21c[2]	No	WT	WT	WT	NK	WT	WT
61	Female	5.73	1.9	BCP	47,XX,del(9)(p?21p?21),i(9)(q10),+21c[8]/47,XX,+21c[7]	No	WT	WT	WT	R	IK6	R
62	Male	3.54	95.7	BCP	49,XY,+X,+9,?add(12)(p1),+21c[12]/47,XY,+21c[8]	No	WT	WT	R	WT	WT	WT
63	Female	18.50	4.2	BCP	47,XX,+21c[13]	NK	WT	WT	TL	NK	Whole gene	R
64	Male	12.34	21.9	BCP	47,XY,der(14)t(8;14)(q11;q32),+21c[5]/47,XY,+21c[5]	No	WT	WT	WT	NK	WT	WT
65	Male	1.89	14.9	BCP	47,XY,del(7)(q22q34),add(20)(q11),+21c[15]/47,XY,+21c[5]	No	R	WT	WT	NK	WT	WT
66	Male	3.28	148	BCP	Fail	NK	R	WT	R neg, TL NK	WT	WT	WT
67	Male	4.11	29.5	BCP	47,XY,+21c[25]	No	WT	WT	R	NK	Other	R
68	Female	6.65	2.5	BCP	58,XX,+X,+4,+6,+8,+10,+14,+14,+17,+18,+21c,+21,+21[5]/47,XX,+21c[5]	HeH	WT	WT	R neg, TL NK	NK	WT	WT
69	Female	4.24	5.1	BCP	47,XX,+21c[20]	No	WT	WT	R	NK	WT	WT
70	Male	5.15	18.6	BCP	46,XY,der(14;21)(q10;q10),+21c[20]	No	R	WT	WT	NK	WT	WT
71	Female	2.39	5.2	BCP	47,XX,+21c[20]	No	WT	WT	WT	NK	WT	WT
72	Female	23.24	14.9	BCP	Fail	No	WT	WT	R neg, TL NK	NK	Whole gene	R
73	Male	4.33	13.5	BCP	47,Y,add(X)(q2?8),+21c/47,XY,+21c[5]	No	WT	WT	R	NK	WT	WT
74	Male	2.50	13.7	BCP	47,XY,+21c[10]	NK	R	NK	WT	NK	WT	WT

Supplementary Table S3B. (Continued)

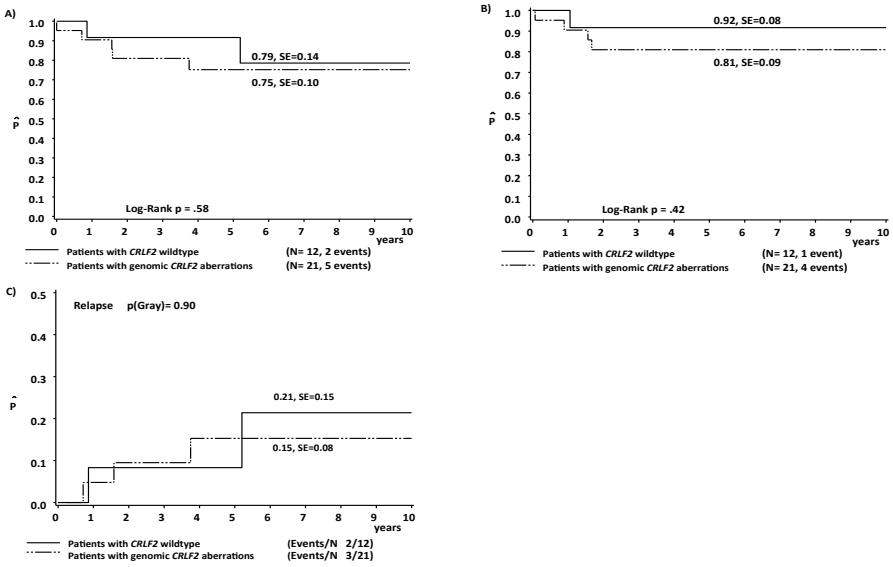
								FISH		PCR and sequencing	MLPA	
75	Male	3.60	57.9	BCP	47,XY,+21c[21]	No	WT	WT	R	NK	WT	WT
76	Male	3.55	14.8	BCP	47,XY,+21c[20]	No	WT	WT	WT	NK	WT	WT
77	Female	18.95	21.2	BCP	48,X,t(X;14)(p2;q32),+der(X)t(X;14)(p2;q32),+21c[7]/47,XX,+21c[16]	No	WT	WT	TL	NK	WT	WT
78	Male	3.43	6.3	BCP	47,XX,+X,i(21)(q10)c,inc[2]/46,XY,i(21)(q10)c,inc[18]	No	WT	WT	R	NK	IK6	R
79	Male	5.98	4.5	BCP	49,XY,+X,del(11)(q22q25),+17,+21c[10]/47,XY,+21c[1]	No	WT	WT	R	NK	WT	WT
80	Male	21.78	17.5	BCP	46,XY,+21c[20]	No	WT	WT	R	NK	IK6	R
81	Female	10.55	83.4	BCP	47,XX,add(12)(p1?),+21c[4]/47,add(6)(q1),-9,+21c,+mar,inc[3]/47,XX,+21c[5]	No	R	WT	WT	NK	WT	WT
82	Male	6.93	60.3	BCP	48,XY,+21c,+21[8]/47,XY,+21c[2]	No	WT	WT	WT	NK	WT	WT
83	Male	4.84	3.2	BCP	47,XY,+21c[20]	No	WT	WT	R	NK	WT	WT
84	Male	19.79	19.6	BCP	47,XY,add(7)(q?11.2),-13,del(14)(q?11.2),+21c,+mar[cp7]/47,XY,+21c[13]	No	WT	WT	WT	NK	Other	R
85	Male	5.41	67.7	BCP	47,XY,+21c[10]	No	R	WT	WT	NK	WT	WT
86	Male	5.21	13	BCP	47,XY,+21c[20]	No	WT	WT	R	NK	WT	WT
87	Female	4.28	6.9	BCP	47,XX,+21c[20]	No	WT	WT	TL	NK	WT	WT
88	Female	2.41	73.2	BCP	47,XX,+21c[14]	NK	R	WT	WT	WT	WT	WT
											9 (10.6%)	23 (27.1%)
Total number of aberrations						4 (5.5%)	15 (17.6%)	-	43 (51.2%)	8 (40%)	23 (27.1%)	

Karyotype nomenclature according to ISCN 2009. Down syndrome acute lymphoblastic leukemia; WBC, white blood cell count; IPT, immune phenotype; BCP, B cell precursor; HeH, High Hyperdiploid (≥51 chromosomes [50 + 21c]), WT, wildtype; R, rearrangement; TL, translocation; NK, not known; IK6, IKZF1 isoform 6.



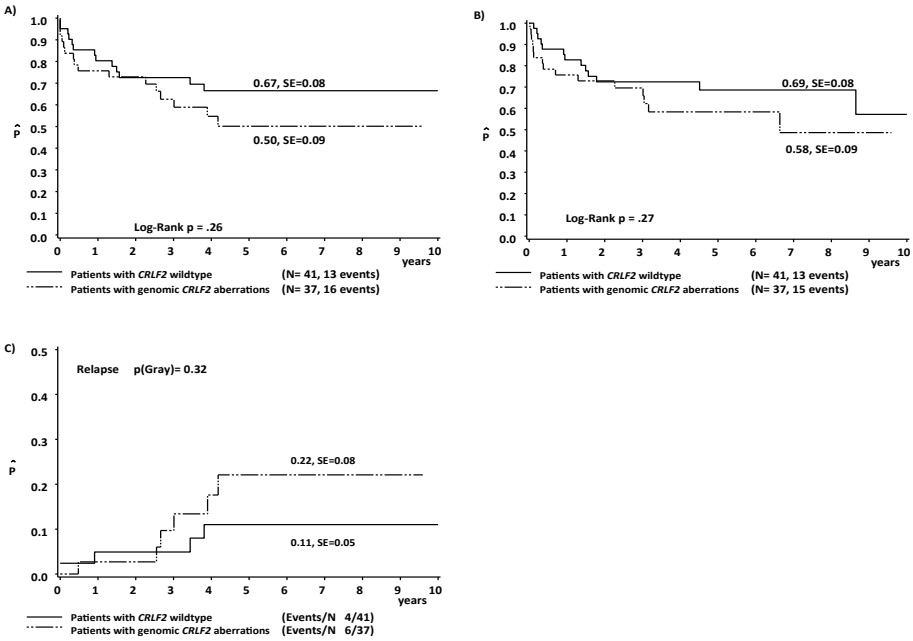
Supplementary Figure S2. Survival of the combined dataset with and without *IKZF1* aberrations

Kaplan-Meier estimates for 6-year EFS (A), OS (B) and CIR (C) for *IKZF1* in the combined dataset (DCOG and UK DS ALL patients). Patients with *IKZF1* deletions have a significantly worse outcome in terms of OS and EFS compared with *IKZF1* wildtype patients. There is no statistically significant difference in CIR.



Supplementary Figure S3. Survival of Dutch childhood oncology group Down syndrome ALL patients with and without *CRLF2* aberrations.

Kaplan-Meier estimates for 6-year EFS (A), OS (B) and CIR (C) for *CRLF2* in DCOG DS ALL patients. There was no statistically significant difference in clinical outcome between patients with or without genomic *CRLF2* aberrations, neither in the DCOG cohort (EFS 75±10% vs. 79±14%; p=0.6, OS 81±9% vs. 92±8%; p=0.42 and CIR 15±8% vs. 21±15%; p (Gray) = 0.9) nor in the UK cohort (EFS 50±9% vs. 67±8%; p=0.26, OS 58±9% vs. 69±8%; p=0.27 and CIR 22±8% vs. 11±5%; p (Gray) = 0.32)



Supplementary Figure S4. Survival of United Kingdom Down syndrome ALL patients with and without *CRLF2* aberrations.
Kaplan-Meier estimates for 6-year EFS (A), OS (B) and CIR (C) for *CRLF2* in MRC UK-ALL DS ALL patients. No significant differences in survival estimates between patients with wildtype *CRLF2* and patients with aberrations in *CRLF2*.

6

BTG1 deletions do not predict outcome in Down syndrome acute lymphoblastic leukemia

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TO THE EDITOR

Children with Down syndrome (DS) have an increased risk of developing acute myeloid (AML) and B-cell precursor acute lymphoblastic leukemia (BCP-ALL).[1] The prognosis of DS ALL is at best similar and often inferior to that of sporadic ALL (non-DS) patients.[2,3] DS ALL is characterized by unique biological features when compared to non-DS ALL. For instance, DS ALL has a lower frequency of the favorable genetic abnormalities t(12;21)(p13;q22) [*ETV6-RUNX1*] and the unfavorable t(9;22)(q34;q11) [*BCR-ABL1*]],[3,4] but a higher frequency of *JAK2* mutations and *CRLF2* rearrangements.[5,6] Using genome wide screening techniques, several (novel) genomic aberrations involved in the pathogenesis of (non) DS ALL were identified.[7,8] The potential prognostic impact of most of these novel aberrations and whether these patients may benefit from specific therapies targeted to these unique genetic features needs to be investigated further.

The B-cell translocation gene 1 (*BTG1*) was recently described as a recurrent lesion in pediatric BCP-ALL.[7,9,10] It was originally identified as a translocation partner of c-*MYC* in B-cell chronic lymphocytic leukemia.[11,12] *BTG1* is a highly conserved gene and belongs to the family of *BTG*/*TOB* genes.[11,13] It plays a role in several crucial cellular processes, such as proliferation, and apoptosis. Recently, Lundin et al. reported on a high frequency of *BTG1* deletions (~29%) in DS ALL.[14] However, the study included a limited number of DS ALL patients (n=17) and hence may not be an accurate estimate of the frequency of *BTG1* deletions in DS ALL. Therefore, we investigated the frequency of *BTG1* deletions in a large series of DS ALL patients and in addition analyzed the prognostic significance of *BTG1* abnormalities.

We screened a population-based cohort of 116 DS ALL patients enrolled in consecutive DCOG and UK treatment protocols (DCOG ALL 8, 9 and 10 and UK ALL ALL97, ALL97/99 and ALL2003).[8] Clinical and cell-biological data, including cytogenetics, were available for all cases. DCOG and UK centrally reviewed diagnosis, classification and clinical follow-up of the patients. The Institutional Review Board approved the investigations, and informed consent was obtained according to local law and regulations.

To identify *BTG1* deletions, we performed Multiplex Ligation-dependent Probe Amplification (MLPA) analysis using the SALSA MLPA kit P335-A3 ALL-IKZF1 (MRC Holland) which contains probes for selected B-cell development and differentiation genes.[8] The *BTG1* gene is covered by four probes, probes to exon 1, exon 2, and 2 additional probes localized to the highly conserved promoter region of the gene (area 1 and 2). The full list and location of the MLPA probes can be downloaded from the MRC Holland website (http://www.mrc-holland.com). Peak heights below 0.7 (0.75 for MRC UKALL samples) and above 1.3 times the control peak height were considered abnormal, with those below 0.7 (0.75) representing deletions, and those above 1.3 representing duplications. The Kaplan-Meier method was used to estimate the 5-year probability of overall survival (OS), and event-free survival (EFS); survival estimates were compared using the log-rank test. OS was measured from the date

of diagnosis to the date of last follow-up or date of death from any cause. EFS was calculated from the date of diagnosis to the date of last follow-up or to the first event, including relapse, failure to achieve CR, and death in complete remission (CR). Cumulative incidence of relapse (CIR) was constructed by the method of Kalbfleisch and Prentice and compared by the Gray test.

All 116 DS ALL patients were classified as B-cell precursor ALL and treated with curative intent. The median age was 5.1 years (range 1.3 – 23.2), the median WBC was 14 x 10^9/L (range 1.2-400), and 65% of the patients were male. The median follow up time for survivors was 4.6 years (range 1.3 months – 15.3 years).

In total we found 8/116 (6.9 %) deletions of *BTG1*, a frequency lower than described by Lundin et al[14] in DS ALL and similar to other non-DS B-cell precursor ALL series.[7,9] Remarkably, all 8 patients showed a nearly identical deletion pattern in which the *BTG1* area 1, area 2, and exon 2 probes were deleted, while exon 1 was retained (6x mono-allelic, 2x bi-allelic). Due to statistical power, we excluded one patient from the analysis who had a mono-allelic amplification of the *BTG1* area 2 probe. This patient also had a *CRLF2* aberration, but the karyotype of this patient showed no other aberration than the constitutional trisomy 21. She remains in complete remission for >10 years. The median age of DS ALL patients with a deletion of *BTG1* was 5.4 years (range 2.7 – 23.2), the median WBC was 59 x 10^9/L (range 11.3-390), and 62% of the patients were male. This is different from the cohort of Lundin et al [14], who reported a high median age of 12 years in patients with a *BTG1* deletion and a WBC of 25x10^9/L. Interestingly they reported that *BTG1* deletions occurred in predominantly male patients (80%), which is different from our cohort (62% male). *BTG1* deletions were not mutually exclusive of other genomic aberrations (e.g. *IKZF1* in 4 patients, *CRLF2* in 4 patients). As previously described [7,9,10], *BTG1* deletions were mainly found in patients with *ETV6-RUNX1* positive ALL (38% vs. 12% in the *BTG1* wildtype group; p=0.02).

Interestingly *BTG1* regulates the glucocorticoid (GC) receptor dependent response in leukemic cells,[15] and it is therefore implicated that loss of this gene perhaps contributes to a poor outcome.[14,15] Unfortunately, data were available on clinical response to one week of prednisone for only 2 patients with a *BTG1* deletion; both were good responders. No significant differences in long-term clinical outcome between wildtype and *BTG1* deleted cases were detected: 5-year EFS 67 ± 5% vs. 60 ± 18% (p=0.4), OS 72 ± 5% vs. 60 ±18%; (p=0.3) CIR 17± 4% vs. 15 ± 15%; (*p*(Gray)= 0.9). A stepwise multivariate Cox regression model was performed which included WBC ≥50x10^9, age ≥10 years, and mutational status of *BTG1*, *IKZF1*, and *ETV6-RUNX1* (Table 1). Deletions of *BTG1* did not predict for prognosis with a hazard ratio (HR) for EFS of 1.97 (95% CI 0.6 – 6.8); p=0.29 and OS HR 2.24 (95% CI 0.6 - 7.9; p=0.2). Instead, *IKZF1* appeared to be the strongest independent risk factor for EFS, with a HR EFS 2.45 (95% CI 1.2–5.1; p=0.02), as previously described by us.[8]

In conclusion, we could not confirm the high frequency of *BTG1* deletions previously described in a small series of DS ALL but found a prevalence similar to non-DS ALL patients.

Table 1. Results of multivariate analysis for event-free survival, overall survival, and relapse free survival

Outcome	Variable	HR	95% CI	*P*-value
EFS	*BTG1*	1.97	0.57 - 6.84	0.29
	IKZF1	2.45	1.18 - 5.07	0.02
	ETV6-RUNX1	0.22	0.03 - 1.74	0.15
	WBC ≥50x10^9	2.55	0.88 - 7.39	0.08
	Age ≥10 years	1.29	0.59 - 2.85	0.52
OS	*BTG1*	2.24	0.63 - 7.91	0.21
	IKZF1	2.02	0.95 - 4,29	0,06
	ETV6-RUNX1	0.21	0.03 -1.67	0.14
	WBC ≥50x10^9	2.49	0.85 - 7.26	0.09
	Age ≥10 years	1.20	0.52 - 2.76	0.66

HR, Hazard ration; CI, Confidence interval; EFS, event-free survival; OS, overall survival; WBC, white blood cell

Moreover, *BTG1* deletions did not predict for dismal outcome in DS ALL patients in contrast to *IKZF1* aberrations. Further research is needed to identify other potential players and cooperating events in the leukemogenesis in DS children.

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7

Down syndrome acute lymphoblastic leukemia is characterized by loss of ETS-related gene (*ERG*) repression

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ABSTRACT

Children with Down syndrome (DS) have an increased risk to develop B-cell precursor acute lymphoblastic leukemia (BCP-ALL). The driving molecular events in DS ALL pathogenesis are as yet unsolved. We compared global gene-expression of 30 DS BCP ALL with 502 non-DS BCP ALL patients to obtain insight into this disease and uncover candidate genes involved in DS ALL leukemogenesis. Interestingly, 25% of the DS ALL patients with genetically unclassified disease (referred to as B-other ALL) were classified as '*BCR-ABL1* like' of which 50% also had a deletion of *IKZF1*, indicating that DS ALL has a prognostic unfavorable genetic profile. In total, we identified 357 probe sets (307 genes) differentially expressed between DS and non-DS B-other-ALL. Most of these genes were not differentially expressed in DS hematopoietic induced pluripotent stem (iPS) cells as compared to non-DS cells. This suggests that deregulation is related to the DS leukemogenesis and not to trisomy 21. Interestingly, the hematopoietic regulator *ERG* was overexpressed in DS-B-other-ALL but strongly repressed in trisomy 21 hematopoietic iPS cells and healthy DS bone marrow. Literature shows that hematopoietic overexpression of *ERG* causes leukemia in mouse models. Considering this oncogenic potential of *ERG* overexpression, we suggest that loss of *ERG* repression in DS early hematopoietic cells may be an important oncogenic driving event in DS BCP-ALL.

INTRODUCTION

Children with Down syndrome (DS) have an increased risk of developing B-cell precursor acute lymphoblastic leukemia (BCP-ALL).[1] The latter differs in presenting phenotypic characteristics as compared non-DS ALL. For instance, an almost complete absence of T cell ALL and infant ALL was shown in DS ALL.[2-5] In addition, the incidence of the well-known (cyto-) genetic subtypes such as *ETV6-RUNX1*, *BCR-ABL1* and *MLL* is low in DS ALL, whereas the frequency of *JAK2*, *CRLF2* and *IKZF1* abnormalities is high as compared to non-DS ALL.[6-10] However, the unique driving genetic event in DS ALL is as yet unknown. Hence, there remains a need to further investigate the molecular aberrations associated with DS ALL, providing more insight in the biological background of DS ALL.

Previous studies in non-DS ALL cases showed that relevant genetic subtypes of ALL can be distinguished by specific discriminative gene expression profiles (GEP).[11-13] In this way, a new clinically relevant group of patients with a '*BCR-ABL1*-like' profile was identified by us and by others in 16% of the non-DS ALL and 38% of the DS ALL patients.[7,10,13,14] These patients cluster together in gene expression profiling with *BCR-ABL1* rearranged ALL, although the leukemic cells do not harbor the *BCR-ABL1* translocation. This '*BCR-ABL1*-like' profile is associated with a poor outcome in non-DS ALL, i.e. event-free survival (EFS) of less than 60% and a cumulative incidence of relapse (CIR) of ~30%.[13,14] To date it is unknown what the frequency and prognostic impact of the '*BCR-ABL1*-like' GEP is in DS ALL.

Interestingly, previous studies have shown that DS ALL cases cluster together with the (cyto-) genetic subgroups of their non-DS ALL counterparts, instead of clustering together as a separate entity from non-DS ALL.[8,15] This suggests that, in contrast to myeloid leukemia (ML) of DS, which is characterized by unique acquired mutations in the transcription factor *GATA1* (globin transcription factor 1),[16-18] DS ALL is a molecularly heterogeneous disease often driven by similar abnormalities as non-DS ALL.[8] To further explore candidate genes and their pathways mediating the leukemogenic effect of trisomy 21, we studied gene expression profiles of DS and non-DS ALL patients, using the Affymetrix Human Genome U133 plus 2.0 platform, to determine whether DS ALL can be characterized by differentially expressed genes or pathways as compared to non-DS ALL.

MATERIALS AND METHODS

Patient material

In total, viably frozen bone marrow (BM) and/or peripheral blood (PB) samples were provided by the Dutch Childhood Oncology Group (DCOG) and by the German Cooperative ALL (CoALL) of respectively 31 and 7 DS ALL patients (BM). Moreover, 10 DS ALL unpaired remis-

sion samples (9 BM, 1 PB), and 18 healthy newborn DS patients (13 PB, 5 BM) were obtained from the DCOG. In total, 502 non-DS ALL samples from the DCOG and the CoALL study group, and 22 healthy non-DS ALL samples from the DCOG were collected. Samples from healthy non-DS patients were taken during diagnostic work up for disease other than leukemia, such as solid tumors. Informed consent was obtained from all patients, after Institutional Review Board approval according to national law and regulations.

The DCOG collected patient characteristics (gender, age, WBC, immunophenotype, and karyotype). No clinical data was available of CoALL DS ALL patients. In our laboratory, samples were analyzed for the presence of a *ETV6-RUNX1* fusion (FISH and RT-PCR), a *BCR-ABL1* translocation (PCR), an *MLL*-rearrangement (split-signal FISH and PCR), a *TCF3* (*E2A*) rearrangement (split-signal FISH), a *TCF3-PBX1* (*E2A-PBX1*) fusion (RT-PCR), and hyperdiploidy by FISH for chromosomes 4, 8, 17, 21, X.

Microarray- based gene expression profiling

After thawing vials with frozen cells, contaminating non-leukemic cells were eliminated as described before,[19] and genomic DNA and total cellular RNA were extracted from leukemic cells using Trizol reagent (Gibco BRL, Life Technologies, Breda, the Netherlands), as previously described.[20] RNA integrity was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA, USA). cDNA and biotinylated cRNA was synthesized and hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. All included samples had a ratio between 3'probe/5'probe for β-actin or glyceraldehyde-3-phosphate dehydrogenase smaller than three, suggesting a minimal breakdown of RNA/cRNA during the experimental procedure.

Preprocessing of microarray data

Samples of *de novo* DS ALL and non-DS ALL patients were hybridized to Affymetrix Gene Chips in different time periods, and for normalization procedures, 8 DS ALL samples were processed in both datasets. Data acquisition was performed using GCOS 1.0 software, and probe set intensities of both DS and non-DS samples were normalized using the variance stabilization normalization (VSN-rma) procedure.[21] Thereafter, the median of the 8 overlapping DS ALL samples were used as a reference to calculate log2 expression ratios for each probe set in the DS ALL and non-DS ALL cohorts separately to correct for batch effects. The 8 overlapping samples were used as reference samples and therefore removed from the two datasets (Supplementary Figure S1). A principal components analysis (PCA) on log2 ratios was carried out as a quality control.

To explore whether differences in gene expression were related to the constitutional trisomy 21 or to the leukemic clone, we compared our data a with the gene expression profile

of induced pluripotent stem cells (iPS) differentiated towards hematopoietic tissue (CD43+, CD41+ and CD235+), and derived from DS and non-DS non-disease tissue (GEO dataset GDS4377),[22] and calculated median log2 ratios per gene, using R, version 3.0.2.[23]

Identification of '*BCR-ABL1*-like' cases

DS ALL patients with a '*BCR-ABL1*-like' signature were identified based on hierarchical clustering using our previous described 110 probes in a non-DS ALL reference cohort (Gene Expression Omnibus accession number GSE13351).[13,14] DS ALL cases that clustered together with '*BCR-ABL1 positive cases*' were identified as '*BCR-ABL1*-like' cases if proven negative for the *BCR-ABL1* translocation. Unsupervised clustering of centralized and scaled expression data, was performed in R using cosine correlations (www.r-project.org).

Quantitative real-time quantitative PCR (RT-qPCR)

The mRNA expression levels of *CRLF2* obtained with GEP were validated in 25/38 DS ALL samples using Taqman. Moreover, expression levels of the *ETS*-related gene (*ERG*) were validated in 36/38 DS ALL, 10 DS ALL remission, 18 healthy newborn DS, 20 non-DS ALL and 22 healthy non-DS samples. The non-DS ALL samples were selected for hyperdiploidy with additional copies of chromosome 21 (n=11) and B-other (n=9). We performed quantitative real-time PCR (RT-qPCR) with primers as previously described and used SYBRgreen (Finnzymes) for expression analysis.[9,24] The average cycle threshold (Ct) value was used to calculate mRNA expression levels relative to the expression level of the reference gene *RPS20* for *CRLF2* and *GAPDH* for *ERG*, by use of the comparative cycle time (ΔCt) method.[25]

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA analysis was performed using the SALSA MLPA kit P335-A3 (MRC Holland). The data were normalized, by dividing the peak area of each probe by the mean peak area of the control probes. Peak heights below 0.7 and above 1.3 times the control peak height were considered abnormal, with those below 0.7 representing deletions, and those above 1.3 representing duplications.

Array-Comparative Genomic Hybridization

To identify copy number changes, we performed 105-K oligonucleotide array-CGH on genomic DNA on the DCOG cohort as previously described.[60] Genomic losses and gains were identified as a minimum of 3 consecutive probes deviating beyond the threshold of -0.8 for

single copy loss and -1.8 for bi-allelic loss (log ratio). Deletions were defined as a loss ≥0.5 million bases, whereas focal deletions were defined as losses < 0.5 million bases.

Statistics

The top most discriminative probe sets between DS and non-DS B-other ALL patients were selected by rank of *p*-values obtained by applying an empirical Bayes linear regression model (LIMMA).[26] Moderated T-statistics *p*-values were corrected for multiple testing using the False Discovery Rate (FDR) method defined by Benjamini and Hochberg.[27] Probe set top lists were used for analysis in the database for annotation, visualization and integrated discovery (DAVID), which can be freely accessed at their website.[28]

To evaluate outcome, statistical analyses were conducted using SAS software (SAS-PC, Version 9.1). Complete remission (CR) was defined as less than 5% blast in the bone marrow, with regeneration of tri-lineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere. Event-free survival (EFS) was calculated from the date of diagnosis to the date of last follow-up or to the first event, including relapse, death in CR, and failure to achieve CR (considered as event on day 0). Early death was defined as any death within the first 6 weeks of treatment, and was considered as an event on day 0. Overall survival (OS) was measured from the date of diagnosis to the date of last follow-up or to the date of death from any cause. The Kaplan-Meier method was used to estimate survival rates, and survival estimates were compared using the log-rank test. The cumulative incidence of relapse (CIR), with other events and death in CR as competing events, was constructed by the method of Kalbfleisch and Prentice and compared using the Gray test. Differences with a P value <0.05 were considered significant.

RESULTS

Patients

A cohort of 38 DS ALL (including the aforementioned 8 reference samples) samples were available for gene expression profiling. All 38 DS ALL patients were B-cell precursor ALL and treated with curative intent according to consecutive DCOG studies (n=31),[29,30] and CoALL studies (n=7).[31] The DCOG DS ALL patients had a median age of 4.5 years at diagnosis (range 2.0 – 17.1), and a median WBC of $8.1x10^9$/L (range 1.2-$112x10^9$/L). Complete cytogenetic data were available for 37 patients. *ETV6-RUNX1* fusions were found in 2 patients; 2 patients had a hyperdiploid (HD) karyotype (51 chromosomes); 1 patient had a *BCR-ABL1* translocation, 1 patient tested negative with molecular methods for *ETV6-RUNX1*, *BCR-ABL1*, *MLL*, and *E2A-PBX1*, but could not be tested for HD due to lack of cytospin preparations for immunological

Table 1. Patient characteristics of included DS ALL patients

ID	Study group	Age	WBC	Subtype	*IKZF1* del	'*BCR-ABL1*-like'	*ERG*
1	DCOG	9.2	29.7	B-other	No	No	No
2	DCOG	4.9	4	B-other	No	No	No
3	DCOG	2.6	18.4	B-other	No	Yes	No
4	DCOG	13.5	1.2	HD	No	No	No
5	DCOG	3.5	22.3	B-other	Total gene	No	No
6	DCOG	15.4	4.3	B-other	No	No	No
7	DCOG	4.2	41.2	B-other	No	No	No
8	DCOG	2.8	32.6	B-other	exon 3-7	No	No
9	DCOG	3.1	7.3	B-other	No	No	No
10*	DCOG	3.9	77.9	B-other	exon 3-6	No	No
11	DCOG	5.6	5	B-other	No	Yes	No
12*	DCOG	7.9	12.9	B-other	exon 2	Yes	No
13*	DCOG	6.8	199	*BCR-ABL1*	exon 3-6	No	No
14	DCOG	3.7	16.5	B-other	No	No	No
15	DCOG	17.1	20.3	B-other	exon 3-6	Yes	No
16	DCOG	7	33	B-other	exon 3-7	No	No
17	DCOG	2	112	B-other	No	No	No
18	DCOG	13.8	1.5	B-other	No	No	No
19*	DCOG	5.5	4.5	B-other	No	Yes	No
20*	DCOG	8.1	390	B-other	exon 3-6	Yes	No
21	DCOG	5.4	3.6	B-other	No	Yes	No
22	DCOG	3.7	8.9	HD	No	No	No
23*	DCOG	3.3	3.6	B-other	No	No	No
24	DCOG	2.3	6.4	*ETV6-RUNX1*	No	No	No
25	DCOG	4.5	5.7	B-other	exon 3-6	Yes	No
26	DCOG	6.2	4.5	B-other	exon 3-6	No	No
27	DCOG	4.5	4.8	B-other	Total gene	No	NK
28	DCOG	2.9	19.6	*ETV6-RUNX1*	No	No	NK
29	DCOG	12.8	39.8	B-other	exon 3-6	No	NK
30*	DCOG	18.1	16.8	B-other	No	No	NK
31*	DCOG	17.1	12.9	B-other	No	No	NK
32	CoALL	NK	NK	B-other	No	No	NK
33	CoALL	NK	NK	B-other	No	No	NK
34	CoALL	NK	NK	B-other	No	No	NK
35	CoALL	NK	NK	B-other	Total gene	No	NK
36	CoALL	NK	NK	B-other	No	No	NK
37	CoALL	NK	NK	Nk#	No	No	NK
38	CoALL	NK	NK	B-other	No	No	NK

* Samples used as a reference sample; HD, hyperdiploid (≥ 51 chromosomes); NK, not known; NK#, this patient tested negative with molecular methods for *ETV6-RUNX1*, *BCR-ABL1*, *MLL*, and *E2A-PBX*, but could not be tested for HD due to lack of cytospins.

staining. The remaining patients (n=32) had no recurrent aberrations and were classified as B-other. No *MLL* rearrangements and *E2A-PBX1* fusions were found in DS ALL samples. Deletions of *IKZF1* were found in 13/38 (34.2%) (Table 1).

Gene expression profiles of DS ALL patients compared to non-DS ALL patients

Microarray based gene expression profiles of DS ALL patients were compared to non-DS ALL patients. To preclude interference by well-known cytogenetic aberrations, only patients with genetically unclassified disease (referred to as B-other) were selected for this comparison, including 25 DS and 141 non-DS B-other ALL patients. In total, 357 probe sets were differentially overexpressed with log fold change (FC) ≥1.5 and *p*FDR ≤0.05 between these 2 groups. The most significant differentially overexpressed gene was *CRLF2* with median log FC 7.3, *pFDR* 6.13E-28 for DS ALL patients, which was validated with RT-qPCR (correlation coefficient of Rs=0.7, p<0.001). Focusing on B-cell development and differentiation genes, besides *CRLF2*, only *VPREB1* (log FC 1.9, *p*FDR 0.001) was significantly overexpressed in DS ALL patients (Table 1). The median expression levels for both probe sets (ps) for *IKZF1* was just below our cutoff (ps 1565818_s_at: log FC 0.9, *p*FDR 0.5 and ps 1565817_at: log FC 1.0, *p*FDR 0.95).

Characteristics of '*BCR-ABL1*-like' DS ALL patients

Eight out of 25 B-other DS ALL patients (32%) were classified as '*BCR-ABL1* like'. The median age of these patients was 5.6 years (range 2.6-17.1) versus 4.7 years (range 2.0-18.1) in the non-'*BCR-ABL1* like' B-other DS patients, p=0.67. The median white blood cell count at diagnosis was $9.3x10^9$/L (range 3.6-390x10^9/L) for '*BCR-ABL1* like' versus $16.7.1x10^9$/L (range 1.5-77.9x10^9/L) for the non-'*BCR-ABL1* like' B-other DS patients, p=0.66. In total, 4 (50%) of the '*BCR-ABL1* like' patients had an *IKZF1* deletion.

We next analyzed whether the '*BCR-ABL1*-like' DS-ALL patients differed in prognosis from DS B-other ALL patients without a '*BCR-ABL1* like' profile. Six out of 8 '*BCR-ABL1* like' patients were in continuous CR with a median FU time of 6.5 years, and 2 patients died of relapse. Kaplan Meier survival estimates showed no differences in 6-year EFS comparing DS ALL with a '*BCR-ABL1* like' profile versus B-other DS ALL patients (EFS 70±18% vs. 70±11%, p=0.8) overall survival (OS 88±12% vs. 77±10%, p=0.5) and cumulative incidence of relapse (30±20% vs. 7±7%, p=0.2), but numbers were small (Supplementary Figure S2).

Overexpression of DNA replication and apoptosis pathways in DS ALL

Using DAVID Gene Ontology[28] to analyze the differentially overexpressed genes for enrichment of pathways, resulted in 17 clusters with a enrichment score (ES) of ≥1.30 (Table 2),

Table 2. Genes involved in B-cell development and hematopoietic differentiation

Gene symbol	Chromosome	Fold-Change	P-value	FDR
Genes involved in hematopoietic lineage determination and differentiation				
CD34	Chr1	1,3	<0.001	0,1
MYB	Chr6	1,2	<0.001	<0.001
GATA1	ChrX	1,0	<0.001	<0.001
GFI1B	Chr9	1,0	0,9	0,9
CSF1R	Chr5	1,0	0,2	0,3
CEBPA	Chr19	0,9	0,5	0,6
Genes involved in B-cell development and differentiation				
CRLF2	ChrX	7,3	<0.001	<0.001
VPREB1	Chr22	1,9	<0.001	<0.001
IKZF1	Chr7	1,4	<0.001	<0.001
EBF1	Chr5	1,3	<0.001	0,1
CDKN2A	Chr9	1,3	<0.001	<0.001
ETV6	Chr12	1,3	0,2	0,3
JAK2	Chr9	1,1	0,2	0,4
CDKN2B	Chr9	1,1	<0.001	0,1
PAX5	Chr9	1,0	<0.001	0,1
P2RY8	ChrX	1,0	0,1	0,2
IL3RA	ChrX	0,8	<0.001	0,1
BTG1	Chr12	0,7	<0.001	<0.001

FDR, false discovery rate; Chr, chromosome; * Fold change between the median of the DS B-other patients versus the median of the non-DS B-other patients.

meaning a p-value less than 0.05. The most significant up-regulated pathway was DNA replication with an ES of 3.47. This cluster comprises 12 genes, including *TERF2* (log FC 3.0, *p*FDR 3.2E-07), which plays a central role in telomere maintenance and protection against end-to-end fusion of chromosomes.[32,33] In addition, the *GINS* complex genes 1 and 2 (*GINS1* log FC 2.34, *p*FDR 1,73E-07, *GINS2* log FC 1.77, *p*FDR 0.0001), 3 mini-chromosome maintenance proteins *MCM2* (log FC 1.88, *p*FDR 3.3E-06), *MCM5* (log FC 1.85, *p*FDR 0.0004) and *MCM6* (log FC 1.6, *p*FDR 0.01), and *CDT1* (log FC 1.65, *p*FDR 0.003), were all over expressed, and play an essential role in initiation of genome replication.[34-37] The 2nd up-regulated cluster (ES 2.9) included the identical protein binding pathway and the 3rd cluster (ES 2.6) comprised the hemoglobin complex. The fourth up-regulated cluster in DS ALL with ES 2.16, included an apoptotic pathway comprising genes such as *POU4F1* (log FC 2.66, *p*FDR 0.006), which is an anti-apoptotic gene, and associated with t(8;21) in adult AML.[38] It also includes *EPHA7* (log FC 2.04, *p*FDR 0.0001), which is a receptor tyrosine kinase and known to be up-regulated in *MLL-AF4* and *MLL-AF9* positive cells.[39] Furthermore, the cluster showed up-regulation of *HRK* (log FC 2.0, *p*FDR 0.01) and *BAX* (log FC 1.77, *p*FDR 1.41E-06), and genes from the TNF family, *TNFAIP3* (log FC 2.0, *p*FDR 0.0007) and *TNFSF12- TNFSF13* (log FC 1.67, *p*FDR 1.62E-06) all

Table 3. Significant annotation clusters in DAVID Gene Ontology

Cluster	Enrichment score	Pathway	Count	P-value	Benjamini
1	3.47	DNA replication	12	1.2E-4	1.0E-1
2	2.93	Identical protein binding	23	9.0E-5	3.5E-2
3	2.56	Hemoglobin complex	5	2.2E-5	5.5E-3
4	2.26	Regulation of apoptosis	26	3.4E-4	1.8E-1
5	1.86	Cell motion	16	4.7E-3	3.4E-1
6	1.79	DNA replication	8	2.3E-4	2.5E-2
7	1.74	Late endosome	6	1.5E-3	1.7E-1
8	1.58	Regulation of DNA recombination	4	6.4E-3	3.4E-1
9	1.46	Response to wounding	17	5.4E-3	3.2E-1
10	1.45	Immune system development	12	2.7E-3	3.1E-1
11	1.44	Positive regulation of osteoblast differentiation	5	4.6E-4	1.3E-1
12	1.42	Transcription repressor activity	12	5.0E-3	3.9E-1
13	1.39	Pigment granule	7	2.1E-3	1.7E-1
14	1.38	Regulation of DNA metabolic process	7	6.9E-3	3.4E-1
15	1.37	Domain: Helix-loop-helix motif	6	2.3E-2	9.6E-1
16	1.33	Domain: MARVEL	5	4.5E-4	3.2E-1
17	1.30	Transcription repressor activity	12	5.0E-3	3.9E-1

Overview of the upper 5 significant enriched annotation clusters in DAVID Gene Ontology, based on a list of 357 differentially over expressed probe sets (FC≥1.5, FDR≤0.05). Only the 3 most significant pathways of each cluster are shown.

Table 4. Genes of the DNA replication and apoptosis regulating pathways according to DAVID Gene Ontology analysis

Gene symbol	Fold-Change	P-value	FDR
Genes involved in DNA replication			
TERF2	3.021	4.08E-09	3.20E-07
GINS1	2.340	1.92E-09	1.73E-07
CTGF	2.292	0.0006	0.0039
MCM2	1.875	7.58E-08	3.30E-06
MCM5	1.852	3.40E-05	0.0004
C16orf75	1.802	1.80E-09	1.64E-07
GINS2	1.769	9.44E-06	0.0002
CDT1	1.653	0.0004	0.0030
FHIT	1.635	1.11E-05	0.0002
MCM6	1.607	0.0023	0.0112
TK1	1.599	1.92E-07	6.86E-06
TYMS	1.470	0.0006	0.0039
Genes involved in apoptosis			
POU4F1	2.661	0.0011	0.0064
TP53INP1	2.296	0.0090	0.0325
CD27	2.237	9.23E-06	0.0001

Table 4. (Continued)

Gene symbol	Fold-Change	P-value	FDR
HSP90B1	2.179	0.0001	0.0011
HSPA1A/B	2.051	3.89E-05	0.0005
TNF-AIP3	2.047	0.0001	0.0007
EPHA7	2.043	3.95E-06	0.0001
HRK	2.034	0.0026	0.0126
TUBB	2.021	4.73E-13	2.39E-10
cystatine B	1.921	2.47E-06	0.0001
CD74	1.898	7.94E-12	2.15E-09
LTB	1.832	0.0100	0.0353
TGFB1	1.794	0.0001	0.0008
BAX	1.777	2.61E-08	1.41E-06
Catalase	1.734	0.0068	0.0263
RAG1	1.703	0.0002	0.0019
TNFSF12-TNFSF13	1.667	3.13E-08	1.62E-06
ID3	1.638	0.0108	0.0376
WFS1	1.635	0.0047	0.0196
HSPB1	1.615	0.0009	0.0052
SQSTM1	1.573	0.0058	0.0232
mutS6	1.547	2.40E-08	1.32E-06
IL6R	1.538	4.78E-06	0.0001
PREX1	1.523	1.97E-07	7.01E-06
CD24	1.512	0.0152	0.0487
RAB27A	1.502	0.0002	0.0016

FDR, false discovery rate

inducing apoptosis via multiple pathways. Table 3 shows genes involved in DNA replication and the apoptotic pathway, including their corresponding values.

Overexpression of chromosome 21 located hematopoietic regulator *ERG*

As DS-ALL is characterized by an extra copy of chromosome 21, it is expected that deregulation of one or more chromosome 21 encoded genes plays a role in driving the oncogenic process. However, the top list of overexpressed genes between DS and non-DS B-other ALL (log FC ≥1.5, *p*FDR ≤0.05) showed only 10 genes located on chromosome 21 (Table 4), of which 2 were hematopoietic regulators (*RCAN* and *ERG*). In addition, the median expression (-0.002, p25: -0.05, p75: 0.005) of all genes located on chromosome 21 was slightly, but statistically significantly, higher for DS ALL patients as compared to non-DS ALL patients (-0.014, p25: -0.06, p75: 0.04, *p*=1.4E-10) (Figure 1A), whereas there was no statistical difference (*p*=0.58) in median expression for the non-chromosome 21 located genes between DS and non-DS

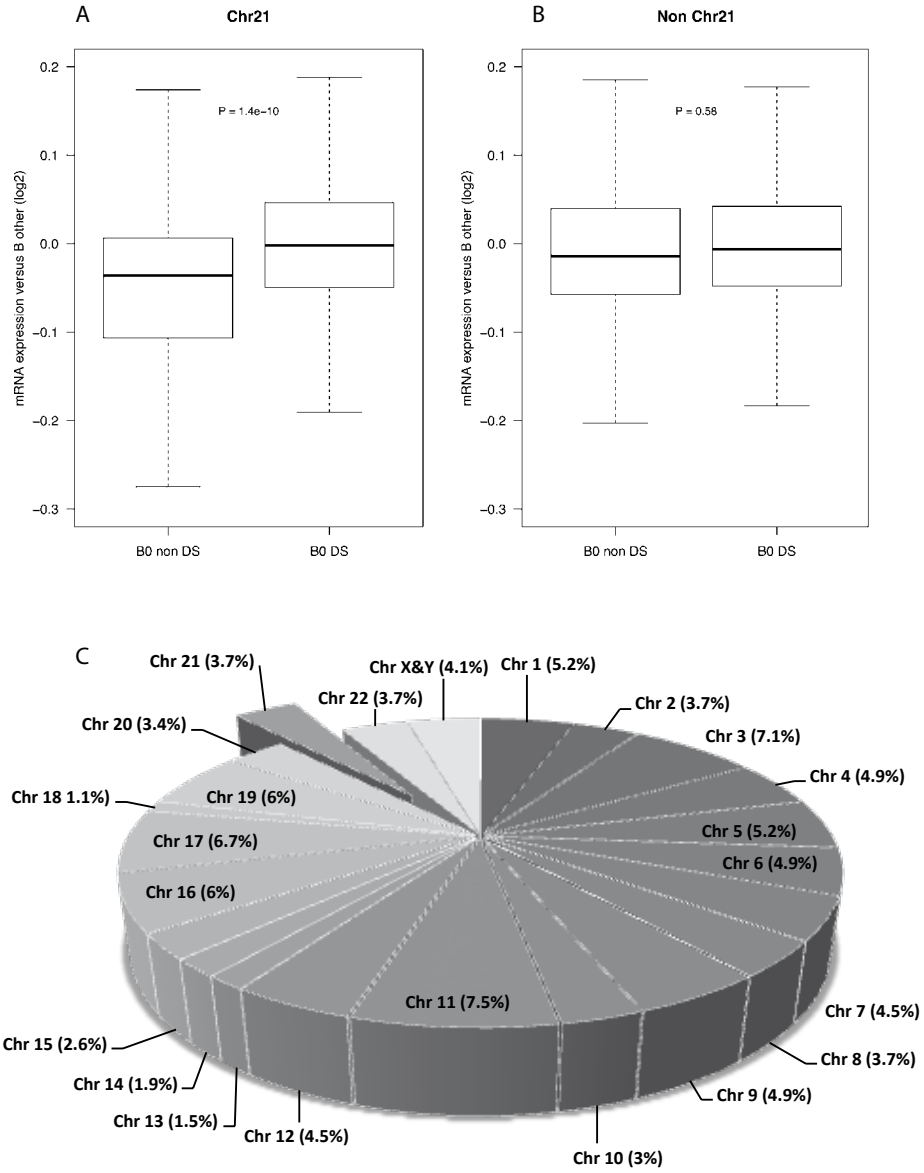

Figure 1. Distribution of differentially expressed genes

Hypergeometric distribution of differentially expressed genes for DS as compared to non-DS B-other ALL patients, showing a 2.4% higher median expression of genes located on chromosome 21, p=1.4e-10 **(A)**, and a 0.5% higher median expression of all other genes, not located on chromosome 21, p=0.58 **(B)**. The pie chart **(C)** illustrates the distribution of 267 differentially over expressed genes from 357 probe sets (FC≥1.5, FDR≤0.05) between DS- and non-DS B-other ALL over the chromosomes. Each part represents a chromosome. The percentage of overexpressed genes per chromosome is shown on the respective part. Chromosome 21 is highlighted because of the constitutional trisomy 21 in DS, and shows an equivalent proportion of genes, χ^2 p=0.28. Chr, chromosome; B0, B-other; DS, Down syndrome.

ALL B-other patients (Figure 1B). Consistent with only a slight overall expression increase, the proportion of significantly differentially over expressed genes (3.7%) on chromosomes 21 in DS ALL cases was not increased when compared to all other chromosomes (4.5%, range 1.1 – 7.5%, χ^2 p=0.28) (Figure 1C).

We next explored whether the overexpression of genes localized on chromosome 21 in DS ALL as compared to non-DS ALL, was mainly due to trisomy 21 or due to the leukemic process promoted by trisomy 21. For that purpose, we compared gene expression changes in DS-ALL with expression changes in DS-derived iPS cells.[22] The vast majority of genes that were found to be overexpressed in DS ALL compared to non-DS ALL patients in our dataset, turned out not to be overexpressed when DS-iPS cells where compared to non-DS ALL cells (p=0.002), indicating that overexpression is not a consequence of merely having an extra copy of chromosome 21 (Figure 2). Interestingly, the chromosome 21 encoded *ERG* gene was strongly reduced in DS-iPS cells, instead of being more highly expressed like the vast majority of genes on chromosome 21 (Figure 3B).

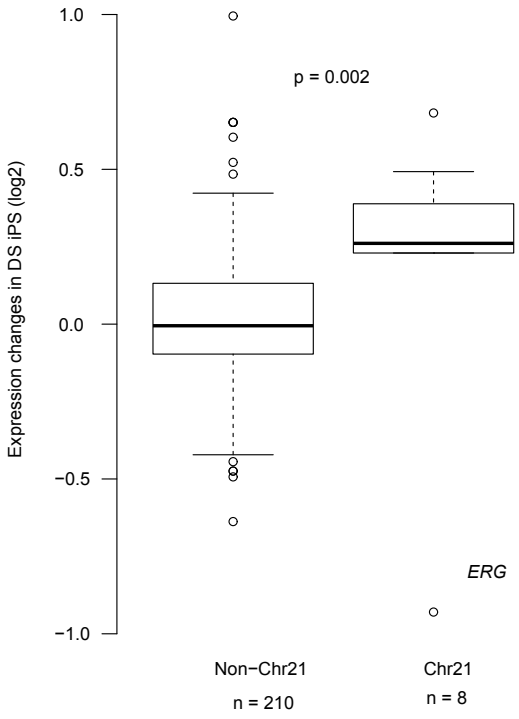

Figure 2. Overexpressed genes in Down syndrome ALL as compared to non-Down syndrome ALL
Plot showing the expression of chromosome 21 localized and non-chromosome 21 localized genes in DS ALL versus expression changes in trisomy 21 hematopoietic derived iPS cells.

To assess whether this reduced expression was also present in patient samples, we measured *ERG* mRNA levels in bone marrow of DS ALL remission samples, healthy DS samples and healthy non-DS individuals. BM samples were taken from 10 DS ALL patients at time of remission 5.4 (range 1.03-14) months after diagnosis. Samples from healthy newborn DS patients

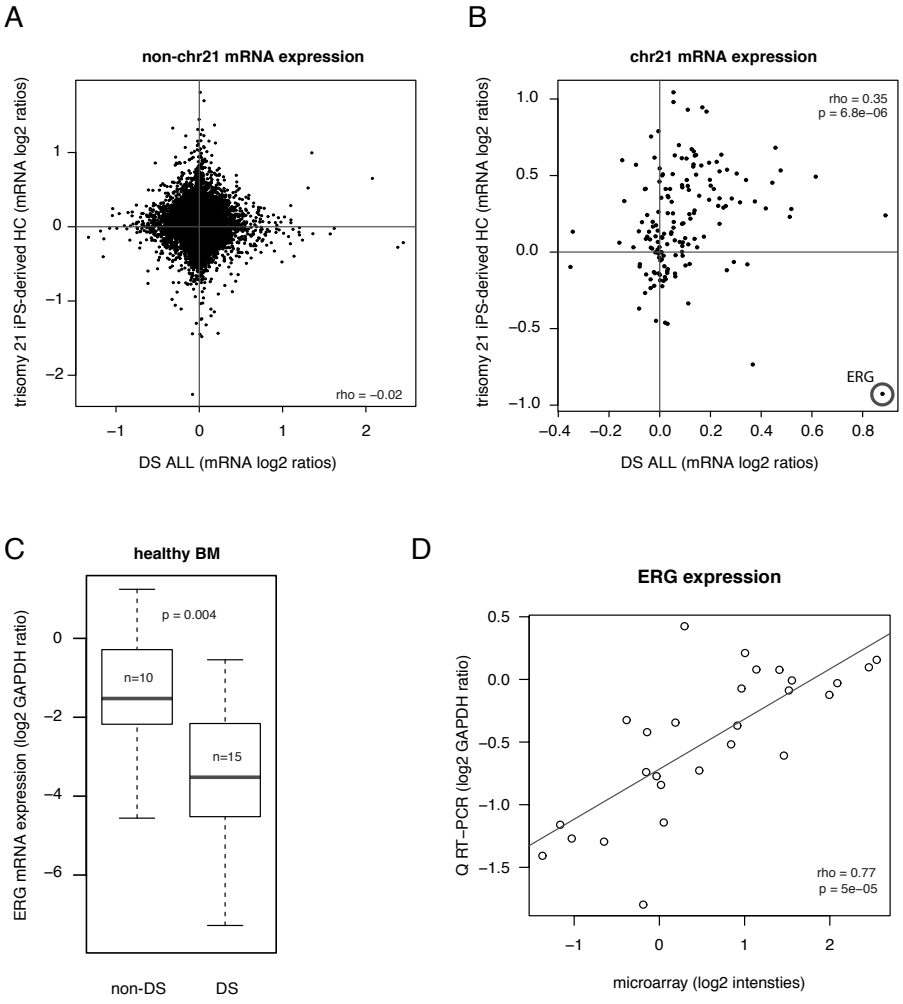

Figure 3. Gene expression in Down syndrome and non Down syndrome hematopoietic differentiated induced pluripotent stem cells

A) Correlation of mRNA expression of all genes, with the exception of chromosome 21 located genes, between DS ALL and trisomy 21 hematopoietic derived iPS cells. **B)** Correlation of mRNA expression of chromosome 21 located genes between DS ALL and trisomy 21 hematopoietic derived iPS cells, showing a low expression of *ERG* in DS ALL cells. **C)** Boxplot showing the median (line) m-RNA expression of *ERG* in healthy DS and non-DS samples, boxes represent 25th and 75th percentile and lines minimum and maximum expression. **D)** Results of RT-qPCR showing the correlation between the mean expression of 4 probe-sets of *ERG* on gene expression arrays and mRNA expression of *ERG* relative to GAPDH. Line represents the linear regression fit.

were drawn within 4 weeks after birth within the context of a DCOG study regarding neonatal prospective screening for Transient Myeloproliferative Disorder (TMD) in DS children. Data on the non-DS BCP ALL patients have been previously reported.[13,14] We showed that, consistent with the DS-iPS data, *ERG* BM mRNA levels in DS remission and healthy samples were reduced (median: -3.5, p25:-4.5, p:75 -2.2) compared to healthy non-DS individuals (median -1.5, p25 -2.2, p75 -0.3, p=0.004) (Figure 3C). This was confirmed with RT-qPCR showing a correlation coefficient of Rs = 0.77, p<0.001 for probe set 213541_s_at (Figure 3D). Array-CGH analysis did not show copy number variations of *ERG* in this DS ALL patient cohort, which potentially could influence *ERG* expression.

As recently was published that *ERG* deletions and *IKZF1* deletions co-occur,[40,41] we explored the correlation between *IKZF1* deletions and increasing *ERG* expression, but the results showed no significant relation (Figure 4).[13]

DISCUSSION

In the present study, we showed that 25% of the B-other cases cluster together with the '*BCR-ABL1* like' non-DS patients. The DS ALL patients with a '*BCR-ABL*1 like profile' had a

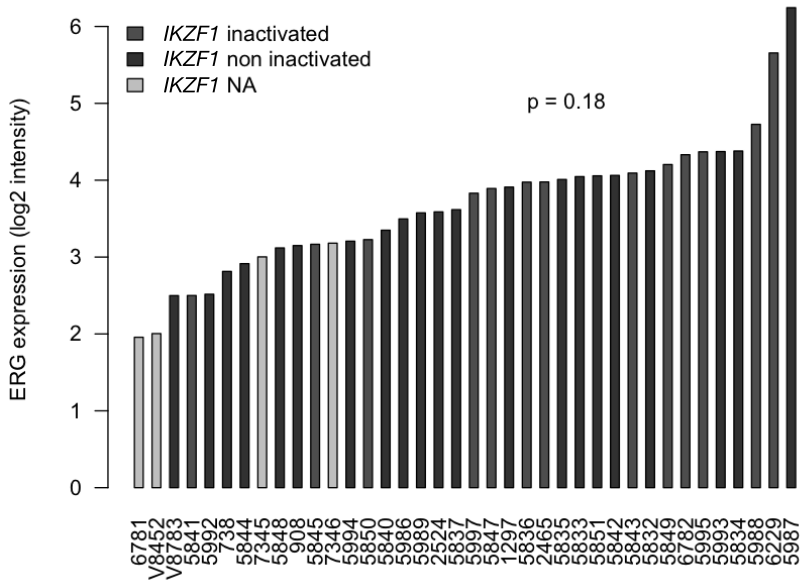

Figure 4. Distribution of *IKZF1* deletions in relation to *ERG* expression in Down syndrome ALL patients

Histogram showing *ERG* expression for DS ALL patients, thereby indicating the *IZKF1* status of patients. *ERG* expression is shown in log2 intensities. Dark grey bars represent patients with *IKZF1* deletions, black bars represent patients with wild type *IKZF1* (including 1 patient with an amplification of exon 2, ID 5835), and light grey bars represent patients with unknown *IKZF1* status.

30% cumulative incidence of relapse, which is similar to their non-DS counterparts.[7,13,14,42,43] Although given the limited sample size, we could not statistically proof that relapse rate was increased compared to DS B-other ALL patients without this signature, these data suggests that the genetic make-up of DS ALL is unfavorable. In an unselected DCOG series of non-DS ALL the frequency of the '*BCR-ABL1*-like' signature was 15%, and 40% of these had *IKZF1* deletions, compared to 20% in 'non-*BCR-ABL1*-like' B-other cases.[13,14] In our series, 50% of the DS ALL patients within the '*BCR-ABL1* like' had an *IKZF1* deletion, which we reported before to confer poor outcome in DS ALL.[7] Interestingly, recent work in non-DS ALL patients showed that *IKZF1* and *ERG* deletions co-occur in 1.5% of non-DS ALL cases, and that patients with concurrent *ERG* deletions have a better outcome.[40,41] Remarkably, none of our DS ALL patients had an *ERG* deletion (95% CI: 0± 10%).

Consistent with previous studies, the expression profiles showed *CRLF2* to be the most significant gene differentially expressed between DS and non-DS B-other ALL patients.[8,9,44] Overexpression arises from *IGH@-CRLF2*, *P2RY8-CRLF2* fusions, or gain of function mutations of *CRLF2* or *IL7R*.[9,44-46] Remarkably, as these abnormalities are present in a substantial proportion (~60%) of all DS ALL patients, *CRLF2* was not present in a specific DS ALL signature,[8] despite the fact that this DS ALL signature was associated with *CRLF2* overexpression in a supervised analysis.[15] Since *CRLF2* abnormalities in DS and non-DS ALL are lacking prognostic relevance,[7,47] the value of *CRLF2* abnormalities in ALL pathogenesis remains to be seen.

Global analysis of gene expression changes with Gene Ontology (GO)[28] revealed an increased expression of DNA replication and apoptosis pathways. For instance, pro-apoptotic genes such as *BAX* and *HRK* in DS ALL were overexpressed in DS ALL. Hypothetically, overexpression of *BAX* results in even more apoptotic activity as it promotes apoptosis through binding and antagonizing B-cell lymphoma 2 (*BCL2*), which is a suppressor of apoptosis.[48] If apoptosis is less well regulated, it could contribute to clonal expansion of hematopoietic cells, thereby facilitating the multistep leukemogenesis of DS ALL. Herztberg et al. also used GO and found a significant enrichment in DNA damage and repair genes in DS ALL with a strong signature of *BCL6*.[8] They hypothesized that either DS is a predisposing factor for ALL through B-cell lymphocytic specific genomic instability involving *BCL6* or that *CRLF2* abnormalities cause a developmental arrest of the pre-leukemic cell in a stage in which *BCL6* is active.[8] Further research is needed to investigate the aforementioned pathways and hypothesis.

Interestingly, while genes localized on chromosome 21 are mostly overexpressed in DS ALL and DS-iPS cells, we saw a reduced expression of *ERG* in BM samples taken from DS children without leukemia, and non-leukemic DS iPS derived HPSCs. *ERG* is a chromosome 21 localized transcriptional regulator of hematopoiesis and is tightly regulated within the hematopoietic stem cell network.[49-51] Within this network, *ERG* is repressed by SCL and ETO2, which are known repressors of genes necessary for erythroid maturation.[52-55] However, these genes are also directly and indirectly induced by *ERG*. Consequently, activation of *ERG* also represses *ERG*. Hypothetically, the extra copy of the *ERG* gene in DS patients may result in

deregulation of this network causing an over-repression of *ERG*.[49-51] Reduced levels of *ERG* have been found to lead to a significant change in cell morphology, increased apoptosis and reduction in hematopoiesis, in particular of the megakaryocyte lineage.[55,56]

In contrast to lower *ERG* levels in DS healthy bone marrow and iPS cells, we found a substantial overexpression of *ERG* in leukemic samples of DS patients. Notably, none of the DS ALL patients in this study had *ERG* abnormalities, other than the constitutional additional copy, which potentially could explain the difference in expression. Until now, the precise mechanism of *ERG* overexpression is unknown, but hematopoietic overexpression of *ERG* has been shown to cause leukemia in mouse models.[57,58] In addition, *ERG* strongly cooperates with the *GATA1s* truncated protein found in myeloid leukemia (ML) of DS, by immortalizing fetal liver megakaryocyte progenitors.[58,59] In view of the clear role of *ERG* overexpression in leukemic proliferation, we suggest that *ERG* is an important chromosome 21 driving oncogene in DS BCP-ALL, paradoxically first characterized by lower expression in healthy DS hematopoiesis.

In conclusion, 25% of the DS B-other ALL patients have a '*BCR-ABL1*-like' profile The CIR for this subset of DS ALL patients was 30%, which is similar as compared to their non-DS counterparts. Furthermore, we found limited up regulation of chromosome 21 localized genes in DS ALL compared to non-DS ALL, and many of these changes are trisomy 21 rather than leukemia specific. One of the few exceptions to this rule is *ERG*, which is overexpressed in DS ALL but repressed in healthy DS BM and DS iPS derived hematopoietic pluripotent stem cells. Further research, studying the role of *ERG* in DS ALL pathogenesis, is warranted.

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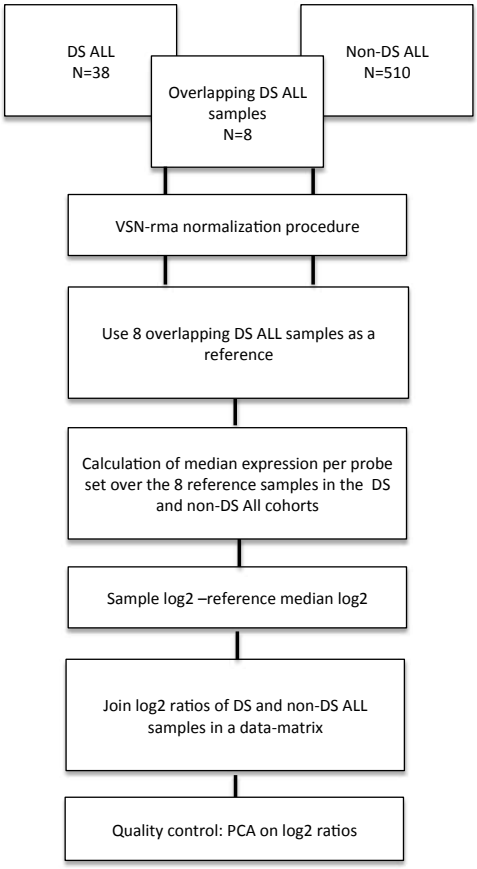
Table 5. Differentially over-expressed genes located on chromosome 21

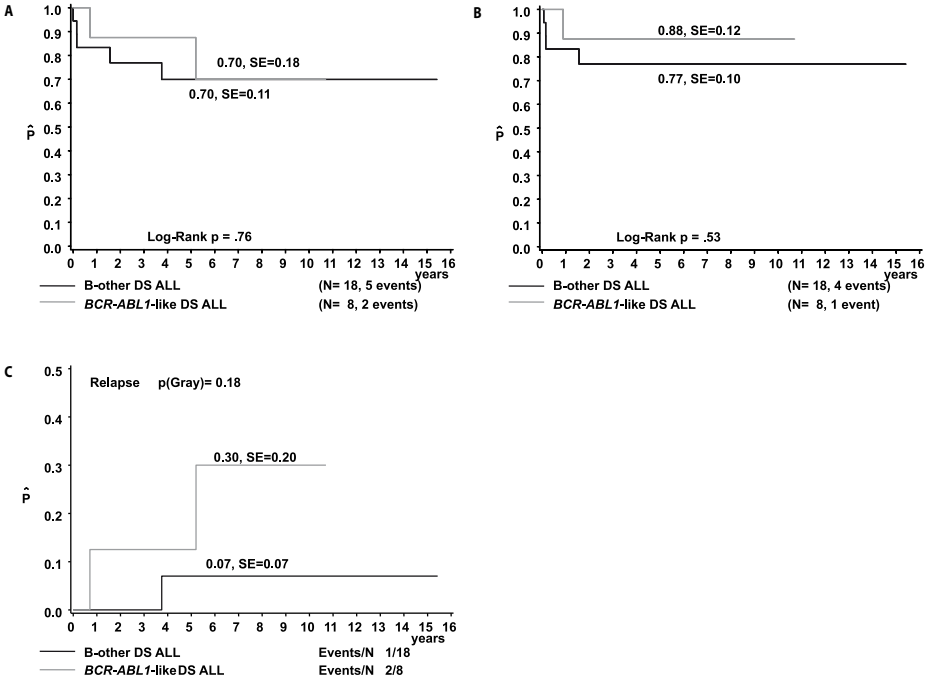
Gene symbol	Chromosome	Fold-Change	P-value	FDR
PDXK	Chr21 (DSCR)	2,340	2,70E-18	1,06E-14
CSTB	Chr21 (DSCR)	1,921	2,47E-06	5,25E-05
NRIP1	Chr21	1,708	2,34E-05	0,0003
HSPA13	Chr21	1,673	7,11E-06	0,0001
SUMO3	Chr21 (DSCR)	1,649	9,68E-12	2,47E-09
MRPS6	Chr21 (DSCR)	1,545	1,58E-05	0,0002
DONSON	Chr21 (DSCR)	1,528	3,16E-05	0,0004
C21orf45	Chr21 (DSCR)	1,509	9,24E-06	0,0001
ERG	Chr21 (DSCR)	1,951	0,0002	0,002
RCAN1	Chr21 (DSCR)	1,507	0,003	0,013

FDR, false discovery rate; Chr, chromosome; DSCR, Down syndrome Critical Region located on chromosome 21q22.1 – 21q22.3.



Supplementary Figure S1. Flowchart of experimental set up and normalization process

Flow chart showing the normalization process of 2 batches of ALL samples. DS, Down syndrome; ALL, Acute Lymphoblastic Leukemia; PCA, Principal Components Analysis.



Supplementary Figure S2. Kaplan-Meier estimates of DS ALL patients with and without a '*BCR-ABL1*-like' signature

The overall survival (B), event-free survival (A), and cumulative incidence of relapse (C) are depicted for the n=8 patients with a '*BCR-ABL1*-like' signature (grey line) versus B-other DS-ALL patients (n=18, black line).

8

General Discussion and future perspectives

In this thesis we aimed at identifying novel genetic abnormalities that characterize DS ALL and described clinically relevant prognostic factors and molecular aberrations found in high frequencies in DS ALL patients. Here we discuss the main findings in the context of current literature, and their implications for future research and treatment approaches.

THE UNRESOLVED ROLE OF TRISOMY 21 IN DOWN SYNDROME LEUKEMIA

Down syndrome is one of the most common congenital chromosomal abnormalities, with a prevalence of ~320 live births in the Netherlands.[1] Children with DS have an increased risk of developing ALL as well as Down syndrome myeloid leukemia (ML DS), which was already discovered in the 1930's.[2 3-7] It is reasonable to suspect that the constitutional chromosome 21 provides a natural model for multistep leukemogenesis. For instance, in studies on abortion material it appeared that trisomy 21 induced an increase in megakaryopoiesis, perhaps as a first step towards myeloid leukemia, which is often megakaryoblastic in nature.[8]

Remarkably, an additional chromosome 21 is one of the most common changes in non-DS ALL cells.[9] Moreover, in approximately 8% of the DS ALL patients there are additional copies of chromosome 21 on top of the +21c. However, the functional consequences of tri- and tetrasomies of genes localized in the "Down syndrome critical region" (DSCR), mapped between the DS21S17 marker and *Myxovirus Resistance1* (*MX1*) on chromosome 21, are still controversial.[10] For instance, it appears that mRNA levels are not always 1.5 fold higher as would be expected by the trisomy state.[11] However, leukemia initiating evens may not be limited to chromosome 21, as for instance in myeloid leukemia of Down syndrome (ML DS) *GATA1* mutations are pathognomonic, and localized on chromosome X, whereas the high frequency of *IKZF1* abnormalities found in DS ALL (reported in this thesis in chapter 5) are localized on chromosome 7.

Furthermore, approximately 25-50% of DS ALL cells harbor an additional chromosome X, which is a common event in hyperdiploid non-DS ALL as well.[12] However, hyperdiploid non-DS ALL often also includes additional copies of chromosome 6, 10, 14, 17, 18 and 21, whereas in DS ALL an additional chromosome X often is a single event. The latter suggests a mechanism between genes localized on chromosome 21 and chromosome X, as also seen in myeloid leukemogenesis in DS children.[13,14] In addition, other cytogenetic abnormalities (discussed in more detail below) may be initiating or additional events in multistep DS ALL leukemogenesis. However, in the end, the underlying responsible mechanism for the high frequency of leukemias in DS children remains unclear. Moreover, as DS patients have a reduced propensity for solid tumors, trisomy 21 is not a cancer susceptible syndrome per se.[2]

CLINICAL CHARACTERISTICS OF ALL IN DOWN SYNDROME

DS-ALL patients differ in presenting phenotypic characteristics from non-DS ALL patients. For instance, the incidence of T cell ALL and CD10-negative ALL (pro-B cell ALL) is lower in DS ALL, but the frequency of these features varies in the literature due the small size of most studies in DS-ALL patients. [14-20] In the large international retrospective study including >650 cases (chapter 2), we indeed showed an almost complete absence of T cell ALL cases (<1%) in DS children while the rate in non-DS ALL is 10-15%.[15,16,18] Hence the increased risk of lymphoblastic leukemia in DS is limited to the B cell lineage. Hypothetically, the rarity of T cell ALL might be due to a block in the NFAT Calcineurin pathway.[21] This pathway is a major regulator of T cell development and function, and is inhibited in DS due to the increased dosage of chromosome 21 localized *RCAN* and *DYRK1A49-50* genes.[22]

We also showed that the age distribution at diagnosis is different as compared to non-DS ALL, as ALL in DS infants did not occur in our large series.[23] The biological background for this is not understood. Furthermore, the absence of DS ALL in infants is in sharp contrast to the transient myeloid disorders (TMD) that are already present at birth in about 5-10% of all DS infants.[24-28] The latter may be derived from a major change in normal multi-lineage myeloid hematopoiesis in the fetal liver, induced by trisomy 21 itself, and thereby creating a window of opportunity for myeloid leukemic transformation in newborn DS children.[8]

In the large PdL working group study, we identified a novel clinically favorable prognostic subgroup of DS-ALL patients, characterized by age <6 years and WBC <10x10^9/L.[23] These criteria predicted outcome more reliably than the classical NCI-criteria (age <10 years and WBC <50x10^9/L).[23,29] Remarkably, DS ALL patients aged ≥6 and <10 year at diagnosis had a relatively poor outcome (EFS 51±3%, OS 70±5%), which was due to a very high frequency of relapse (CIR 41± 6%). This might have contributed to the reduced ability of the classical NCI criteria to risk-stratify DS ALL patients (age cut off ≥10 years). The subgroup of DS ALL patients aged ≥6 and <10 years did not differ in clinical characteristics from patients less than 6 year or ≥10 year at diagnosis. Moreover, no recurrent cytogenetic aberrations were reported for the vast majority of patients within this age group and 42% had a normal karyotype, which is similar to other age groups. This subgroup of DS ALL patients, characterized by a normal karyotype and a poor prognosis, should be explored further by using next generation sequencing to identify the underlying genetic abnormalities that drive the disease.

Unfortunately MRD was not routinely determined during the era of the PdL working group study, hence it is unknown whether MRD would be a better predictive biomarker for outcome than these revised NCI-criteria, as has been shown for non-DS ALL in multiple studies.

ROLE OF CYTOGENETIC AND MOLECULAR ABERRATIONS OF ALL IN DOWN SYNDROME

Over the past decades, genetic features of leukemic cells have become an integral part of classification and prognosis of pediatric leukemia.[30] To better stratify DS ALL patients into appropriate risk-adapted treatment strategies, we searched for molecular aberrations with modern techniques such as bi-directionally sequencing and multiplex ligation-dependent probe amplification (MLPA). In addition we used array-comparative genomic hybridization (CGH) to search for copy number variations (CNVs) in DS ALL, and gene expression profiling (GEP) and compared DS patients to non-DS ALL patients.

The frequency of the genetic abnormalities of the leukemic cells that have well established favorable and unfavorable prognostic relevance in non-DS ALL, is lower in DS ALL (Figure 1).[13,17,18,30-32] Also, the precise impact of these abnormalities on treatment outcome in DS ALL was unknown, as all published series lack a sufficient sample size to draw clear conclusions.[13,16-18] In this thesis (chapter 2), we demonstrated that the genetic abnormalities predicting favorable outcome in non-DS ALL similarly predict outcome in DS-ALL.[23] For instance, DS patients having *ETV6-RUNX1* positive ALL or HeH ALL with trisomy 4&10 abnormalities have an excellent prognosis, but comprise only ~12% of all DS ALL patients, whereas in non-DS ALL they comprise ~35% of patients. The low incidence of MLL rearrangements and *BCR-ABL1* translocations, has precluded us from a definitive answer regarding the predictive value of these aberrations, although we did not find evidence that the prognostic impact of these aberrations would differ from non-DS ALL.

When focusing on novel molecular aberrations as described in this thesis, the first abnormality is a mutation in the pseudokinase domain of *Janus Kinase 2* (*JAK2*).[33] Point mutations of *JAK2* at R683 were found in ~20% of the DS ALL patients (Chapter 4),[33-36] and differ from the well-known activating *JAK2* V617F mutation found in myeloproliferative neoplasms (MPN).[37] Initially, the *JAK2* R683 mutation was thought to be unique for DS ALL, in a similar fashion as *GATA-1* mutations are for ML-DS.[38,39] However, a little later the same *JAK2* mutations were also reported in less than 10% of *high-risk* non DS ALL patients.[40] *JAK* belongs to a family of intracellular non-receptor protein tyrosine kinases that transduce cytokine-mediated signals through the STAT family of transcription factors, and plays an important role in regulating the processes of cell proliferation.[33,41] In vitro experiments showed immortalization of hematopoietic progenitor cells and constitutive activation of the JAK-STAT pathway in *JAK2* mutated mouse BaF3 cells. Expression of the *JAK2* mutants, but not the wild-type gene, conferred cytokine-independent proliferation of BaF3 mouse cells, indicating that *JAK2* R683 mutations are gain-of-function driver mutations.[33] As a consequence it is suggested that *JAK2* inhibition might be a useful therapeutic approach in *JAK2* mutated DS ALL.[41]

Shortly thereafter, genomic abnormalities of the cytokine receptor like factor 2 (*CRLF2*), have been reported both by others and by our group, in approximately 60% of DS ALL

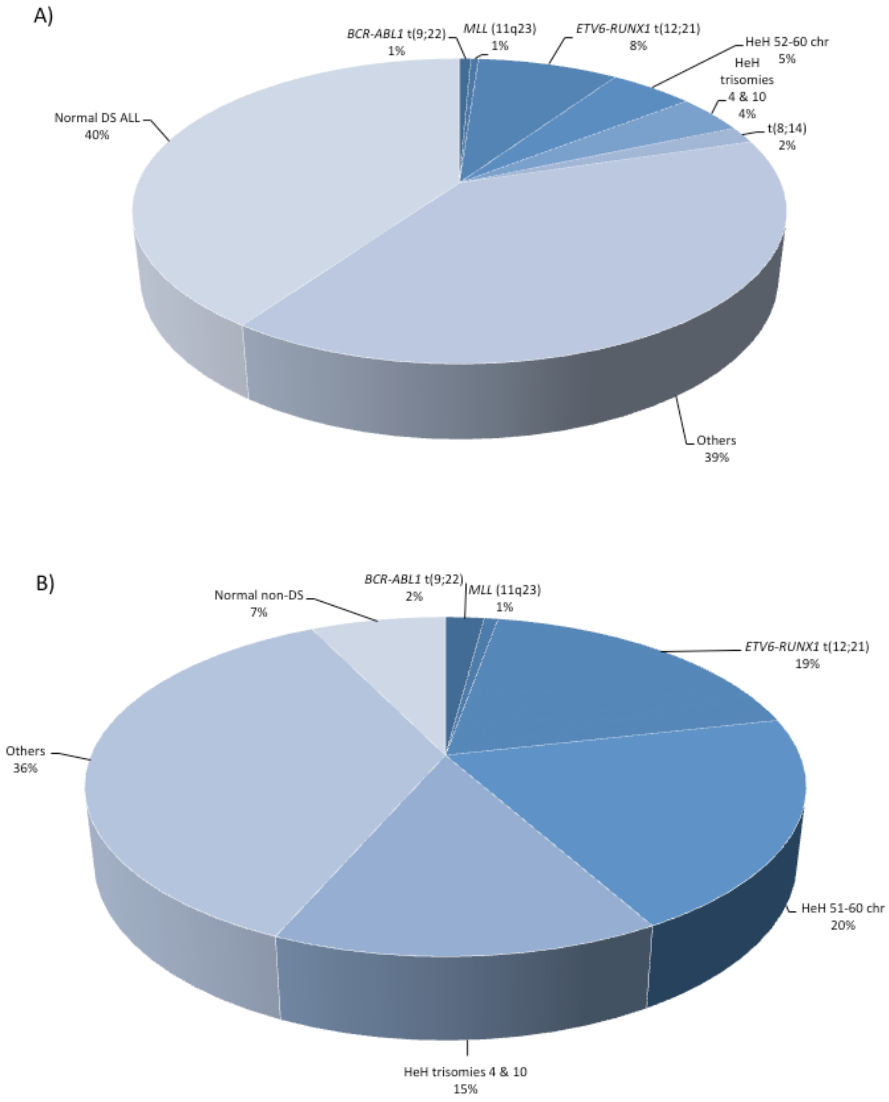


Figure 1. Cytogenetic aberrations of Down syndrome and non Down syndrome ALL patients

Pie chart depicting the genetic heterogeneity of DS-ALL patients (A) and non-DS ALL patients (B). HeH, hyperdiploid: >51 chromosomes in DS ALL, >50 chromosomes in non-DS ALL.

patients (chapter 2 and 5). This is remarkably high as compared to the 15% observed in high-risk non-DS ALL patients.[42-46] These aberrations include a) translocations of *CRLF2* with the Immunoglobulin heavy chain locus (*IGH@*) at chromosome 14q32 and b) interstitial

deletions in the pseudo autosomal region 1 (PAR1) resulting in *P2RY8-CRLF2* fusion, c) gain of function mutations of *CRLF2*, and d) gain of function mutations of *IL7R*. *CRLF2*, together with the α-chain of the *IL7R*, forms a receptor for *TSLP*, and is known to mediate allergic and inflammatory responses.[47] Interestingly in ~50% of the DS ALL patients with *CRLF2* over-expression, there are additional genomic mutations in *IL7R*, or in the signaling components downstream, that include the enzymes *JAK2* and *JAK1*.[33,35,43,47-49] Functionally, aberrations of *CRLF2* lead to constitutive activation of the JAK-STAT pathway, enabling increased survival and proliferation.[43-46] However the exact role of *CRLF2* abnormalities in ALL and in particular in DS ALL leukemogenesis is still unclear.

In an attempt to obtain insight in the underlying causal relation between the high frequency of *CRLF2* aberrations and the biological background of DS ALL, we hypothesized that *CRLF2* alterations are already present prenatally, analogous to non-DS patients with *ETV6-RUNX1* positive ALL which in combination with post-natal genetic changes gives rise to leukemia.[50] For that purpose, we performed, in collaboration with the Institute of Cancer Research (UK), a pilot study in 111 peripheral blood samples of healthy newborn DS patients, but none of the patient samples showed a *P2RY8-CRLF2* fusion transcript (unpublished data). This can be due to either *CRLF2* being an unstable transcript, which is also sometimes seen in prenatal *ETV6-RUNX1* rearrangements, or due to a low sensitivity of the used method (RT-qPCR), or due to *CRLF2* aberrations occurring as a second hit occurring later during leukemogenesis. It could also indicate that genomic *CRLF2* aberrations are passengers or late clonal events, which is reflected by the fact that others and we could not confirm the prognostic significance of *CRLF2*.[42,43,45,51]

The third molecular aberration investigated in this thesis emerging from our array comparative genomic hybridization (CGH) studies were abnormalities in B-cell development and differentiation genes (chapter 5). In non-DS BCP ALL, such aberrations recently appeared to be common, occurring in a frequency of 67%-82% in high-risk and *BCR-ABL1*-like non-DS ALL patients.[52-57] We found deletions in B-cell development genes in approximately 50% of unselected DS ALL children, hence also in 'low risk' patients as defined by classical NCI-criteria. We showed that deletions in *IKZF1*, encoding the lymphoid transcription factor IKAROS, were the most prominent aberrations, being present in 35% of our DS ALL patients, as compared to 15% of non–DS B-other ALL.[42,55,58] Intriguingly, this frequency is comparable to the incidence in high-risk non-DS ALL patients, and to non-DS ALL patients with a '*BCR-ABL1* like' gene-expression signature.[52,55] The '*BCR-ABL1* like' signature denote a new clinically relevant entity which is associated with a poor outcome and clusters together in gene expression profiling with *BCR-ABL1* positive ALL, although these leukemic cells do not harbor the *BCR-ABL1* translocation. Of interest, the '*BCR-ABL1* like' signatures as reported by North-American and European investigators appear to classify different patients as '*BCR-ABL1* like'.[52,59,60]

Approximately 25% of the DS ALL as compared to 16% of the non-DS ALL patients has a '*BCR-ABL1*-like' profile.[61] This profile is associated with poor outcome, resulting in an EFS of

70% for DS ALL as compared to less than 60% in non-DS ALL.[52,61,62] The cumulative incidence of relapse is around 30% for both DS and non-DS '*BCR-ABL1*-like' ALL.[61] Within '*BCR-ABL1*-like' DS ALL, 40% has a deletion of *IKZF1*, which is similar to the frequency of *IKZF1* deletions observed in non-DS '*BCR-ABL1*-like' cases.[57] The high frequency of '*BCR-ABL1*-like' and *IKZF1* in DS ALL, suggests that a relatively large proportion of DS ALL patients may have a genetic signature with characteristics of high-risk BCP non-DS ALL. This suggestion fits with previous published outcome estimates showing that DS ALL patients treated according to a high-risk protocol showed similar outcome as compared to non-DS ALL patients, in contrast to what was considered standard risk DS ALL patients by classical NCI criteria who experience inferior outcome on 'standard risk' protocols.[17,18]

Using next generation sequencing, Roberts et al., have shown that activating tyrosine kinase and cytokine receptor signaling rearrangements, are a hallmark of '*BCR-ABL1*-like' ALL.[62] Furthermore, genomic lesions affecting lymphoid transcription factors were found in all (n=15) studied cases with '*BCR-ABL1*-like' ALL, including 2 cases with NUP214-ABL1, one case with insertion of the erythropoietin receptor gene (*EPOR*) into the immunoglobulin heavy chain locus (*IGH@-EPOR*), and 1 case each with the in-frame fusions *EBF1-PDGFRB*, *BCR-JAK2*, *STRN3-JAK2*, *PAX5-JAK2*, *ETV6-ABL1*, *RANBP2-ABL1*, and *RCSD1-ABL1*.[62] Moreover, abnormalities of lymphoid transcription factors, the vast majority being *IKZF1* deletions, were found in all studied cases.[62] Considering the high percentage of '*BCR-ABL1* like' patients in DS ALL, it would be very interesting to investigate if *PDGFRB* and other targetable fusions are also present in '*BCR-ABL1* like' DS ALL.

Interestingly, recent work in non-DS ALL patients showed that *IKZF1* and *ERG* deletions may co-occur, and that patients with concurrent *ERG* deletions, do not have a poor clinical outcome.[63,64] Approximately 3% of non-DS ALL patients harbor *ERG* deletions, and 40% of these patients also have *IKZF1* deletions. Therefore the combination of the latter two is rare, as it only comprises ~1.5% of the non-DS patients.[63,64] Remarkably, we did not find any *ERG* deletions (95% CI 0±10%) in our DS ALL cohort.

The role of *IKZF1* deletions in ALL and in particular in '*BCR-ABL1* like' ALL is yet unsolved. However, as disruption of normal lymphoid development by deletions of *IKZF1* and rearrangements of cytokine receptor signaling by *CRLF2* abnormalities, frequently occur in both DS and non-DS '*BCR-ABL1*-like' ALL. This suggests that these two pathways may cooperate in inducing precursor B ALL in DS ALL, and this pleads against a unique event initiating DS ALL. Another interesting observation is that the expression of the immunoglobulin joining chain (IGJ) gene, which is involved in maturation of immunoglobulin's, is deregulated in *IKZF1* deleted and in '*BCR-ABL1*-like' ALL. Further research is needed to elucidate whether *IKZF1* deletions functionally affect this maturation process or that *IKZF1* deletions cooperate with activating JAK-STAT pathway alterations occurring in ~60% of DS ALL patients.

The last molecular aberration investigated in this thesis concerns deletions of *BTG1*, which was reported to occur in high frequency (27%) in DS ALL.[65] We found a prevalence of only

7% of *BTG1* deletions in DS ALL, which is similar to non-DS ALL patients.[66] Deletions of *BTG1* were mainly found in *ETV6-RUNX1* positive ALL, which might explain the lack of prognostic impact of *BTG1* deletions in our cohort of DS ALL patients. Remarkably, recent work in non-DS ALL patients, showed that *BTG1* deletions are mainly detected in the prognostic unfavorable *BCR-ABL1* positive subset of ALL, and less frequent in hyperdiploid ALL, which in general has a good prognosis.[66] All deletions of *BTG1* DS ALL were mono-allelic, and localized in the second exon at the telomeric site of *BTG1*, however exon 1 was retained. Interestingly, Waanders, et al. identified multiple additional *BTG1* deletions at a subclonal level, by using a sensitive PCR-based screening.[66] In some cases these subclones grew out into the major clone at relapse.[66] From literature it is known that at both sites of the *BTG1* deletion, a VD(J) recombination signal sequence is present, suggesting that illegitimate RAG1/RAG2-mediated recombination is the responsible molecular mechanism for multiclonal evolution.[66] Interestingly, the same mechanism is suggested to play a role in *IKZF1* deletions.[67,68] Since the frequency of *IKZF1* deletions in DS ALL is as high as 35%, it strengthens our hypothesis that *IKZF1* deletions are an important driving event in DS ALL leukemogenesis.

None of the discussed molecular aberrations was mutually exclusive of other genomic abnormalities, which is depicted in Figure 2. For instance, DS ALL patients having *JAK2* mutations as the sole aberration were not found; instead, *JAK2* mutations occur frequently with

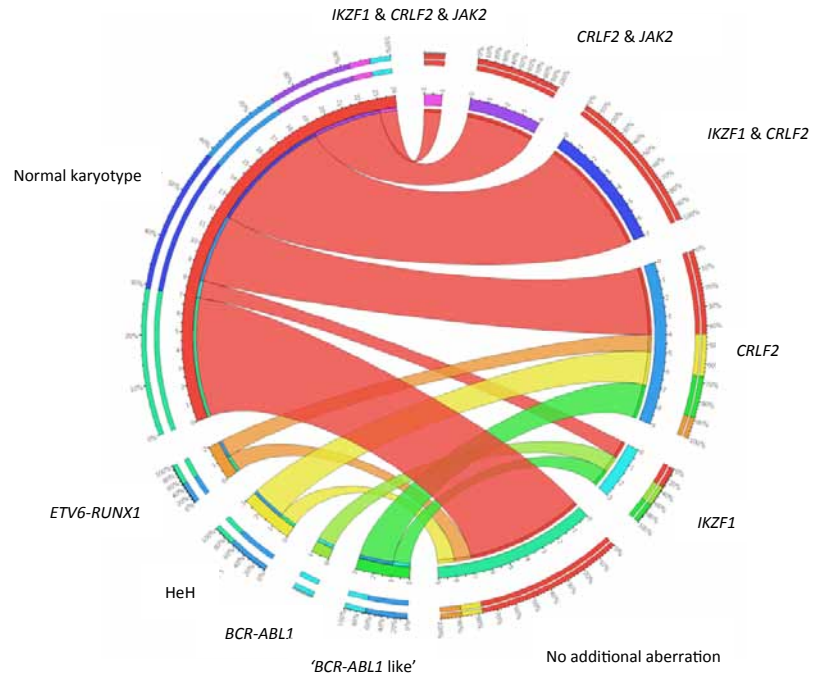


Figure 2. Circosplot depicting connections between (cyto-) genetic and molecular aberrations in Down syndrome acute lymphoblastic leukemia

IKZF1 deletions and *CRLF2* gene rearrangements. Notably, a large subset of patients (40%) has no known aberrations. This subset of patients would be an ideal group to apply RNA sequencing to identify potential players and cooperating events that underlie leukemogenesis in DS children.

OVEREXPRESSION OF ETS-RELATED GENE IN DOWN SYNDROME ALL

Previous studies in non-DS ALL cases showed that gene-expression profiling (GEP) is a powerful diagnostic tool to classify relevant genetic subtypes of ALL.[52,59,60] However, in previous studies, it was shown that clustering of DS ALL cases by GEP did not seem feasible, as they clustered together with the genetic subgroups of their non-DS ALL counterparts.[43,53] This suggests that the cytogenetic mark adds stronger to the profile than the constitutional chromosome 21.[43,69] .

Altered gene expression levels still may play a role in DS ALL leukemogenesis, as an extra copy of chromosome 21 disturbs hematopoietic regulating genes encoded by chromosome 21. Interestingly, in this thesis we show that as compared to non-DS patients, ETS-related gene (*ERG*) has a reduced expression in non-leukemic DS samples, and a high expression in leukemic DS ALL samples **(Chapter 7)**. In healthy cells, activation of *ERG* also represses *ERG* by an indirect and direct feedback loop. As *ERG* is a chromosome 21 localized transcriptional regulator of hematopoiesis, the constitutional extra copy of the *ERG* gene, may result in disruption of this feedback loop and subsequently in deregulation of the hematopoietic stem cell network.[70-72]

Our data suggest that *ERG* up-regulation occurs in the leukemic state and may play a role in leukemogenesis. It is known that hematopoietic overexpression of *ERG* causes T-cell ALL in mouse models,[73,74] and, as *ERG* cooperates with the *GATA1* mutated protein by immortalizing megakaryocyte progenitors, it is suggested that the additional constitutional copy of *ERG* in trisomy 21 may play a role in ML DS.[74] In vitro experiments with induced *ERG* expression in the B cell lineage, showed that *ERG* expression confers a differentiation arrest of pro-B cells to pre-B cells, accompanied by enhanced proliferation of precursor B-cells.[75] However, mouse models overexpressing *ERG*, were incapable of inducing B cell ALL. [75] Further research of molecular mechanisms regulating inhibition of differentiation and promoting proliferation of B-cells through overexpression of *ERG*, is an important area of further investigations. If silencing of *ERG* expression in B-cells attenuates proliferation or even induces apoptosis, *ERG* might be an interesting target for molecular therapy.

TREATMENT STRATEGIES

It is well known that DS patients have a poor prognosis as compared to their non-DS ALL counterparts.[15-19,23,42] In addition, although overall prognosis improved significantly over the past decades, we showed no improvement in survival for DS ALL patients treated from 2000-2005 as compared to patients treated from 1995-2000. This may be due to the delicate balance between treatment intensity, which is required given the high-risk genetic profile of many DS ALL cases, and treatment-related mortality. To improve outcome for DS ALL patients, either different risk group stratification and/or novel treatment strategies need to be considered for future treatment protocols, especially targeted treatment options which specifically exert effect on the leukemic cell population, but not on healthy tissues.

The clinically favorable prognostic subgroup of DS-ALL patients, characterized by age <6 years and WBC <10x10^9/L, had an overall survival of 87%, but still a relatively high cumulative incidence of relapse (17%) with a low rate of TRM (3%). This is inferior to what is reported for non-DS ALL patients with NCI-SR,[76-85] or SR patients in BFM protocols, which use a stratification strategy based on MRD in 3 arms.[86] This clinical subset of DS ALL patients may need an intensification block following induction and CNS-directed therapy and might therefore be classified into the intermediate MRD risk group.

However, although less common as compared to non-DS ALL, *ETV6-RUNX1* fusions (in 8.3%) and hyperdiploid (HeH) trisomies 4 and 10 (in 12%) predict an excellent outcome in DS ALL in our large PdL working group study.[23] Notably, TRM rather than relapse was the major cause of treatment failure in these patients. Therefore, this group of DS ALL patients might be eligible for treatment reduction rather than intensification, such as withholding from anthracyclines, and/or stratification in the MRD SR group.

Contrary to this, *IKZF1* deletions, which are present in 35% of the DS ALL patients, predict for dismal outcome and in particular for an increased risk of relapse. This subset of patients might benefit from treatment intensification, although therapy efficacy needs to be carefully balanced against the enhanced toxicity. Notably, as a result of our studies in DS and non-DS ALL patients,[42,57] patients with *IKZF1* deletions are now stratified in an intensified treatment arm of the current DCOG ALL-11 treatment protocol.

FUTURE PERSPECTIVES FOR TREATMENT STRATEGIES

In general, treatment intensification using conventional cytotoxic chemotherapy is not preferred in DS ALL as they have an increased risk for TRM. To further improve prognosis in DS ALL patients, novel non-myelosuppressive targeted therapies are an interestingly therapeutic option to be explored in the near future.

First, a substantial proportion (~60%) of the DS ALL patients have genomic *CRLF2* aberrations, which signals together with *IL7R* through *JAK* 1-2, the latter being mutated in 18% of the DS ALL patients. The functional consequence of these abnormalities is a constitutive activation of the JAK-STAT pathway, thereby promoting survival of leukemic cells, which makes it an interesting target for inhibitors. However, the major side effect of *JAK2* inhibitors is myelosuppression, which is due to co-suppression of wildtype *JAK2*, and which is required for normal hematopoiesis. This may render it difficult to combine *JAK* inhibitors with conventional chemotherapy in DS-ALL patients who already suffer from higher TRM rates. At this moment, the Children's Oncology Group is completing phase I trials with *JAK1*/*JAK2* inhibitors such as Ruxolitinib, for children with relapsed or refractory solid tumors, leukemia or myeloproliferative disease (Clinical Trial NCT01164163).[87,88] In addition, inhibitors of the mTOR-signaling pathway, which is also activated through *CRLF2*/*IL7* signaling, might be another novel strategy in the subset of *CRLF2* positive DS ALL patients, but it is remains to be determined whether inhibiting these pathways will benefit DS ALL patients.

Deletion of *IKZF1* is the only aberration that clearly predicts poor survival. However, to date there is no targeted approach for *IKZF1* deleted DS or non-DS ALL. Since *IKZF1* deletions are often found in '*BCR-ABL1*-like' patients, this might be an interesting subgroup for novel therapies. Recently, within '*BCR-ABL1*-like' ALL, rearrangements of *PDGFRB* and other drugable targets were identified.[62] This is probably of clinical value, as for instance patients with chronic myeloproliferative disease and activating *PDGFRB* rearrangements show complete hematologic and molecular responses to Imatinib treatment.[62] If so, the combination of tyrosine kinase inhibitors such as Imatinib, Dasatinib, or Ruxolitinib and conventional chemotherapeutics might improve outcome for '*BCR-ABL1*-like' DS ALL patients.[62,89 90] In addition, Blinatumomab which belongs to a new class of monoclonal antibodies, bi-specific T-cell engagers, is a promising new drug in leukemia in general. Presently, phase 1 and phase 2 clinical trials in are ongoing for ALL patients.[91] Blinatumomab enables the patient's T cells to recognize the malignant B cells by a linking molecule which binds to CD19 and CD3.[92] Other antibody options may be unlabeled anti-CD22 antibodies such as Epratuzumab or CD20-antibodies such as Rituximab.

Notably, as a result of our PdL working group study, an initiative is underway to establish an international prospective registry to collect demographic, biological and clinical outcome data on a large group of DS ALL patients, and to establish uniform immune monitoring and supportive care recommendations. The longer-term aspiration is to develop an international treatment protocol for DS ALL patients, allowing sufficient power to prospectively evaluate new risk-adjusted strategies, and aiming at improving prognosis of future DS ALL patients by reducing both relapse and treatment related mortality (TRM).[21]

THE LIMITATIONS OF TREATMENT INTENSIFICATION OF ALL IN DOWN SYNDROME

DS ALL patients are notorious for their enhanced vulnerability for chemotherapy-induced side effects.[11,48,93-95] Fatal infections are the major cause of TRM, of which the vast majority is of bacterial origin.[15,16,19,23,96] The causes of infectious toxicities are multifactorial and have been linked to decreased phagocytic activity of granulocytes, lower number of B-cells, decreased proliferation of T- cells, and low serum immunoglobulin levels in DS children.[96,97] Furthermore, certain chemotherapeutic drugs, such as Methotrexate (MTX), can lead to breakdown of cellular barriers, increasing the rate and severity of mucositis and creating a window of opportunity for infectious diseases.

Intriguingly, the large sample size of our PdL cohort provided evidence that relapse and not TRM was the major cause of treatment failure in children with DS ALL. Even in the 18 (2.8%) patients who received a SCT, relapse was the main factor contributing to dismal outcome. Furthermore, our large retrospective PdL working group study could not associate TRM with any specific chemotherapeutic agent. For instance, the comparison between patients treated on a 3-drug regimen (CCG/COG/UK, n=193) and patients treated on an anthracycline containing 4-drug regimen (AIEOP/BFM, n=106), did not show differences in TRM, nor in EFS, OS and CIR. Although there was a trend towards higher TRM for 4-drug induction, the hypothesis that anthracycline containing induction regimens had higher rates of TRM could not be clearly confirmed in the PdL study. In addition, in the same PdL working group study, we could not specifically link high dose MTX courses to TRM. This was remarkable, as it is well known that DS ALL patients are more susceptible to MTX-induced side effects than non-DS ALL patients.[11,48,93-95] One important caveat in interpreting the data from the PdL study is that we cannot verify whether toxicity was really well documented in all patients in this retrospective study, and hence there may be underreporting.

Although TRM could not be related to a specific drug in our PdL working group study, we showed in chapter 3 that grade 3-4 gastro intestinal toxicity was more frequent in DS than in non-DS children (25% vs. 4%), but this was not related to differences in plasma MTX concentrations between DS and non-DS ALL patients. Hence, these toxic effects are most likely explained by differences in pharmacodynamics and not by pharmacokinetic effects between DS and non-DS children. For instance, decreased folate levels resulting in enhanced polyglutamation and MTX-induced cell kill,[98,99] or polymorphisms in genes linked to the pharmacodynamics of MTX[100,101], or a gene dosage effect as the responsible MTX transport folate carrier is localized on chromosome 21, could underlie the enhanced susceptibility of the healthy tissues for MTX.[11,99,102] The latter one perhaps being less likely as passive diffusion of MTX across the cell-membrane also occurs using high-dose MTX schedules.[103,104] Interestingly, the Children's Cancer Group-1991 study showed that DS ALL patients who received intensified MTX therapy had better survival than those who did not.[105] New developments,

such as therapeutic drug monitoring, in which the dose of MTX is based on the clearance from the prior course,[106] could be a solution to balance enhanced toxicity against therapy efficacy if TRM is also taken into account in the model.

Furthermore, we show in this thesis that TRM is not related to a certain phase of therapy, but occurs throughout the treatment period, with about half of the deaths occurring during maintenance therapy (Figure 3). We therefore recommend improving supportive care measurements for DS ALL patients throughout therapy. The current DCOG ALL-11 treatment protocol integrated specific supportive care guidelines for DS ALL patients, including better surveillance during induction, such as admission in the hospital from diagnosis until in very good clinical condition, and more frequent outpatient visits with additional blood tests.[107] However, additional measurements during maintenance are lacking.

It is intended that DS ALL patients receive the same treatment intensity as non-DS ALL patients. However, a study from the Nordic society of Pediatric Hematology and Oncology (NOPHO) reported that the median administered dose of methotrexate (MTX) and 6-mercaptopurine (6-MP) was 25% lower for DS as compared to non-DS ALL patients.[48] As some study suggest that DS patients (without leukemia) have lower median WBC and absolute neutrophil count (ANC) values as compared to their healthy non-DS counterparts,[97,108] chemotherapy should result in lower WBC and ANC counts for DS ALL patients. However, Bohnstedt et al. showed however that the median WBC and ANC were similar during maintenance in DS ALL as compared to non-DS ALL patients.[48] This is either the result of treatment reduction or it reflects a reduced sensitivity for 6-MP and MTX in DS patients as compared to non-DS ALL

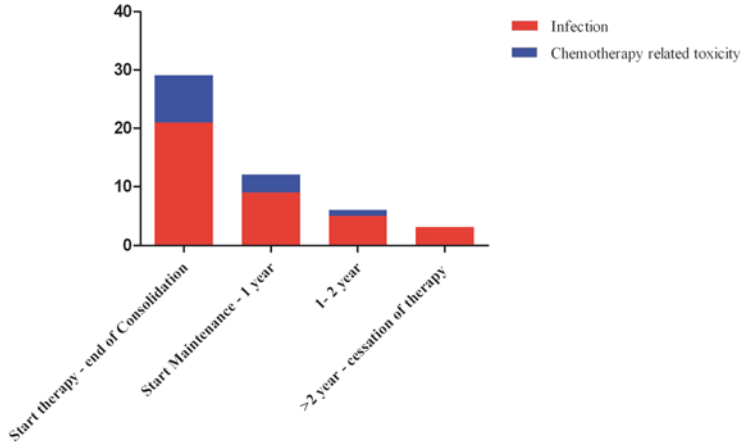


Figure 3. Distribution of treatment related mortality in relation to therapy phases for Down syndrome ALL patients
Distribution of treatment related mortality in the retrospective Ponte di Legno working group study. Treatment related mortality was cause of death in 50 patients and is depicted for 4 different time points on the X-axes. Y-axes shows the absolute number of patients. Infection is represented in red, other chemotherapy related toxicity in blue.

patients,[48] suggesting that in order for the treatment to be effective, a similar target WBC for DS and non-DS patients may not be appropriate.

CONCLUSION

In this thesis we provided evidence that to date there is no evidence of a unique genetic lesion occurring in DS and not in non-DS ALL patients. Moreover, we showed that DS ALL is a genetically '*high risk*' disease, as a substantial proportion of DS ALL patients has a '*BCR-ABL1*-like' gene expressions profile and/or *IKZF1* deletions. We therefore recommend being cautious in treatment reduction for DS ALL patients, as relapse and not TRM is the major cause of treatment failure. The exception to this rule may be the small group of 12% of patients with *ETV6-RUNX1* rearrangements or hyperdiploid trisomies 4 and 10. To further improve outcome, DS-ALL patients may benefit from 1) improved risk-group stratification with current available therapy, 2) from the elucidation of the genetic background, especially in the normal karyotype cases, 3) the development of targeted therapies that improves prognosis and reduces acute and long-term toxicity as well as TRM for all patients, and 4) from enhanced intensive supportive care throughout treatment. This requires several novel studies, such as the role of MRD in DS ALL, the role of JAK-inhibitors in DS ALL, unraveling the genetic background by using novel deep-sequencing procedures, and studies evaluating antibiotic/antifungal and/or immunoglobulin prophylaxis in DS ALL. As DS ALL is a rare disease, international collaboration is essential to further elucidate the driving mutation or pathway.

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9

Summary
Samenvatting

SUMMARY

Children with DS have an increased risk in developing leukemia, including acute myeloid (AML) as well as acute lymphoblastic leukemia (ALL). When assessing overall outcome, children with DS-ALL have fared less well than their non-DS counterparts due to a high relapse rate and an increased treatment related mortality (TRM) throughout the treatment period. Hence, there is a need to unravel the biological background of DS ALL, which in turn may lead to the development of new and non-myelosuppressive therapies for this vulnerable group of patients. In this thesis we studied the clinical and biological features of DS ALL that determine prognosis (chapter 2-3), and used several genome wide techniques to identify molecular aberrations involved in the pathogenesis of DS ALL (chapter 4-7).

In chapter 2 we aimed at the identification of clinically relevant outcome parameters, determination of the prognostic relevance of well-established and novel (cyto-) genetic aberrations, and at the detection of causes of treatment failure in DS ALL. As almost all published series lack sufficient power to provide definitive answers to these issues, we undertook a large retrospective study within the international Ponte di Legno (PdL) working group. We included 653 DS-ALL patients enrolled in 16 international trials from 1995-2004, and used non-DS BCP-ALL patients from the Dutch childhood oncology group (DCOG) and Berlin, Frankfurt Münster (BFM) group as reference cohorts. DS-ALL patients had a higher 8-year cumulative incidence of relapse (CIR) and treatment-related mortality (TRM) than non-DS patients, resulting in lower event-free survival (and overall survival (OS), once more confirming the poor outcome of DS ALL in general.

Within DS-ALL, we identified good prognosis subgroups, i.e. *ETV6-RUNX1* positive ALL or ALL with hyperdiploid (HeH) ALL with trisomy 4&10 abnormalities. The CIR for both these subgroups was very low and TRM was the major cause of dismal outcome patients with HeH trisomy 4&10. This suggest that these patients might be eligible for treatment reduction rather than intensification, such as withholding from anthracyclines, and/or stratification in the National Cancer Institute standard risk group (NCI SR), preferably with minimal residual disease (MRD) confirmation of excellent early response. In addition, we identified a novel clinically favorable prognostic subgroup of DS-ALL patients, characterized by age <6 years and with blood cell (WBC) count $<10x10^9$/L, which differs from the classical prognostic factors in non-DS ALL according to the NCI criteria. However, these patients still had a relatively high CIR, suggesting they may need an intensification block following induction and CNS-directed therapy, and might therefore be classified into the intermediate MRD risk group.

In general, infection associated TRM was not related to a specific treatment-phase or regimen, but interestingly about half occurred during maintenance therapy. While relapse remains the major cause of poor survival, vigilant supportive care is necessary to improve outcome. We therefore recommend improving supportive care measurements for DS ALL patients throughout therapy with aggressive treatment of infections. Moreover, DS ALL patients should receive

the same treatment intensity as non-DS patients in order to prevent relapse but with prompt interruptions for aplasia and with more intensive surveillance than non-DS children.

It is well known that DS ALL patients are vulnerable for excessive toxicity. Especially Methotrexate (MTX), which is a key agent in current ALL treatment, is notorious for its side effects in DS ALL. In chapter 3 we describe the results of a retrospective case-control study in which we used non-linear mixed effect modeling to study the pharmacokinetics and toxicity induced by high dose (1-5 g/m^2) MTX courses in 44 DS and 87 matched non-DS ALL patients. In total, we analyzed 468 MTX courses and observed significantly more grade 3-4 gastro-intestinal (GI) toxicity in DS patients as compared to non-DS ALL patients. Interestingly, the occurrence of grade 3-4 GI toxicity was not related to plasma MTX area under the curve (AUC). MTX-clearance was 5% lower in DS-ALL patients, however no significant differences in MTX plasma levels were detected at T=24 and at T=48 hours. Therefore, we feel that this small difference in clearance is probably clinically not relevant. In conclusion, we did not find evidence for differences in MTX pharmacokinetics between DS and non-DS patients which could explain the higher frequency of GI toxicity, and the greater need for MTX dose reductions. Hence, these differences are most likely explained by differential pharmacodynamic effects in the tissues between DS and non-DS children.

In chapter 4, we described mutations of the kinase and pseudokinase domain of *Janus Kinase* 1-3 (*JAK*), identified with bi-directionally sequencing. We screened 6 DS patients with transient myeloproliferative disease (TMD), 15 ML-DS and 35 DS ALL patients. *JAK1* mutations were found in 1 ML-DS patient and 1 DS ALL patient. *JAK2* R683 mutations, which activate the JAK-STAT pathway, were found in ~20% of the DS ALL patients, and *JAK3* mutations were found in 1 TMD and 1 ML-DS patient. *JAK2* mutations lack prognostic significance in DS ALL, as no relapses occurred. Furthermore, a meta-analysis of published data did not show any differences in survival of *JAK2* mutants compared to wildtype patients.

In chapter 5, we used array Comparative Genomic Hybridization (CGH) and Multiplex Ligation-dependent Probe Amplification (MLPA) to find molecular aberrations in genes involved B-cell development and differentiation. We studied a population-based cohort of 34 DCOG DS ALL patients and validated our results in 84 DS ALL children from United Kingdom trials. In total, 50% of DS ALL patients had ≥1 deletion in a B-cell development or differentiation gene. Interestingly, *IKZF1* encoding the lymphoid transcription factor IKAROS, was deleted in 35% of all DS ALL patients, which is a proportion similar to non-DS high-risk ALL patients. *IKZF1* deletions independently predicted dismal outcome. Remarkably, 62% of all patients had genomic Cytokine Receptor Like Factor 2 (*CRLF2*) rearrangements but this abnormality lacked prognostic relevance.

In Chapter 6, we established the frequency and the prognostic significance of B-cell translocation gene 1 (*BTG1*) deletions in a large series of DS ALL patients. We found *BTG1* aberrations in ~7% of the DS ALL patients and they all showed a nearly identical deletion pattern in which the *BTG1* area 1, area 2, and exon 2 probes were deleted, while exon 1 was retained. Interestingly, *BTG1* deletions were mainly found in patients with *ETV6-RUNX1* positive ALL.

Moreover, *BTG1* deletions did not predict for poor clinical outcome in DS ALL patients. In short, we could not confirm the high frequency of *BTG1* deletions previously described in smaller series and found the prevalence to be similar to non-DS ALL patients.

In Chapter 7 we aimed at identifying genes differentially expressed between DS and non-DS ALL, which are involved in DS ALL leukemogenesis. Therefore we compared global gene-expression profiles (GEP) of 38 DS ALL with 502 non-DS BCP ALL patients. We identified 357 probe sets (307 genes) being significantly differentially expressed between DS and non-DS BCP-ALL. Interestingly, the vast majority of genes overexpressed in DS as compared to non-DS ALL, are not differentially expressed in differentiated hematopoietic (CD43+, CD41+ and CD235+) "induced pluripotent stem cells (iPS)" derived from DS tissue. This indicates that differences in gene expression are not only a consequence of a trisomy 21 but are related to the DS leukemogenesis. In addition, we showed that as compared to non-DS patients, ETS-related gene (*ERG*) has a reduced expression in DS bone marrow samples taken from DS children without leukemia, but is overexpressed in bone marrow samples of DS ALL patients. As *ERG* is a chromosome 21 localized transcriptional regulator of hematopoiesis, the constitutional extra copy of the *ERG* gene, may result in deregulation of the hematopoietic stem cell network and therefore be an important driving event in DS BCP-ALL.

Furthermore, we showed that 25% of the DS ALL cases clustered together with '*BCR-ABL1*-like' non-DS patients. Patients with a '*BCR-ABL1*-like' profile cluster together with *BCR-ABL1*–positive cases, although the leukemic cells do not harbor the *BCR-ABL1* translocation. Non-DS ALL patients with a '*BCR-ABL1* like' signature denote a new clinically relevant entity having poor outcome. The DS ALL patients with a '*BCR-ABL*1 like profile' had a CIR of 30%, which is similar to their non-DS counterparts. In addition, 50% of the '*BCR-ABL1*-like' DS ALL patients have deletions of *IKZF1*, providing further evidence that genetic make-up of DS ALL is unfavorable.

In conclusion, we provided evidence that to date there is no evidence of a unique genetic lesion occurring in DS and not in non-DS ALL patients. Moreover, we showed that DS ALL is a genetically 'high risk' disease, as a substantial proportion of DS ALL patients has a 'BCR-ABL1-like' gene expressions profile and/or IKZF1 deletions. We therefore recommend being cautious in treatment reduction for DS ALL patients, as relapse and not TRM is the major cause of treatment failure. The only exception to this rule may be the small group of 12% of patients with ETV6-RUNX1 rearrangements or hyperdiploid trisomies 4 and 10. To further improve outcome, DS ALL patients may benefit from 1) improved risk-group stratification with current available therapy, 2) from the elucidation of the genetic background, especially in the normal karyotype cases, 3) the development of targeted therapies that improves prognosis and reduces acute and long-term toxicity as well as TRM for all patients, and 4) from enhanced intensive supportive care throughout treatment. This requires several novel studies, such as the role of MRD in DS ALL, unraveling the genetic background by using novel deep-sequencing procedures, the role of JAK-inhibitors in DS ALL, and studies evaluating antibiotic/antifungal and/or immunoglobulin prophylaxis in DS ALL. As DS ALL is a rare disease, international collaboration is essential to achieve these goals.

NEDERLANDSE SAMENVATTING

Kinderen met het syndroom van Down hebben een verhoogde kans op het krijgen van acute lymfatische (ALL) en acute myeloide leukemie (AML). Ondanks een duidelijke verbetering in de prognose van kinderen met ALL in de laatste 30 jaar, blijft het toekomstperspectief voor leukemie patiënten met het syndroom van Down minder gunstig dan voor patiënten zonder Down syndroom. Dit proefschrift behandeld de studies die we verricht hebben op basis van klinische data van patiënten met DS ALL (hoofdstuk 2-3) en de moleculaire afwijkingen die voorkomen bij DS ALL (hoofdstuk 4-7).

Hoofdstuk 2 beschrijft een grote internationale retrospectieve studie in samenwerking met de Ponte di Legno (PdL) werkgroep. Doel van deze studie was om de prognostische waarde van klinische en celbiologische kenmerken en oorzaken van falen van de behandeling te bestuderen bij kinderen met DS ALL. In totaal hebben we 653 DS ALL patiënten geïncludeerd die behandeld zijn tussen 1 januari 1995 en 1 januari 2005 en hebben deze vergeleken met non-DS B cel voorloper (BCP) ALL van de Stichting Kinderoncologie Nederland (SKION) en de Berlin Frankfurt Münster (BFM) groep. DS ALL patiënten hadden een hogere kans op het krijgen van een recidief dan non-DS ALL patiënten en tevens een hogere kans op therapie-gerelateerde mortaliteit [Treatment Related Mortality (TRM)] wat resulteerde in een lagere gebeurtenis-vrije overleving' [Event Free Survival (EFS)] en overleving [overall survival (OS)]. In de analyse kwam naar voren dat DS ALL patiënten met een leeftijd jonger dan 6 jaar in combinatie met minder dan $10x10^9$/L witte bloedcellen ten tijde van de diagnose, een nieuwe groep patiënten vormden met een goede prognose (EFS: 78%, OS: 87%). Deze afkappunten van leeftijd en aantal witte bloedcellen zijn anders dan de criteria van het Amerikaanse National Cancer Institute (NCI), die boven of onder de 10 jaar en WBC groter of kleiner dan $50x10^9$/L gebruikt. Opvallend was echter dat patiënten <6 jaar met minder dan $10x10^9$/L witte bloedcellen nog een relatief hoge kans op het terugkeren van de ziekte (CIR 17%) hadden. Dit suggereert dat zij na de inductie therapie nog een extra blok met intensieve therapie nodig hebben, eventueel aangevuld met therapie voor leukemiecellen in het centraal zenuwstelsel.

Tevens vonden we dat patiënten met *ETV6-RUNX1* fusies in de leukemiecellen of een hoog hyperdiploid (HeH) karyotype (≥51 chromosomen) een goede prognose hebben, wat overeenkomt met ALL in kinderen zonder DS. Circa 45% van de HeH DS ALL patiënten hadden zowel 3 kopieën van chromosoom 4 als van chromosoom 10. Deze laatste groep van patiënten had een EFS van 88% en geen van hen kreeg een recidief. Ook het aantal kinderen met *ETV6-RUNX1* fusies had een zeer laag risico voor het recidiveren van de ALL. Dit suggereert dat deze patiënten mogelijk in aanmerking zouden kunnen komen voor reductie van therapie door bijvoorbeeld minder of geen anthracyclines toe te dienen. Een andere strategie zou zijn om deze patiënten alleen te behandelen in de standaard risico arm van een behandelprotocol, waarbij bij voorkeur de excellente respons op therapie bevestigd wordt

met een snelle afname van minimale rest ziekte (minimal residual disease [MRD]) in het begin van de behandeling.

Ook hebben we gevonden dat TRM niet gerelateerd was aan een specifieke fase van de therapie maar werd gezien gedurende de hele behandelperiode. Tevens vonden we dat TRM niet gerelateerd was aan een bepaald chemotherapeuticum. Opvallend was dat het gebruik van toxische anthracyclines tijdens de inductie geen duidelijke invloed had op TRM. De mortaliteit als gevolg van infectieuze TRM was ook verhoogd in DS ALL. Dit impliceert dat betere ondersteunende behandelingen (supportive care) gedurende de hele behandelperiode nodig zijn voor deze kwetsbare groep patiënten. Opvallend was echter dat recidiverende ziekte en niet TRM de grootste factor van de slechtere prognose van DS ALL patiënten is. Om het terugkeren van de ziekte te voorkomen zou het aantal witte bloedcellen gedurende de therapie in DS ALL patiënten net zo laag moeten zijn als in ALL patiënten zonder DS, maar met frequente controles en acute onderbreking van therapie indien er sprake is van aplasie. Naar aanleiding van bovengenoemde resultaten is er een internationale werkgroep opgezet die zich bezig houdt met het verzamelen van gegevens van DS ALL patiënten om van daaruit een specifiek behandelprotocol voor kinderen met DS ALL te ontwikkelen.

Methotrexaat (MTX) is een van de belangrijkste componenten van de ALL behandeling in Nederland. DS ALL patiënten ervaren meer MTX gerelateerde toxiciteit dan non-DS ALL kinderen en met name de gastro-intestinale toxiciteit staat op de voorgrond. In hoofdstuk 3 wordt de retrospectieve case-control studie beschreven die verricht is op een groep van 44 DS ALL patiënten en 87 non-DS ALL controle patiënten. De ervaren toxiciteit tijdens protocol M (meerdere kuren hoge dosis MTX intraveneus) en de bijhorende MTX-spiegels in het bloed werden verzameld en geanalyseerd door gebruik te maken van non-linear mixed effect modeling (NONMEM). Op deze manier wilden we onderzoeken of het mogelijk was om de toxiciteit terug te voeren op een veranderde farmacokinetiek in DS ALL patiënten t.o.v. non-DS ALL patiënten. In totaal verzamelden we gegevens van 468 MTX kuren variërend van 1-5 g/m^2 in 44 DS en 87 non-DS ALL patiënten. Graad 3-4 gastro-intestinale toxiciteit werd significant vaker waargenomen in DS patiënten in vergelijking met non-DS ALL patiënten maar dit was niet gerelateerd aan de hoeveelheid plasma MTX onder de curve. De klaring van MTX door DS ALL patiënten was 5% lager dan non-DS ALL patiënten. Echter dit verschil lijkt klinisch niet relevant te zijn aangezien er geen verschil werd gezien in de MTX plasma spiegel op tijdstip 24 en 48 uur na het starten van de hoge dosis MTX. Hieruit kan worden geconcludeerd dat het niet waarschijnlijk is dat DS ALL patiënten een andere farmacokinetiek hebben die verantwoordelijk is voor de verhoogde toxiciteit. Het is dan ook meer waarschijnlijk dat de farmacodynamiek in de verschillende lichaamsweefsels anders is in DS ALL kinderen t.o.v. non-DS kinderen. Aangezien DS ALL kinderen die in ons cohort behandeld werden met een gemiddelde dosis MTX (1-3 gram/m^2) weinig toxische problemen ondervonden bevelen wij deze dosis aan voor toekomstige behandelschema's van DS ALL kinderen.

Al geruime tijd spelen genetische afwijkingen van de leukemiecel een belangrijke rol in prognose en maken ze deel uit van de classificatie voor het te volgen behandelprotocol. Meer recent is het met nieuwe technieken mogelijk geworden om op moleculair niveau afwijkingen van de leukemiecel te detecteren. Deze moleculaire afwijkingen gaan een steeds belangrijker rol spelen in de moderne tijd waarin men streeft naar therapie op maat. In hoofdstuk 4 hebben we de frequentie van mutaties van de Janus Kinase (JAK) familie genen JAK1, JAK2 en JAK3 in kaart gebracht van 6 DS patiënten met transiënte myeloproliferatieve leukemie (TMD), 15 ML-DS en 35 DS ALL patiënten. Veel studies richtten zich met name op de reeds bekende mutaties waardoor het werkelijke aantal afwijkingen mogelijk onderschat werd. Het volledige kinase en pseudokinase domein van *JAK1*, *JAK2* en *JAK3* werd met behulp van polymerase chain reaction (PCR) geamplificeerd, gesequenced en geanalyseerd. *JAK1* mutaties werden gevonden in 1 ML-DS patiënt en in 1 DS ALL patiënt. In *JAK2*, zijn in exon 16 op positie R683 mutaties gevonden in 6 (~20%) DS-ALL patiënten. Deze mutatie activeert het JAK-STAT pathway en komt ook voor in een lagere frequentie in high risk non-down ALL. *JAK3* mutaties werden aangetoond in 1 patiënt met TMD en in 1 ML-DS patiënt. Het muterende effect van de mutaties in zowel *JAK1* als *JAK3* is onbekend. De prognose van patiënten met een *JAK2* mutatie bleek niet beter of slechter te zijn dan voor patiënten zonder deze mutatie. De '10-jaar gebeurtenis-vrije overleving' [Event Free Survival (EFS)] was 100% voor patiënten met een *JAK2* mutatie versus 75% in patiënten zonder afwijking. Hierin worden alle gebeurtenissen zoals refractaire ziekte, recidief, dood door welke oorzaak dan ook, verwerkt. Ook werd geen verschil gezien in de 10-jaars OS en de cumulatieve incidentie van recidief (CIR) was eveneens gelijkwaardig. Deze resultaten werden bevestigd door een meta-analyse van 171 DS ALL patiënten. Concluderend, *JAK* mutaties zijn zeldzaam in DS gerelateerde leukemie, met uitzondering van *JAK2* mutaties in DS ALL en deze hebben geen prognostische waarde.

Kinderen met DS ALL hebben een lagere frequentie van de veelvoorkomende (cyto-) genetische afwijkingen bij non-DS ALL, waardoor we op zoek zijn gegaan naar moleculaire afwijkingen die specifiek betrokken zijn bij de pathogenese van DS ALL. Hiervoor is gebruik gemaakt van array Comparative Genomic Hybridization (CGH) and Multiplex Ligation-dependent Probe Amplification (MLPA), waarvan de resultaten in hoofdstuk 5 worden beschreven. Met zowel array-GCH als met MLPA is het mogelijk kleine amplificaties of deleties in het DNA op te sporen die niet zichtbaar zijn met standaard genetisch onderzoek. Bij de analyse is met name gekeken naar afwijkingen in genen die betrokken zijn bij de ontwikkeling van B-cellen en naar afwijkingen van *CRLF2* en *JAK2*. De resultaten voortvloeiend uit analyse van een Dutch Childhood Oncology (DCOG) 'population based' cohort van 34 DS ALL samples werd gevalideerd door een UK 'population based' cohort van 84 DS samples . In totaal had 50% van de DS ALL patiënten ≥1 afwijking in een B-cel ontwikkelings-gen: *PAX5* (12%), *VPREB1* (18%), en *IKZF1* (35%). *JAK2* was gemuteerd in 15% van de patiënten en gnomische afwijkingen van *CRLF2* werden gevonden bij 62% van de DS ALL patiënten. De overleving van patiënten met een afwijking in *IKZF1*, dit is het gen dat codeert voor de transcriptiefacto IKAROS,

was significant slechter dan voor patiënten zonder deze afwijking (6-year EFS 45±16% vs. 95±4%; p=0.002) en dit werd bevestigd in het validatiecohort van de UK (6-year EFS 21±12% vs. 58±11%; p=0.002). In een multivariaat analyse bleek dat deleties van *IKZF1* een sterke voorspellende waarde hadden voor een slechte prognose. Dit zou kunnen betekenen dat DS ALL patiënten gelijkenis vertonen met non-DS ALL patiënten met een high risk profiel en dat we daar in de toekomst met de behandeling rekening mee moeten houden.

In hoofdstuk 6 wordt de frequentie en de prognostische relevantie van afwijkingen van het B cel translocatie gen 1 (*BTG1)* in DS ALL besproken naar aanleiding van een publicatie waarin werd beschreven dat deleties van dit gen vaak voorkomen in DS ALL, maar een klein aantal patiënten had geïncludeerd. *BTG1* speelt een cruciale rol in cel processen zoals proliferatie en geprogrammeerde celdood. Dit gen werd onderzocht in een 'population based' cohort van 116 DS ALL patiënten van de DCOG en de UK. In totaal vonden we in 6.9% van de DS ALL patiënten een deletie van *BTG1*, een frequentie die overeenkomt met non-DS ALL patiënten en duidelijker lager dan beschreven in de kleine studie. Opvallend was dat alle afwijkingen een nagenoeg identiek patroon van deleties lieten zien waarin de *BTG1* regio 1 en 2 en exon 2 was gedeleteerd maar exon 1 nog in tact was. De mediane leeftijd van patiënten met een *BTG1* deletie was 5.4 jaar versus 12 jaar in de en 62% had het mannelijk geslacht wat duidelijk verschilt van de resultaten in de gepubliceerde studie (12 jaar en 80% man). Mutaties van *BTG1* kunnen samen met andere (cyto-) genetische afwijkingen voorkomen en werden met name gezien in de groep patiënten met een *ETV6-RUNX1* afwijking. Afwijkingen van *BTG1* hebben geen prognostische waarde wanneer ze vergeleken worden met DS ALL patiënten met een normaal *BTG1* gen. Concluderend konden we de hoge frequentie van *BTG1* deleties in een grote groep DS ALL patiënten niet bevestigen.

In hoofdstuk 7 hebben we gebruik gemaakt van 'gen-expressie profiling' (GEP). Met behulp van deze gen expressie profielen hebben we gezocht naar nieuwe genen die karakteristiek zijn voor DS ALL patiënten, en die ons mogelijkerwijs iets kunnen leren over de pathogenese van DS ALL, en daardoor interessant zijn voor de ontwikkeling van specifiek gerichte therapie (targeted therapy). In de analyse hebben we een groep van 38 DS ALL patiënten vergeleken met 502 non-DS ALL patiënten. We vonden dat 357 probe sets (307 genen), ≥ 1.5 keer tot expressie kwamen in de vergelijking tussen DS en non-DS patiënten. Opmerkelijk was dat de genen die tot over-expressie kwamen in deze vergelijking niet tot over-expressie kwamen in hematologische pluripotente stamcellen (CD43+, CD41+ and CD235+) afkomstig van DS weefsel. Dit impliceert dat veranderingen in expressie van genen niet alleen veroorzaakt worden door de extra kopie van chromosoom 21 in het DS, maar gerelateerd zijn aan het ontstaan van leukemie. Verder hebben we aangetoond dat in vergelijking met non-DS ALL patiënten, het *ERG* (ETS-related gene) gen een lagere expressie heeft in het beenmerg van DS patiënten zonder leukemie, maar een hogere expressie in het beenmerg van DS patiënten met leukemie. Aangezien *ERG* gelegen is op chromosoom 21 en de transcriptie van hematopoietische factoren reguleert, zou de extra kopie van *ERG* kunnen resulteren in het ontregelen

van het hematopoietische netwerk waar *ERG* onderdeel van uitmaakt. Dit suggereert dat *ERG* een belangrijke rol speelt in het ontstaan van ALL in kinderen met het syndroom van Down.

Tevens hebben we aangetoond dat 25% van de DS ALL patiënten samen clustert met ALL patiënten zonder DS met een '*BCR-ABL1*-like' profiel. Patiënten met dit profiel clusteren samen met patiënten die een *BCR-ABL1* translocatie hebben zonder dat deze afwijking in de leukemiecellen wordt aangetoond. ALL patiënten zonder DS met het '*BCR-ABL1*-like' profiel vormen een groep met slechte prognostische vooruitzichten. De kans op het terugkeren van de ziekte is voor zowel kinderen met het DS als kinderen zonder het DS ongeveer 30%. Ten slotte zagen we dat 50% van de kinderen met een '*BCR-ABL1*-like' profiel ook een deletie van *IKZF1* hebben. Dit ondersteunt de hypothese dat leukemie in kinderen met DS ALL een ongunstig profiel heeft.

Concluderend hebben we aangetoond date er tot op heden geen bewijs is voor een unieke mutatie die wel voorkomt in DS ALL maar niet in non-DS ALL. Verder hebben we aangetoond dat DS ALL een ziekte is met een hoog risico profiel aangezien een substantieel deel van de DS ALL patiënten een 'BCR-ABL1-like' profiel heeft en / of IKZF1 deleties. Terughoudendheid ten aanzien van het reduceren van behandeling is dan ook geboden, temeer omdat in dit proefschrift is aangetoond dat het recidiveren van de ziekte de belangrijkste oorzaak van het falen van de behandeling is en niet de toxiciteit van de behandeling. De enige uitzondering hierop zijn DS ALL patiënten met ETV6-RUNX1 fusies of met 3 kopieën van chromosoom 4 en 10. De prognose van DS ALL patiënten zou verbeterd kunnen worden indien zij zouden kunnen profiteren van 1) verbeterde stratificatie van DS ALL patiënten in de huidige behandelprotocollen 2) opheldering van de genetische achtergrond van DS ALL en dan met name in de groep van DS ALL patiënten waarin op dit moment nog geen afwijkingen zijn aangetoond, 3) de ontwikkeling van doelgerichte (targeted) therapieën die de prognose verbeteren en tegelijkertijd de acute en lange termijn toxiciteit en mortaliteit verminderen en 4) van intensieve 'supportive care' gedurende de hele behandeling. Hiervoor zijn nieuwe studies nodig die de waarde en de rol van MRD in DS ALL bepalen, studies die de met nieuwe technieken de genetische achtergrond van DS ALL verder onderzoeken, studies naar de rol van JAK-inhibitors in DS ALL en studies die de waarde van antibiotica, antischimmelmedicatie en immunoglobulines als profylaxe in de behandeling van DS ALL evalueren. Aangezien DS ALL een zeldzame ziekte is, is internationale samenwerking nodig om bovenstaande te bewerkstelligen.

10 About the author

CURRICULUM VITAE

Trudy Buitenkamp werd op 4 juni 1975 geboren te Ten Post. Na de middelbare school startte zij de opleiding HBO-Verpleegkunde te Groningen, waarbij zij haar afstudeerstage van 6 maanden in het 'Holy Famlily Hospital' te Berekum in Ghana deed. Na het voltooien van de HBO-V ging zij in het najaar van 1998 als verpleegkundige aan het werk in het Sophia Kinderziekenhuis te Rotterdam. Hier kwam zij in aanraking met het vakgebied kinderoncologie en al snel behaalde zij de specialistische vervolgopleidingen kinderverpleegkunde en kinderoncologie. Tijdens haar werkzaamheden als kinderoncologie verpleegkundige kwam zij in aanraking met wetenschappelijk onderzoek met als ultiem doel de overleving en kwaliteit van leven te verbeteren van kinderen met een oncologische aandoening. Dit was een belangrijke factor in het besluit om geneeskunde te gaan studeren. Na het behalen van haar VWO certificaten startte zij in 2003 haar studie geneeskunde te Rotterdam. Vanaf het derde jaar van de studie volgde zij daarnaast de master of science in "Clinical Research" die zij in 2008 afrondde. Daarna startte zij haar promotietraject waarvan de resultaten zijn beschreven in dit proefschrift. In april 2012 hervatte zij haar studie geneeskunde en startte met de coschappen. Tevens heeft zij gedurende haar coschappen actief geparticipeerd in de ontwikkeling van een internationaal behandelprotocol voor kinderen met het syndroom van Down. Zij is getrouwd met Martijn Arkesteijn en samen hebben zij een dochter: Sterre (2010).

LIST OF PUBLICATIONS

International publications

1. **Buitenkamp TD**, Mathôt RA, de Haas V, Pieters R, Zwaan CM. Methotrexate-induced side effects are not due to differences in pharmacokinetics in children with Down syndrome and acute lymphoblastic leukemia. Haematologica. 2010 Jul; 95(7): 1106-13
2. Blink M*, **Buitenkamp TD***, van den Heuvel-Eibrink MM, Danen-van Oorschot AA, de Haas V, Reinhardt D, Klusmann JH, Zimmermann M, Devidas M, Carroll AJ, Basso G, Pession A, Hasle H, Pieters R, Rabin KR, Izraeli S, Zwaan CM. Frequency and prognostic implications of JAK 1-3 aberrations in Down syndrome acute lymphoblastic and myeloid leukemia. Leukemia. 2011 Aug; 25(8): 1365-8. *Shared first authorship
3. **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 Oct; 26(10): 2204-11.
4. **Buitenkamp TD**, Pieters R, Zimmermann M, de Haas V, Richards SM, Vora AJ, Mitchell CD, Schwab C, Harrison CJ, Moorman AV, van den Heuvel-Eibrink MM, Zwaan CM. BTG1 deletions do not predict outcome in Down syndrome acute lymphoblastic leukemia. Leukemia. 2013 Jan; 27(1): 251-2.
5. **Trudy D. Buitenkamp**, Shai Izraeli, Martin Zimmermann, Erik Forestier, Nyla A.Heerema, Marry M. van den Heuvel-Eibrink, Rob Pieters, Carin M. Korbijn, Lewis B. Silverman, Kjeld Schmiegelow[8] Der-Cheng Liang, Keizo Horibe, Maurizio Arico, Andrea Biondi, Giuseppe Basso, Karin R. Rabin, Martin Schrappe, G.unnar Cario, Georg Mann, Maria Morak, Renate Panzer-Grümaye, Veerle Mondelaers, Tim Lammens, Helene Cave, Batia Stark, Ithamar Ganmore, Anthony V. Moorman, Ajay Vora, Stephen P. Hunger, Ching-Hon Pui, Charles G Mullighan, Atsushi Manabe, Gabriele Escherich, Jerzy R. Kowalczyk, James A. Whitlock, and C. Michel Zwaan. Acute lymphoblastic leukemia in children with Down syndrome: a retrospective analysis from the Ponte di Legno study group. Blood. 2014 Jan 2; 123(1): 70-7.
6. Blink M, van den Heuvel-Eibrink MM, Gallimore NE, **Buitenkamp TD,** den Boer ML, Balgobind BV, Sanders MA, de Haas V, Klusmann JH, Reinhardt D, Hasle H, Muzikova K, Pieters R, Zwaan CM. Gene expression profiling of transient myeloproliferative disorder and myeloid leukemia of Down syndrome. Submitted.
7. T**.D. Buitenkamp**[1], M.M. van den Heuvel – Eibrink[1], M. Zimmerman[2], J.M. Boer[1], M. Blink[1], T.C.J.M. Arentsen-Peters[1], V. de Haas[3], R. Pieters[1], M.L. den Boer[1], M. Fornerod[1], C.M. Zwaan[1] Dow syndrome acute lymphoblastic leukemia is characterized by loss of ETS-related gene repression Manuscript in preparation.

National publications

1. Blink M, **Buitenkamp TD**, van Wering ER, Zwaan CM Down Syndroom en Leukemie. Down Up (uitgave Stichting Down Syndroom) 2008.
2. Blink M, **Buitenkamp TD**, van Wouwe JP, van Wering ER, van der Velden VHJ, Zwaan CM. Ontwikkelingen in de diagnostiek en behandeling van leukemia bij Kinderen met het Down Syndroom. Tijdschrift voor Kindergeneeskunde. April 2009.

PHD PORTFOLIO

Name PhD student: Trudy D. Buitenkamp	
Erasmus MC department: Pediatric Oncology-Hematology	
Research School: Molecular Medicine	
PhD period: February 2008 – June 2014	
Promotor: Prof. Dr. R. Pieters	
Supervisors: Dr. C.M. Zwaan, Dr. M.M. van den Heuvel-Eibrink	
General academic courses	**Year**
Classical Methods for Data Analysis (CC02) (NIHES)	2009
Biomedical English Writing and Communication	2011
Photoshop and illustrator (CS5)	2011
Specified courses	
Basic and Translational Oncology (MM)	2008
Biomedical Research Techniques	2008
Basic course on Gene Expression Arrays	2009
Basic course on 'R'	2010
Seminars and workshops	
Annual PhD day, Erasmus MC	2008
Annual Pediatric research Day, Erasmus MC	2008-2010
Annual Molecular Medicine Day, Erasmus MC	2008-2012
Annual Pediatric Oncology Symposium	2008-2011
2nd AML-BFM Research Symposium, Hannover, Germany	2008
International meeting Acute Lymphoblastic Leukemia, London, UK	2009
(Inter)national conferences	
International Symposium "Acute Leukemias XII", Munich, Germany	2008
19th Annual Meeting of the I-BFM Study Group, Glasgow, UK (oral presentation)	2008
40th SIOP Annual Meeting, Berlin, Germany (oral presentation)	2008
6th Biennal I-BFM Childhood Leukemia Symposium, Glasgow, UK	2008
50th ASH annual Meeting, San Francisco, USA (2 poster presentations)	2008
51th ASH annual Meeting, New Orleans, USA (1 poster presentation)	2009
53rd ASH annual Meeting, San Diego, USA (1 poster presentation)	2011
43rd SIOP Annual Meeting, Auckland< New Zealand (oral presentation)	2011
22nd Annual Meeting of the I-BFM Study Group, Gdansk, Poland (oral presentation)	2011
8th Biennial Childhood Leukemia Symposium, Santiago, Chili (oral presentation)	2012
17th Congress of EHA, Amsterdam, The Netherlands (oral presentation)	2012
Other	
Travel grant for the 50th and 51th ASH annual meeting awarded by the ASH committee	2008, 2009
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Martin, despite a very busy agenda and the existence of corrupt excel files, you managed to make an uncountable number of Kaplan Meier curves for me. Thank you once again.

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Trudy

LIST OF ABBREVIATIONS

Abbreviations	
ABL1	C-abl oncogene 1, non-receptor tyrosine kinase (gene)
AEIOP	Associazione Italiana di Ematologia ed Oncologia Pediatrica
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
ANC	Absolute neutrophil count
AUC	Area under the curve
B-other	Genetically unclassified B-ALL
BCL2	B-cell lymphoma 2
BCP	B cell precursor
BCR	Breakpoint cluster region (gene)
BFM	Berlin-Frankfurt-Münster
BM	Bone marrow
BSA	Body surface area
BTG1	B-cell translocation gene 1 (gene)
CCG	North American Children's Oncology Group including the Children's Cancer Group and the Pediatric Oncology Group studies
CCLG	Childhood Cancer and Leukaemia Group
CCR	Continuous comple remission
CD	Cluster of differentiation
CDKN2A/B	Cyclin-Dependent Kinase Inhibitor (gene)
cDNA	Complementary DNA
CEBPA	CCAAT/enhancer binding proetin (C/EBP), alpha (gene)
CGH	Comparative Genomic Hybridization
CI	Confidence interval
CIR	Cumulative incidence of relapse
CL	Central compartment
CLL	Chronic Lymphoblastic Leukemia
CML	Chronic Myeloid Leukemia
CN	Cytogenetically normal
CNS	Central nervous system
CNV	Copy number variation
CoALL	Cooperative study-group for childhood acute lymphoblastic leukemia
Cobs	Observed concentration
COG	Children's Oncology Group (North America)
Cpred	Predicted concentration
CRCL	Creatinine clearance
CRLF2	Cytokine Receptor Like Factor
Ct	Cycle treshold

CTCAE	Common Toxicity Criteria for Adverse Events
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DCOG	Dutch Childhood Oncology Group
DFCI	Dana Faber Cancer Institute
DNA	Deoxyribonucleic acid
DS	Down Syndrome
DSCR	Down syndrome critical region
E2A	Transcription Factor E2-Alpha (gene)
EFS	Event-free survival
EORTC	European Organization for Research and Treatment of Cancer (Belgium)
ERG	v-ETS erythroblstosis virus E26 oncogene homolog (gene)
ETV6	Ets variant 6 (gene)
FC	Foldchange
FDR	False discovery rate
FISH	Fluorescence in situ hybridization
GATA1	Globin transcription factor 1 (gene)
GC	Glucocorticoid
GEP	Gene expression profiling
GO	Gene Ontology
HB	Hemoglobine
HeH	High hyperdiploid
HR	Hazard ratio
HR(G)	High risk (group)
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IGH@	Immunoglobuline heavy chain (gene)
IKZF1	IKAROS family zinc finger 1
iPS	Induced pluripotent stem cells
ISCN	International System for Human Cytogenetic Nomenclature
ITT	Intrathecal triple therapy
JACLS	Japan Association of Childhood Leukemia Study
JAK	Janus kinase (gene)
JMML	Juvenile myelomonocytic leukemia
kDa	kilo Dalton, measurement of atomic mass commonly used to indicate protein mass
KIKA	KinderKankervrij
KOCR	Kinderoncologisch centrum rotterdam (funding organization for pediatric oncology research and training)
LIMMA	Llinear models for microarray analysis
MDS	Myeodysplastic syndrome
ML	Myeloid leukemia
MLL	Myeloid/lymphoid or mixed -lineage leukemia

MLPA	Multiplex ligation-dependent probe amplification
6-MP	6-Mercaptopurine
MPD	Myeloproliferative disorders
MPL	Myeloproliferative leukemia virus oncogene (gene)
MPN	Myeloproliferative neoplasms
MR(G)	Medium risk (group)
MRC-UKALL	Medical Research Council United Kingdom
MRD	Minimal residual disease
MTX	Methotrexate
MX1	Myxovirus Resistance 1 (gene)
NCI	National Cancer Institute
NCRI	National Cancer Research Institute
NK	Natural killer
NONMEM	Non-linear mixed effect modeling
NOPHO	Nordic Society for Pediatric Hematology and Oncology
NR	Non responder
OFV	Objective function value
OS	Overall survival
P2RY8-	Purinergic Receptor P2Y, G-Protein Coupled, 8 (gene)
PAR1	Pseudoautosomal region 1
PAX5	Paired box 5 (gene)
PB	Peripheral blood
PBX1	Pre-B-cell leukemia homeobox 1 (gene)
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PdL	Ponte di Legno
PK	Pharmacokinetics
POG	Pediatric Oncology Group
PPLLSG	Polish Paediatric Leukaemia and Lymphoma Study Group
PS	Probe set
Q	Inter-compartmental clearance
RFC	Reduced folate carrier
RFS	Relapse free survival
RNA	Ribonucleic acid
RPS20	ribosomal protein S20 (gene)
RT-PCR	Reverse transcription PCR
RT-qPCR	Quantitative real time PCR
RUNX1	Runt-related transcription factor 1 (gene)
SCT	Stem cell transplantation
SJCRH	St. Jude Children's Research Hospital
SNP	Single-nucleotide polymorphism

SR(G)	Standard risk (group)
T	Time point
TCCSG	Tokyo Children's Cancer Study Group
TCF3	Transcription factor 3 (gene)
TL	Transient Leukemia
TMD	Transient myeloproliferative disorder
TPOG	Taiwan Pediatric Oncology Group
TRM	Treatment related mortality
V1	Volume of distribution of the central compartment
V2	Volume of distribution of the peripheral compartment
VPREB1	Pre-B lymphocyte 1 (gene)
VSN	Variance Stabilzation Normalization
WBC	White blood cell count
WHO	World Health Organization
WT	Wild-type

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