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DISSERTATION

The clinical relevance of aneuploidy in relapses of pediatric B-cell precursor acute lymphoblastic leukemia

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ABSTRACT (deutsch)

Die Aneuploidie ist ein charakteristisches Merkmal leukämischer Zellen der Akuten Lymphoblastischen Leukämie der B-Zellreihe (Englisch: *B-cell precursor acute lymphoblastic leukemia*, *BCP-ALL*) im Kindesalter. Zwei wesentliche Aneuploidie-Untergruppen, die sogenannte *high hyperdiploidy* und *hypodiploidy* (Englisch), sind mit einer unterschiedlichen Prognose nach der Leukämie-Ersterkrankung assoziiert. *High hyperdiploidy*, allgemein definiert als Aneuploidie mit >50 Chromosomen oder einem DNA-Index ≥1.16, tritt bei etwa 30% der Patienten auf und sagt eine hohe Überlebenswahrscheinlichkeit voraus. Im Gegensatz dazu ist die *hypodiploidy* mit <45 Chromosomen eher selten (<2%) und betroffene Patienten haben sehr schlechte Heilungschancen nach der Erstbehandlung. Für Kinder mit einem Rückfall (Rezidiv) der BCP-ALL ist die klinische Relevanz der Aneuploidie noch weitgehend unbekannt. Wir untersuchten daher eine große Patientenkohorte (n=413) mit erstem BCP-ALL-Knochenmarkrezidiv, die in Deutschland im Rahmen der multizentrischen ALL-REZ BFM 2002-Rezidivstudie behandelt wurden, auf Aneuploidien. Da die konventionelle Karyotypisierung bei Leukämiezellen des Rezidivs häufig nicht erfolgreich ist, wurde ein kombinierter Ansatz aus DNA-Index-Messungen mittels Durchflusszytometrie und einer Kopienzahl-Analyse der Zentromere mittels *multiplex ligation-dependent probe amplification* (MLPA) angewendet. Die Ergebnisse der MLPA-Methode zeigten eine hohe Konkordanz zu Kopiezahl-Daten aus Fluoreszenz-in-situ-Hybridisierungen und zu DNA-Index-Messungen. Eine nachfolgende Clusteranalyse der MLPA-Daten identifizierte anschließend die verschiedenen Aneuploidie-Untergruppen anhand ihres Musters an chromosomalen Zugewinnen. Die *high hyperdiploidy* wurde in 16% der Rezidive (n=64/413) festgestellt, und die Patienten zeigten einen günstigen Verlauf nach der Rezidivbehandlung mit einer 10-Jahres-Wahrscheinlichkeit des ereignisfreien Überlebens (pEFS) von etwa 70%. Rezidive mit *low hyperdiploidy* (n=42/413) hingegen erreichten ein signifikant schlechteres pEFS von nur 40% (*P*=0.007). Drei Patienten mit scheinbarer *high hyperdiploidy* zeigten ein ungewöhnliches Muster ihrer hinzugewonnenen Chromosomen sowie zusätzliche *TP53*-Mutationen. Eine anschließende Array-basierte Allelotypisierung offenbarte einen hypodiploiden Ursprung dieser drei Rezidive, jedoch konnte der hypodiploide Klon nicht mit DNA-Index, Karyotypisierung oder Fluoreszenz-in-situ-Hybridisierung nachgewiesen werden (sogenannte maskierte *hypodiploidy*). Insgesamt hatten die Patienten mit maskierter (n=3) oder offensichtlicher (n=8) *hypodiploidy* beim Rezidiv ein besonders schlechtes pEFS von 9% nach der Rezidivbehandlung (P=0.001). Bemerkenswert ist, dass die aktuelle Behandlungsstrategie für BCP-ALL-Rezidive solche maskierten *hypodiploidy*-Fälle aufgrund ihres günstigen klinischen Erscheinungsbildes beim Rezidiv nicht als Hochrisikopatienten identifizierte. In der multivariaten Analyse zeigte sich die *hypodiploidy* zudem als unabhängiger Prognosefaktor für ein schlechtes ereignisfreies- sowie Gesamt-Überleben. Diese Ergebnisse legten nahe, dass BCP-ALL-

Rezidive mit *hypodiploidy* zukünftig einem Hochrisiko-Behandlungsarm bzw. alternativen Therapieansätzen zugewiesen werden sollten. Die vorliegende Studie war eine Grundlage dafür, dass die Identifikation von *hypodiploidy* in die klinische Routine-Diagnostik von BCP-ALL Rezidiven aufgenommen wurde und dieser genetische Marker sowie *TP53*-Mutationen in der nächsten internationalen Rezidiv-Studie in die Risikostratifizierung einfließen werden.

ABSTRACT (english)

Aneuploidy is a cytogenetic hallmark of leukemic cells from pediatric B-cell precursor (BCP) acute lymphoblastic leukemia (ALL). Two major subgroups, i.e. high hyperdiploidy and hypodiploidy, are associated with outcome of patients after frontline treatment. High hyperdiploidy, generally defined as >50 chromosomes or a DNA index ≥1.16, occurs in about 30% of patients and is associated with favorable prognosis. In contrast, hypodiploidy with <45 chromosomes is rare (<2%) and patients suffer from a very poor outcome after frontline treatment. For relapsed BCP-ALL, however, the clinical relevance of aneuploidy is largely unknown. Therefore, we investigated aneuploidy in a large cohort of patients with a first bone marrow relapse of BCP-ALL (n=413) registered in the German ALL-REZ BFM 2002 relapse trial. As conventional karyotyping was not successful for most relapse samples, we used a combined approach of DNA index by flow cytometry and centromere copy number analyses by multiplex-ligation dependent probe amplification (MLPA). MLPA data showed a high concordance with available copy number data from fluorescence-in situ hybridizations and with the DNA index. A subsequent cluster analysis of MLPA data identified distinct aneuploidy subgroups according to their pattern of chromosomal gains. High hyperdiploidy was identified in 16% of relapses (n=64/413) and patients showed a most favorable outcome after relapse treatment with a 10-year probability of event-free survival (pEFS) of 70%. The group of low hyperdiploid relapses (n=42/413) had a significantly inferior pEFS of only 40% (*P*=0.007). Three patients with apparent high hyperdiploidy showed an unusual pattern of chromosome gains and *TP53* mutations. Subsequent array-based allelotyping revealed a hypodiploid origin of these three cases; however, the hypodiploid clone was not detected by DNA index, karyotyping or fluorescence-in situ hybridization (masked hypodiploidy). Collectively, patients with masked (n=3) or evident (n=8) hypodiploidy had a very poor pEFS of 9% after relapse treatment (P=0.001). Notably, the current treatment stratification for relapsed ALL did not identify masked hypodiploid cases as high-risk due to their favorable clinical presentation at relapse. In multivariate analysis, hypodiploidy was an independent prognostic factor for poor event-free and overall survival suggesting stratification of hypodiploid BCP-ALL relapses into high-risk treatment arms in future trials or allocation to alternative treatment approaches. With this study, SNP arrays were implemented in the diagnostic procedures for relapsed ALL to facilitate the diagnosis of (masked) hypodiploidy. Furthermore, hypodiploidy and *TP53* mutations will be included as markers for stratification of patients to a very-high-risk group in the upcoming international relapse trial.

SUMMARY

Current state of research

Pediatric acute lymphoblastic leukemia

With 23% of all pediatric malignancies, acute lymphoblastic leukemia (ALL) is the most frequent cancer diagnosed in children and adolescents under the age of 18 years (Figure 1A) with a peak incidence in children between one and four years of age.[2] The majority (ca. 87%) of all cases have a precursor B-cell immunophenotype.[3] Treatment of children with newly diagnosed ALL in Germany is optimized by randomized clinical trials initiated in the 1970's by the Berlin-Frankfurt-Münster (BFM) group. As a result of steady advances in the risk-adapted treatment strategies of these consecutive BFM-trials, the prognosis of ALL patients has significantly improved during the last decades (Figure 1B). Today, newly diagnosed pediatric ALL is highly curable with long-term survival rates of approximately 90% in Germany, Austria and Switzerland[3,4] (Figure 1B) as well as in other international trials.(reviewed in Pui *et.al* 2011[5])

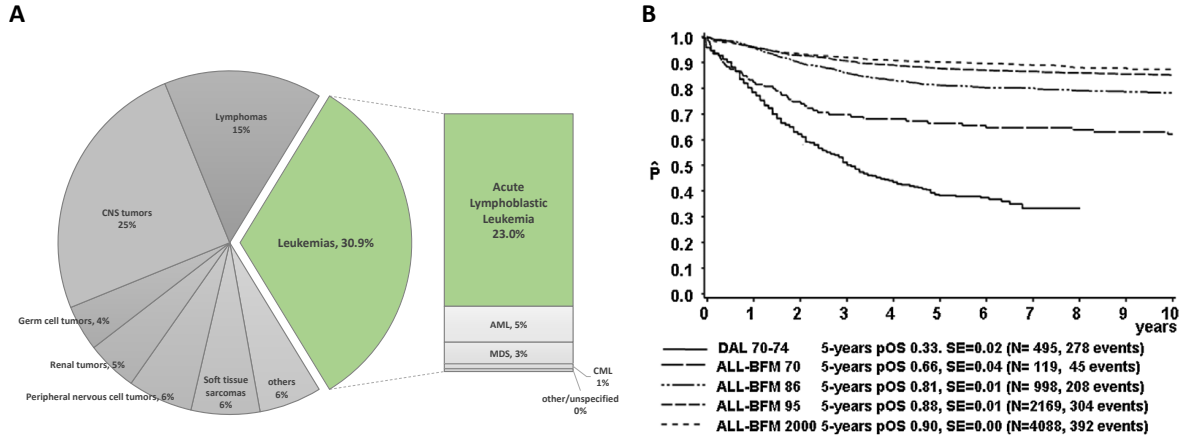

Figure 1. Relative frequencies of pediatric malignancies in Germany and long-term survival of ALL patients in five consecutive treatment trials. (A) Acute lymphoblastic leukemia is the most common malignancy during childhood. Data adapted from [2] including 10.440 cases with pediatric ALL diagnosed between 2009-2013 in Germany. (B) Increasing probability of overall survival (pOS) for patients with ALL treated in clinical trials in Germany since 1970 (from [4]). Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BFM, Berlin-Frankfurt-Münster; CML, chronic myeloid leukemia; CNS, central nervous system; DAL, Studien der Deutschen Arbeitsgemeinschaft für Leukämieforschung und –behandlung im Kindesalter; MDS, myelodysplastic syndrome.

Relapse of pediatric B-cell precursor ALL

Relapse of ALL is the recurrence of the disease after complete remission and the leading cause of treatment failure occurring in about 10-20% of patients after frontline ALL treatment.[6,7] By contrast to the dramatically improved cure rates for newly diagnosed ALL since the 1970s, strategies for the

treatment of relapsed ALL were missing at that time and most patients succumbed to the disease within one year after relapse[8]. The first clinical trials for the treatment of ALL marrow relapses were initiated in the early/mid-1980s in Germany (BFM group)[9,10], USA (POG group)[8] and Italy (AIEOP group)[11] using mainly intensified chemotherapy regimens with drugs from frontline therapy in higher doses and/or alternative settings. By this, a second complete remission could be achieved in 82-92% of relapsed patients[8,10,11] and a 6-year-EFS rate of 31% was reported from the BFM group[10]. In these and subsequent studies, three main risk factors were identified to predict outcome after first relapse, which are still the basis for risk-stratification in modern relapse treatment protocols: (I) time to relapse, i.e. the duration of first complete remission after frontline treatment, (II) site of relapse and (III) the immunophenotype of leukemic cells.[8,10-14] More specifically, patients with an ALL relapse soon after or even during frontline treatment have a particularly poor prognosis. Isolated bone marrow relapses fare worse than relapses that involve extramedullary compartments and, regarding the immunophenotype, relapses of the T cell lineage have a worse prognosis than those of the precursor B-cell lineage. By a combination of these three factors, patients are assigned to either low-/intermediate- or high-risk relapse treatment arms (see next paragraph for details on the ALL-REZ BFM 2002 protocol). In general, patients with low- and intermediate-risk relapses receive chemotherapy alone, while additional hematopoietic stem cell transplantation (HSCT), as the most intense treatment option in current state-of-the-art relapse treatment protocols, is essential for patients with high-risk relapses.[15,16] Further treatment optimization during the last decades included investigations of alternative chemotherapy drugs, combinations and dose intensities as well as the improvement of supportive care especially for HSCT.[12] More recently, the molecular response after induction treatment, i.e. minimal residual disease (MRD), has been integrated into risk-stratification to indicate which patients from the large heterogenous group of intermediate-risk relapses would also benefit from allogeneic HSCT.[17] Despite these advances in risk-adapted treatment strategies for relapsed ALL, the cure rates of patients after ALL relapse remain low with approximately 40% EFS and OS with current therapies,[13,15] and relapsed ALL still accounts for the majority of cancer-associated deaths in children and adolescents.[6]

The major challenge in the treatment of relapsed ALL is to carefully balance treatment efficacy versus drug toxicity. Leukemic blasts at relapse are more resistant to chemotherapy than those from initial diagnosis, and therefore treatment of relapsed ALL needs to be more intense[18]. However, drug related toxicities limit the extent of intensification and, furthermore, patients after relapse are generally less tolerant to chemotherapy due to the previous front-line treatment of their primary ALL. Hence, to further improve the survival of patients after ALL relapse, new alternative treatment strategies are needed that also incorporate information on specific genetic alteration in this genetically heterogenous malignancy. The identification of suitable genetic markers may, on the one hand, aid in

refining the risk stratification of relapsed ALL and, on the other hand, elucidate biologic mechanisms to develop more targeted treatment approaches.

The ALL-REZ BFM 2002 protocol for treatment of patients with relapsed ALL in Germany

In Germany, most patients with an ALL relapse are treated within consecutive randomized phase III trials aiming at the optimization of relapse therapy. These trials were established in 1983 by the BFM study group for relapsed ALL (ALL-REZ BFM) headed by the Department of Pediatric Hematology/Oncology at the Charité Universitätsmedizin Berlin[12]. In the recently closed, multi-centric relapse trial ALL-REZ BFM 2002, risk assessment of patients was based on the three established clinical factors time to relapse, site of relapse and immunophenotype of leukemic blasts.[19] The time to relapse was categorized as *very early* (<18 months after initial diagnosis), *early* (≥18 months after initial diagnosis) or *late* (≥6 months after completion of initial therapy). The site of relapse was defined as either *isolated BM relapse* (≥25% blasts in the BM without extramedullary involvement), *combined BM relapse* (≥5% blast in the BM and at least one extramedullary manifestation of ALL) or *isolated extramedullary relapse* (<5% blasts in the BM with extramedullary involvement). Using a combination of these risk factors (Table 1), patients were assigned to either a standard-risk (S1), intermediate-risk (S2) or high-risk group (S3/S4) which directed intensity and duration of ALL relapse treatment (Figure 2).

Table 1. Risk-stratification of patients with relapsed ALL within the ALL-REZ BFM 2002 trial.

		Immunophenotype: non-T			Immunophenotype: (pre-) T		
Site of relapse		isolated extramedullary	Bone marrow combined	Bone marrow isolated	isolated extramedullary	Bone marrow combined	Bone marrow isolated
Time to relapse	Very early	S2	S4	S4	S2	S4	S4
	Early	S2	S2	S3	S2	S4	S4
	Late	S1	S2	S2	S1	S4	S4

From [19].

The ALL-REZ BFM 2002 treatment courses included multi-drug systemic chemotherapy, intrathecal administration of cytostatic drugs and local irradiation of extramedullary sites if necessary. The treatment represented a conventional chemotherapy comprising the following key elements: prednisone and dexamethasone (corticosteroid drugs), daunorubicin and idarubicin (anthracyclines), cytarabine, methotrexate, 6-thioguanine, and 6-mercaptopurine (antimetabolites), cyclophosphamide and ifosfamide (alkylating agents), vincristine and vindesine (vinca alkaloids) and L-asparaginase. After the first four weeks of common intensive induction treatment, patients were randomly assigned to either intensive block- or low-dose continuous chemotherapy (Figure 2).

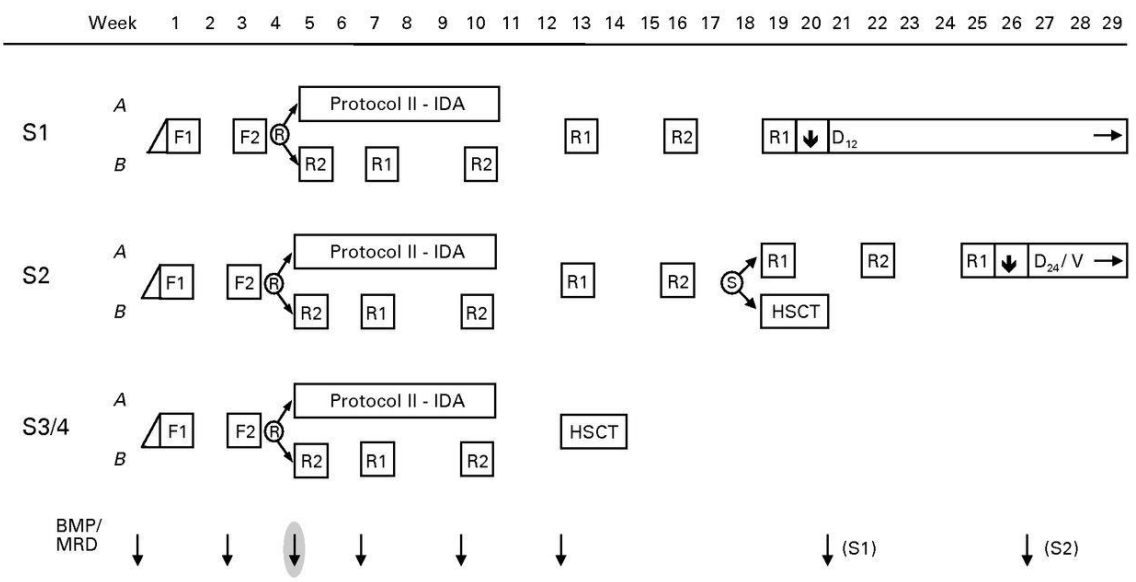

Figure 2. ALL-REZ BFM 2002 treatment scheme for children with first relapse of acute lymphoblastic leukemia. Patients with relapsed acute lymphoblastic leukemia diagnosed between 2002 and 2012 in Germany were treated according the ALL-REZ BFM 2002 protocol. Patients received a common induction treatment and were subsequently randomized either to low dose continuous consolidation treatment (Prot-II-IDA; arm A) or to conventional intensive block treatment (R1, R2 blocks; arm B). The treatment intensity is directed by risk-stratification (S1, standard-; S2, intermediate-; S3/S4, high-risk) using clinical factors at diagnosis. A hematopoietic stem cell transplantation (HSCT) is intended for S3/S4 patient as well as for S2 patients with high minimal residual disease (MRD≥10^{-3}, i.e. one or more leukemic cells in 1000 healthy mononuclear cells) after induction treatment (week 5). Arrows in the bottom row indicate time points of bone marrow punctures (BMP). Picture taken from [19].

The effectiveness of chemotherapy was monitored at different time points during treatment (Figure 2, black arrows) by (I) examination of peripheral blood and BM smear preparations (cytological response) and (II) quantification of minimal residual disease (MRD) in BM aspirates (MRD response). Cytological remission was defined as less than 5% leukemic blasts in regenerating bone marrow, absence of peripheral blasts and no extramedullary involvement. MRD was quantified by real-time quantitative PCR analysis of immunoglobulin and T cell receptor rearrangements.[17] High-risk (S3/S4) patients require a further intensification during consolidation treatment by allogeneic HSCT as soon as a complete remission is achieved (Figure 2). For intermediate-risk (S2) patients, the MRD level after the induction phase (week 5) indicates the necessity of further treatment intensification by allogeneic HSCT (cut-off MRD≥10^{-3}).[19]

Genetic alterations in pediatric B-cell precursor ALL

Distinct genetic alterations characterize the leukemic cells of BCP-ALL (Figure 3A), and several of those lesions have shown to be associated with prognosis after frontline treatment (Figure 3B).(reviewed in [20,21] About 75% of BCP-ALL cases harbor gross cytogenetic alterations that can be detected by karyotyping and/or fluorescence in-situ hybridization (FISH).[21] These include high hyperdiploidy (>50 chromosomes), hypodiploidy (<45 chromosomes), intra-chromosomal amplification of chromosome 21 (iAMP21), the chromosomal translocations *ETV6-RUNX1* [t(12;21)], *BCR-ABL1* [t(9;22)], *TCF3-PBX1*

[t(1;19)], *MLL-AF1* [t(4;11)] and other *MLL* rearrangements (Figure 3A). Hyperdiploidy and *ETV6-RUNX1* are the two most common alterations in BCP-ALL and associated with excellent outcome after frontline treatment (Figure 3A/B)[20,21]. In contrast, hypodiploidy, iAMP21, *BCR-ABL1* or *MLL* rearrangements are less frequent and associated with poor prognosis (Figure 3A/B)[20,21]. These cytogenetic alterations are usually mutually exclusive, although rare cases have been reported of, for example, simultaneous hyperdiploidy and *ETV6-RUNX1* or *BCR-ABL1*[22,23]. In the last decade, a range of secondary sub-microscopic genetic deletions were identified in genes involved in B-cell development (*IKZF1*, *PAX5*, *EBF1*) or cell cycle control and tumor suppression (*CDKN2A/2B, RB1*).[24,25] More recently, recurrent mutations in the *TP53* gene as well as in genes of the cytokine receptor, tyrosine kinase and RAS signaling pathways have been identified(reviewed in [26]) For the scope of this thesis, two of the major cytogenetic subgroups, i.e. 'high hyperdiploidy' and 'hypodiploidy' are described in more detail in the following sections.

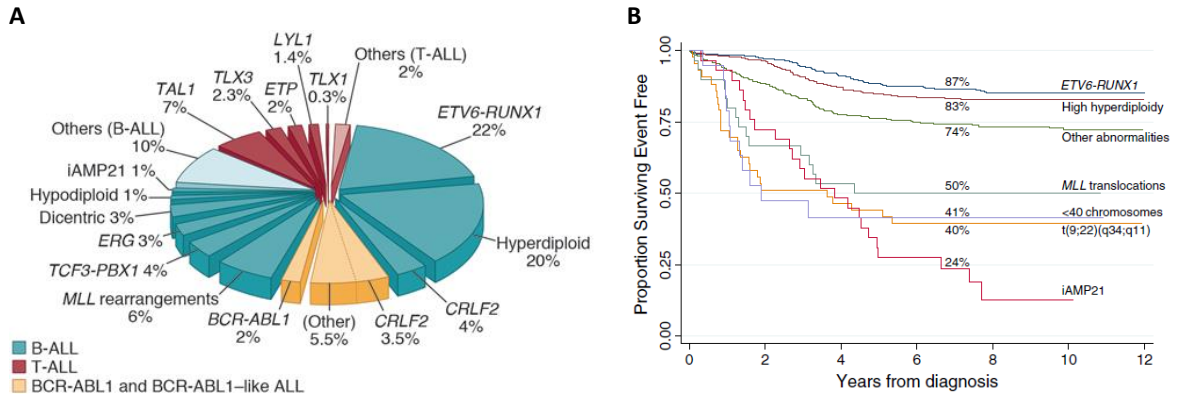

Figure 3. Cytogenetic subtypes in pediatric ALL and association with prognosis. (A) Pediatric acute lymphoblastic leukemia (ALL), and in particular B-cell precursor ALL, is characterized by diverse cytogenetic alterations such as aneuploidies (hyper-/hypodiploidy) and chromosomal translocations like *ETV6-RUNX1*, *BCR-ABL1* or MLL rearrangements. (B) Several alterations are associated with outcome after frontline treatment. Pictures taken from [21] (A) and [20] (B).

High hyperdiploidy in B-cell precursor ALL

High hyperdiploidy is detected in about one third of patients with newly diagnosed BCP-ALL and hence, is one of the most frequent cytogenetic abnormalities in pediatric ALL.([*reviewed* in [29]]) It is generally defined by the presence of more than 50 chromosomes per cell and has been associated with most favorable outcomes in numerous studies of pediatric ALL (see paragraph below). The pattern of extra chromosomes in a high hyperdiploid karyotype is not random, but typically includes some or all of chromosomes 4, 6, 8, 10, 14, 17, 18, 21 and X. However, gains of other chromosomes can be observed at lower frequencies as well. The earliest findings of high hyperdiploidy in pediatric ALL date back to 1967 when Fritz Lampert performed DNA measurements in ten pediatric ALL cases and observed that patients with an increased DNA content had a particularly long survival.[30] In the mid-1980s, high hyperdiploidy was recognized as a distinct cytogenetic subgroup in pediatric ALL associated with favorable prognosis.[27,31-33] With current state-of-the-art ALL treatments, patients with high hyperdiploidy achieve EFS and OS rates of about 80% and 90%.([34,35] *and reviewed in* [29]) However, there is no clear-cut consensus on the exact cytogenetic definition of high hyperdiploidy. While the International System for Human Cytogenetic Nomenclature (ISCN) precisely defines the term "hyperdiploidy" as the presence of 47-57 chromosomes, the definition of high hyperdiploidy in ALL varies between different studies/trials. The bottom limit of '>50 chromosomes' is based on a karyotype study by *Williams et.al*[27], who observed a bimodal distribution of the chromosome number (Figure 4A). In their study, patients with >50 chromosomes had the best overall treatment response (no induction treatment failures, no deaths) and authors suggested a natural division at this point. In another study by *Look et.al*[28], the ploidy of blast cells was assessed by the

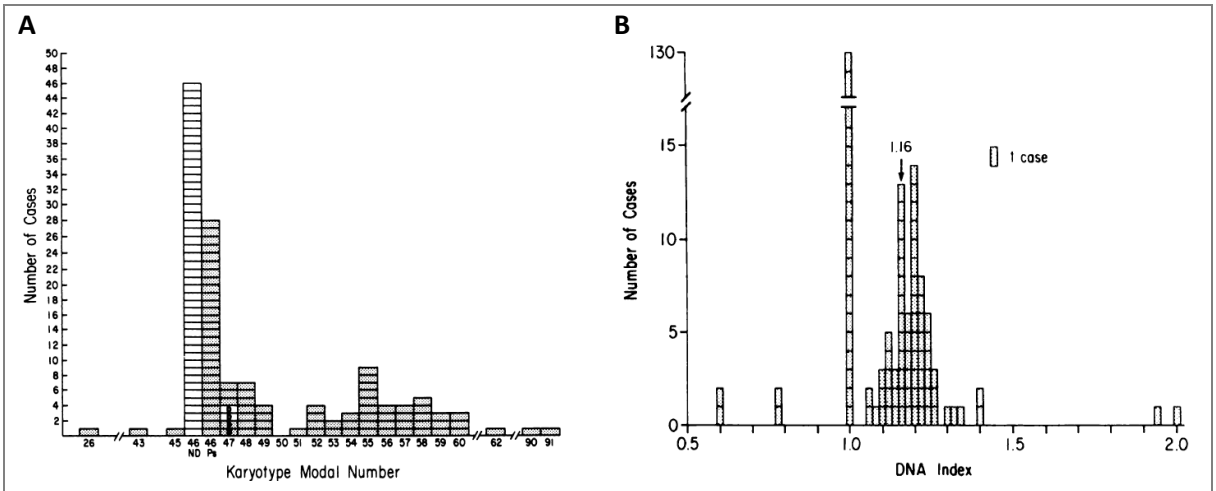

Figure 4. Earliest definitions of high hyperdiploidy in acute lymphoblastic leukemia. The cytogenetic subgroup of high hyperdiploidy in pediatric acute lymphoblastic leukemia was defined by a modal number of >50 chromosomes (A, from Williams *et.al* [27]) or a DNA-Index of ≥1.16 (B, from Look *et.al* [28]). (A) The modal chromosome number of leukemic cells from 136 patients with acute lymphoblastic leukemia showed a bimodal distribution with a hiatus at 50 chromosomes. When separating the hyperdiploid group at this point, patients with >50 chromosomes showed most favorable clinical features and the best treatment response. (B) The cellular DNA content was assessed by flow cytometry in leukemic cells from 205 children with newly diagnosed acute lymphoblastic leukemia. The DNA-Index (DI) of 1 corresponds to normal diploid cells. The authors observed the lowest rate of treatment failure in the group of hyperdiploid patients with DI≥1.16.

DNA index (DI), i.e. by flow cytometric analysis of the blast cell DNA content. They identified the most favorable prognostic group of patients at DI≥1.16 (Figure 4B), which corresponds to approximately ≥53 chromosomes.[28,35]

Despite their generally good prognosis, still about 20-30% of high hyperdiploidy cases suffered a relapse.[22,36-38] Therefore, several study groups aimed at refining the high hyperdiploidy definitions and suggested an even higher modal chromosome number (MCN) and/or a gain of specific chromosomes to confer a survival advantage. Jackson *et.al*[39] (table 2, POG) observed an improved EFS in hyperdiploid patients with an extra chromosome 6, which they found to be correlated with >51 chromosomes. Harris *et.al*[40] (table 2, POG) saw that patients with a combined trisomy of chromosome 4 and 10 had a superior EFS of 95% - even if the patients had an unfavorable DI of <1.16. In the study by Heerema *et.al*[38] (table 2, CCG), a trisomy of chromosome 5 led to a worse outcome while trisomy of either chromosome 10, 17 or 18 or a simultaneous gain of chromosomes 10 and 17 were associated with improved outcome. In multivariate analysis, only trisomy of chromosome 10 remained an independent predictor for superior outcome. In a meta-analysis of the data from the latter two study groups, the best prognosis was observed if the leukemic cells had a simultaneous gain of +4/+10/+17 (triple trisomy)[41] (table 2, COG). Moorman *et.al*[22,23] (table 2, MRC-UK ALL) could not confirm the results on triple trisomy +4/+10/+17, nor did they find a worse prognosis for patients with +5. Instead, in two successive UKMRC ALL studies[22,23], a trisomy of chromosome 18 had the most significant effect on the outcome with increased pEFS, pOS and lower risk of relapse. Additionally, authors found a survival advantage for patients with MCNs of 54-65 and stated that this prognostic impact was influenced by the effects of trisomies +4, +10 and +18, as these were more common in high MCN karyotypes. In a study by Paulsson *et.al*[34] (table 2, NOPHO), multiple cytogenetic features were associated with higher pEFS such as individual gains of chromosomes 4, 6, 17, 18, or 22, presence of triple trisomy +4/+10/+17 or an MCN>55, but none of these features also correlated with an improved pOS. In a study by Dastugue *et.al*[35] (table 2, EORTC-CLG), presence of trisomies +4, +18 as well as several combinations of +4, +10, +17, +18 had a favorable prognostic impact. Nonetheless, the best indicator for excellent outcome in their study was the ploidy by modal chromosome number and they observed the best prognosis for patients with 58-66 chromosomes or a DI≥1.24. In summary, the published data on how to identify high hyperdiploidy and prognostic subgroups therein is complex and diverse between different studies. This suggests a strong influence by the study setting, i.e. different methodological strategies, the composition of the study cohorts and differences in treatment protocols.

Table 2. Studies on high hyperdiploidy in patients with initial diagnosis of B-cell precursor ALL from different international study groups.

Study group	Definition of high hyperdiploidy	Number of patients	Subgroup with improved survival (pEFS) within high hyperdiploidy	Other findings; remarks	Reference
POG	MCN >46	239	Trisomy +6 or MCN >51	Strong association between +6 and MCN >51	*Jackson et.al, 1990*[39]
POG	Abnormal karyotype	1021	Combined trisomy +4/+10	+4/+10 was associated with superior pEFS even if the DI was lower than 1.16	*Harris et.al, 1992*[40]
CCG	MCN 51-68	480	MCN 54-58 or individual trisomies +10, +17 or +18 or combined trisomy +10/+17	strongest predictor for improved outcome: +10 worse outcome: trisomy +5	*Heerema et.al, 2000*[38]
COG (meta-analysis)	MCN >50	4985	Combined trisomy +4/+10/+17 (triple trisomy) in standard-risk patients		*Sutcliffe et.al, 2005*[41]
MRC UKALL	MCN 51-65	700	Individual trisomies +4, +10 or +18 or MCN 54-65	Trisomies 4, 10 and 18 were enriched in karyotypes with MCN 54-65. Only trisomies 4 and 18 remained independent indicators for improved survival in a multivariate analysis.	*Moorman et.al, 2003*[22]
MRC UKALL	MCN 51-65	562	Trisomy +18	No different outcome of patients with or without triple trisomy +4/+10/+17.	*Moorman et.al, 2010*[23]
NOPHO	MCN 51-67	688	Trisomies +4, +6, +17, +18, or +22 or triple trisomy +4/+10/+17 or MNC >55	None of the factors also conferred a better pOS. Triple trisomies highly correlated with MCN>55.	*Paulsson et.al, 2013*[34]
EORTC-CLG	MCN >50	541	Trisomies +4 or +18 or several combinations of +4, +10, +17, +18 or MCN≥58 or DI≥1.24	Strongest factor for excellent outcome: MCN 58-66.	*Dastugue et.al, 2013*[35]

Study groups: POG, Pediatric Oncology Group; CCG, Children's Cancer Study Group; COG, Children's Oncology Group (merge of POG, CCG and others, since 2000) (North America, Australia, New Zealand); UKMRC ALL, Medical Research Counsil Working Party on Childhood Leukaemia (United Kingdom); NOPHO, Nordic Society of Peadiatric Heamatology and Oncology (Sweden, Denmark, Norway, Finland, Island, others); EORTC-CLG, European Organisation for Research and Treatment of Cancer – Children's Leukemia Group (France, Belgium, Portugal). Abbreviations: DI, DNA index; MCN, modal chromosome number

Hypodiploidy (<46 chromosomes) in B-cell precursor ALL

Hypodiploidy is defined by the ISCN as a karyotype with less than 46 chromosomes. In ALL, hypodiploidy is a rare cytogenetic subtype occurring in less than 7% of patients.[42-45] The majority (>80%) of all hypodiploid cases are near-diploid (45 chromosomes) and survival of these patients is intermediate with an EFS of 65-75%.[43,44] In contrast to this, the remaining small proportion of patients with more severe hypodiploidy (<45 chromosomes) has an overall incidence in ALL of approximately 1.2% and an extremely poor outcome with EFS rates of merely 30%.[43-47] In the treatment of newly diagnosed ALL, hypodiploidy is used as high-risk marker.[48,49] Within this group of <45 chromosomes, two major subtypes have been recognized (i) by their characteristic chromosomal patterns,[45,50] and (ii) by distinct sub-microscopic lesions and transcription profiles[51]. In near-haploid cases (24-30 chromosomes), the majority of chromosomes are monosomic, however, chromosomes 21, 14, X, 18, 8 and 10 are frequently retained disomic.[43,45-47,50,51] Furthermore, about 70% of near-haploid cases harbor genetic alterations in genes of the Ras- and receptor tyrosine kinase signaling pathways (i.e. in *NF1*, *NRAS*, *KRAS*, *MAPK1*, *FLT3*, and *PTPN11*).[51] In the second major hypodiploidy subgroup, i.e. low-hypodiploidy (31-39 chromosomes), retention of chromosomes 1, 5, 6, 8, 10, 11, 14, 18, 19, 21, 22, and X/Y is common[45,50,51] and alterations of *TP53*, *IKZF2* and *RB1* were observed in 91%, 53% and 41% of cases.[51] In both, near haploidy and low hypodiploidy, doubling of the hypodiploid clone was reported in up to 60% and 90% of cases, respectively.[43-47,50,51]

Scope of the thesis

Treatment of ALL relapse is generally more intense than the treatment at initial diagnosis. Still, a significant number of relapse patients suffer a second event and long-term outcome of patients after relapsed ALL remains unsatisfactory low.[15] In the most recent protocol for treatment of relapsed ALL, ALL-REZ BFM 2002, clinical factors at relapse diagnosis and the molecular response after induction treatment were used to guide treatment intensity. The identification of additional molecular markers may improve risk-adapted treatment stratification. Such prognostic factors may be provided by recurrent genetic alterations that have been identified and described for initial ALL. While, for instance, hypodiploidy is used as high-risk factor in the treatment of newly diagnosed ALL,[48,49] the clinical relevance at relapsed disease is unknown. And, although patients with high hyperdiploid ALL have a generally favorable prognosis, the high prevalence of this cytogenetic subgroup, means that a substantial number of high hyperdiploid leukemias will relapse.[52-56] Yet, there are only few reports on clinical associations of aneuploidy in relapsed ALL,[55,57] and patient cohorts are either relatively small (n≤100 patients) and/or the patients did not receive a uniform relapse treatment.[53,55,56] We therefore aimed to investigate the clinical importance of aneuploidy in relapsed BCP-ALL in a large cohort of patients all treated according to the ALL-REZ BFM 2002 protocol.

Aneuploidy is commonly detected by karyotyping. However, karyotyping frequently fails for ALL cells due to low cell viability in culture and poor banding resolution.[58] In our study cohort, informative karyotyping data were obtained in only 18% of patients. Ploidy was therefore determined prospectively by the DNA index within the ALL-REZ BFM 2002 trial and data were available for approximately 60% of the patients. For a more in-depth analysis of aneuploidy, a copy number screening using multiplex-ligation dependent probe-amplification (MLPA) was established and used to determine changes in chromosome copy number. Both, MLPA and DI data were then used to investigate the clinical importance of aneuploidy in first bone marrow relapses of BCP-ALL.

Methods

Patients and samples

Patients included in this study were diagnosed with first isolated or combined bone marrow relapse of pediatric BCP-ALL between 2002 and 2012 in Germany. Isolated extramedullary relapses were not considered due to scarce availability of extramedullary specimen. All patients were treated according to the protocol of the German multi-center trial ALL-REZ BFM 2002 clinical trial (ClinicalTrials.gov identifier NCT00114348) approved by the local ethics committee and written informed consent was obtained from patients and/or guardians. Patients were considered for the present study if the following criteria were met (n=413): availability of high-quality DNA from relapse diagnosis, sufficient amount of leukemic blasts at relapse diagnosis (see below "Establishing MLPA P181") and successful MLPA analysis of the DNA sample from relapse diagnosis. Patients who were excluded from the present study (n=139) showed no selection bias for time-point and site of relapse, gender and event-free survival after relapse (table 3). The median follow-up time of patients in complete continuous remission after first relapse was 9.4 years (range 1.4-15.3). DNA from bone marrow or peripheral blood samples at relapse diagnosis was prepared after enrichment for mononuclear cells by Ficoll density gradient centrifugation as previously described.[59]

Table 3. Distribution of clinical/outcome parameters between all patients with marrow relapse enrolled in the ALL-REZ BFM 2002 trial (n=552) and patients excluded from this study (n=139).

	ALL-REZ BFM 2002		Patients excluded		
Parameter	**n**	**%**	**N**	**%**	**P**
	552	100	139	100	
Time-point of relapse					0.136
Very early	98	17.8	30	21.6	
Early	136	24.6	42	30.2	
late	318	57.6	67	48.2	
Site of relapse					0.832
Isolated BM	428	77.5	109	78.4	
Combined BM	124	22.5	30	21.6	
Sex					0.534
Males	314	56.9	75	78.3	
Female	238	43.1	64	60.7	
10-year event-free survival ± standard error					0.529
	48.2% ± 2.2%		45.2% ± 4.4%		

Methods to determine aneuploidy / chromosome copy number

DNA index (DI)

The DI is the ratio of the cellular DNA content from a sample to be determined to the cellular DNA content of normal cells from healthy donors. A DI=1 defines diploidy, while DI<1 or DI>1 describe hypo-

and hyperdiploidy, respectively. The DI was determined prospectively in the framework of the ALL-REZ BFM 2002 trial for 309 relapse samples. Briefly, cells were fixed in ice-cold 70% ethanol, stored at 4 °C, then washed and re-suspended in a volume of 200 µl of phosphate buffered saline with 22.5 µl of 1.0 mg/ml propidium iodide (Sigma-Aldrich, München, Germany) and 10µl of 10 mg/ml RNase A solution (Sigma-Aldrich). The DI was assessed by a BD FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) in accordance with consensus guidelines[60] and data were analyzed with ModFit LT 2.0 software (Verity Software House, Topsham, ME, USA). Re-analysis of selected DI data were done using Kaluza Analysis Software v1.3/v2.1 (Beckman Coulter Life Sciences., Indianapolis, IN, USA).

Karyotyping

Karyotyping is a cytogenetic method to determine the number and structural appearance of chromosomes in a cell. The leukemia samples from patients enrolled in the ALL-REZ BFM 2002 trial were processed for karyotyping in the Department of Pathology, University Gießen, Gießen, Germany and in the Labor Berlin, Charité Vivantes GmbH, Berlin, Germany. In brief, bone marrow and/or peripheral blood cells of patients were cultivated unstimulated for 24 and/or 48 h and chromosome banding analysis was performed according to standard procedures. Informative karyotypes were available for 76/413 patients of our study.

Fluorescence in-situ hybridization (FISH)

Interphase FISH uses fluorescently labelled DNA probes to detect aberrant copy numbers of specific chromosomes (centromere FISH probes) or genes (locus-specific FISH probes). Locus-specific FISH probes can be further used to determine genomic translocations. Interphase FISH was performed as described previously[61] for selected patients using Vysis copy number enumeration and locus-specific identifier probes (Abbott Molecular, Abbott Park, IL, USA). FISH data were used to validate MLPA results as described below.

Establishing the MLPA P181 assay to determine the copy number status of leukemic cells

MLPA is a PCR-based method for simultaneous quantification of up to 50 different DNA sequences. In this study, the P181 probe mix (MRC Holland, Amsterdam, The Netherlands) was used to detect centromeric copy number alterations in leukemic samples. Except for chromosome Y, each chromosome is targeted by two MLPA probes that detect regions close to the centromere. Nine additional probes enable the determination of DNA quantity and XY-status of the sample as well as the assessment of MLPA assay quality. Each target-specific MLPA probe consists of two individual oligonucleotides that hybridize immediately adjacent to each other to the DNA sequence of interest

(Figure 5A). The long oligonucleotide further contains an unspecific, non-hybridizing stuffer sequence that is variable in length. Universal PCR primer binding sequences at the end of each oligo pair allow for multiplex amplification. The forward primer is fluorescently labelled (6-FAM), which enables the

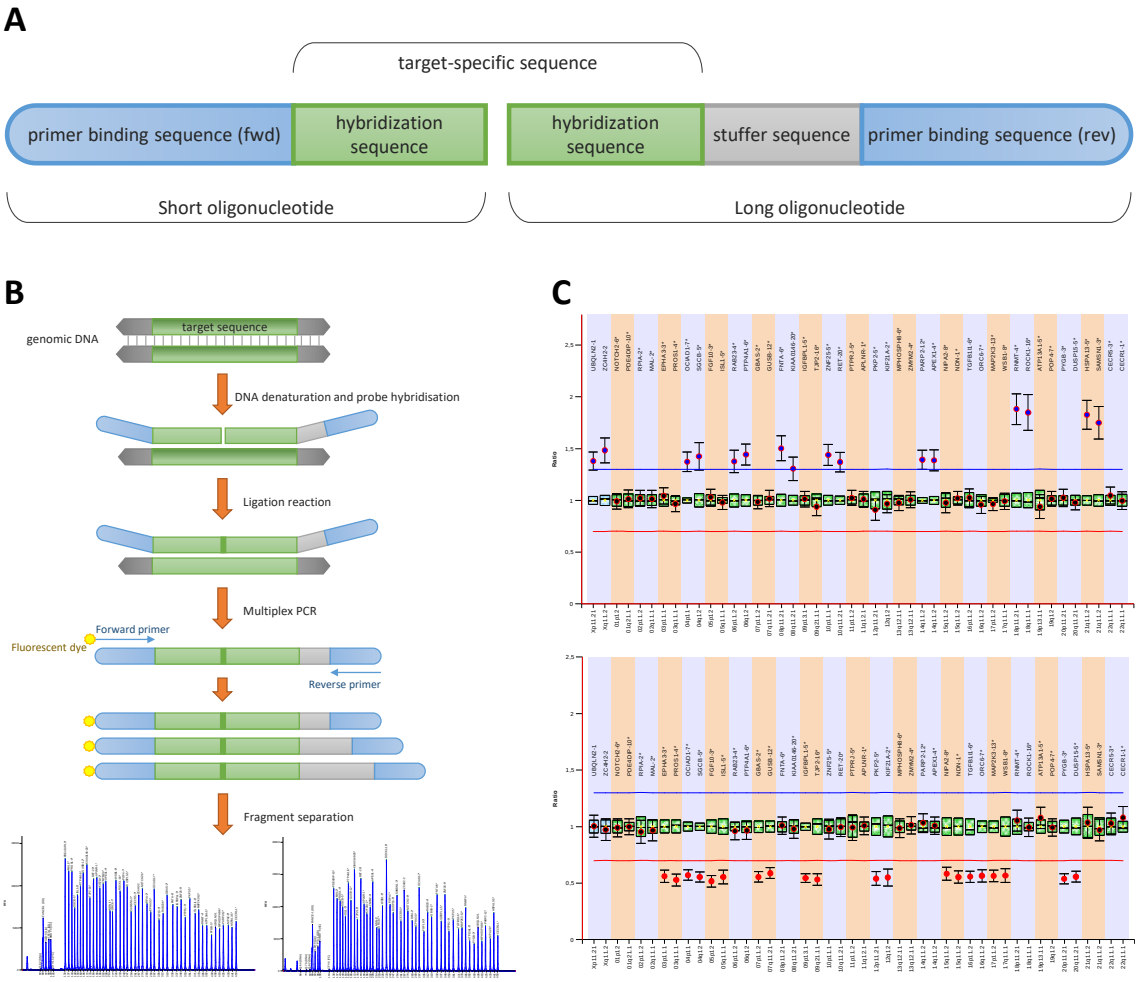

Figure 5. General MLPA probe design, procedure and data analysis. (A) General design of an MLPA oligo probe pair. (B) MLPA experimental procedure comprising denaturation/hybridization, ligation, multiplex PCR and fragment separation. Two representative electropherograms (X-axis: fragment size [number of nucleotides]; Y-axis: relative fluorescence units) of a reference sample (left) and a patient sample (right) are shown. (C) Representative MLPA P181 ratio bar charts after data normalization using Coffalyser.NET software (X-axis: probe location; Y-axis: MLPA probe ratio; green bars: variation within healthy reference samples; circles: MLPA ratios of the patient sample). The upper panel shows a hyperdiploid relapse with gains of chromosomes X, 4, 6, 8, 10, 14, 18, and 21. The lower panel shows a hypodiploid relapse with monosomies of chromosomes 3, 4, 5, 7, 9, 12, 15, 16, 17, and 20. Figures A and B were adapted from MRC Holland, Amsterdam, The Netherlands.

detection of amplification products by capillary electrophoresis using an ABI 3730 DNA Analyzer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). A standard MLPA procedure for copy number analysis is performed in five steps (Figure 5B): (i) overnight hybridization of oligo probes to heat-denaturized DNA at 60°C, (ii) enzymatic ligation of DNA-bound oligo probes, (iii) amplification of ligation products, (iv) detection of amplicons by capillary electrophoresis, and (v) MLPA data analysis. As normal references, six DNA samples from mononuclear cells of healthy blood

donors or of bone marrow samples from patients with isolated extramedullary ALL relapses were used in each experiment. MLPA data analysis (i.e. DNA fragment analysis, peak sizing, and intra- and inter-sample normalization) was performed using Coffalyser.NET software (MRC Holland). An MLPA probe ratio (leukemic *vs* healthy sample) of 1.0 is considered a normal/diploid copy number and ratios of ≤0.7 or ≥1.3 indicate a heterozygous deletion (monosomy) or gain (trisomy). Ratios ≤0.5 or ≥1.5 indicate a homozygous deletion or a multiple gain (tetrasomy and higher), respectively. The sensitivity of the MLPA P181 kit was determined by serial dilutions of DNA from the leukemia cell line MHH-cALL2 with DNA from a blood sample of a healthy donor. The karyotype of this cell line has been described as 51,XX,+X,+18,+der(18)t(15;18)(q13.1;q22.1),+21,+21,[62] and MLPA P181 confirmed trisomy of chromosome X and tetrasomies of chromosome 18 and 21. Serial dilution experiments showed that 40% of abnormal DNA is required to detect an aneuploidy by MLPA. From 34 patients, MLPA P181 data were compared with 92 available FISH analyses with centromere enumeration probes. A theoretical DI was calculated from whole chromosome changes as detected by MLPA using the formulas developed by Rachierou-Sourisseau *et.al*[63]. The MLPA-based theoretical DI was then compared to the flow-cytometrically measured DI for n=305 patients by linear regression and Bland-Altman analysis.

LOH analysis by CytoScan HD SNP array

Genome-wide SNP array analyses were performed for selected samples using the CytoScan™ HD array (Affymetrix Santa Clara, CA, USA). Samples were processed in the Department of Human Genetics, Universitätsklinikum Erlangen, Erlangen, Germany. Data were then analyzed using the Chromosome Analysis Suite 3.1.0.15 software (Affymetrix Santa Clara, CA, USA) with default filter settings to determine chromosomal loss of heterozygosity (LOH).

Detection of additional genetic alterations

The following additional genetic information was available from previous studies: mutations of *TP53*[64,65], mutations of *NRAS*, *KRAS*, *FLT3*, *PTPN11*[65,66] and *CREBBP*[65], focal deletions of *EBF1*, *IKFZ1*, *CDKN2A/B*, *PAX5*, *ETV6*, *BTG1*, *RB1*, PAR1 region and recurrent fusion genes *ETV6-RUNX1*, *BCR-ABL1*, *KMT2A-AFF1*[59]. Furthermore, presence of *KMT2A-MLLT1* fusion gene was detected by reverse transcriptase PCR during the ALL-REZ BFM 2002 trial. For a subset of patients, data on *KMT2A* rearrangement were available from nuclear FISH using a *KMT2A* break apart probe (n=28) and/or from karyotyping (n=76) during the ALL-REZ BFM 2002 trial (data from Department of Pathology, University Gießen, Gießen, Germany and Labor Berlin, Charité Vivantes GmbH, Berlin, Germany).

Statistical analysis

Equality of categorical variables was analyzed by Pearson's Chi-square or Fisher's exact test and for continuous variables by Mann-Whitney U or Kruskal-Wallis test. Kaplan-Meier analyses were used to estimate probabilities of event-free (pEFS) and overall survival (pOS). Differences were compared with log-rank test. EFS time was defined as the time from relapse diagnosis to the date of a subsequent event (second relapse, secondary malignancy or death in complete remission) or, for patients in complete continuous remission, to the date of analysis. EFS time was set to zero in case of death during induction treatment or non-response to chemotherapy. OS time was defined as time from relapse diagnosis to the date of death or, for surviving patients to the date of analysis. Multivariate Cox regression modelling for EFS and OS was done using backward stepwise selection to remove non-significant factors and differences were assessed by the Wald test. Statistical calculations were performed using IBM SPSS Statistic 22.0 software for Windows (SPSS Inc., Chicago, IL, USA). Unsupervised cluster analysis of MLPA P181 was performed in cooperation with Dr. rer. nat. Michael P. Schröder (Department of Hematology/Oncology, Charité Universitätsmedizin Berlin). The copy number matrix comprising P181 probe information as categorical variable was converted into a dummy matrix of binary measurements. Thus, each MLPA probe column resulted in three binary columns, one for 'loss', one for 'diploid/normal', and one for 'gain' in which '1' codes for true and '0' for false. From the dummified MLPA data, a cosine similarity matrix (1-cosine distance matrix) was obtained, which was then used to calculate a complete linkage using Python (Anaconda Inc., Austin, TX, USA). The resulting hierarchical clustering was divided into clusters at a distance threshold of 0.38.

Essential findings

If not indicated otherwise, all references to figures and tables in this paragraph refer to those in the selected publication Groeneveld-Krentz et.al 2019 **Aneuploidy in children with relapsed B-cell precursor acute lymphoblastic leukaemia: clinical importance of detecting a hypodiploid origin of relapse**.

The MLPA method is suitable to detect chromosomal changes in leukemic cells of relapsed BCP-ALL

MLPA P181 was performed to detect chromosome copy number changes in leukemic samples from patients with first relapse of BCP-ALL. The copy number data obtained from MLPA P181 were systematically compared to available ploidy data from FISH analyses and DI measurements as follows.

For 34 patients, FISH analyses with centromere enumeration probes (CEP) were available (n=92) (Supplementary Methods and Supplementary Table SI). MLPA P181 and CEP-FISH were 100% concordant in all cases with no more than one major aneuploidy clone (n=72) (Supplementary Table

SI). Nine other cases were multi-clonal by CEP-FISH, i.e. had two or more aneuploid cell populations with tri-, tetra- and/or pentasomies of the same chromosome. In all nine cases, MLPA correctly detected the chromosome status as 'gain'. However, as the MLPA method measures bulk DNA of a sample, a precise copy number of the gained chromosome could not be deduced from MLPA data in these cases (Supplementary Table SI). Relapsed ALL is frequently multi-clonal and therefore we chose to define the status of each MLPA probe as categorical variable (i.e. as diploid/normal, loss, gain) instead of a definite copy number value. Discrepancies between MLPA and FISH were found in 11/92 comparison points (12%). Four of these were subclonal chromosome gains below the MLPA P181 detection limit of 40%. In the remaining seven cases, MLPA identified alterations of the 17p arm that were undetected by CEP-FISH. In all of these seven cases, additional FISH analyses using an LSI p53 probe confirmed the MLPA P181 result. In summary, FISH and MLPA were 100% concordant in 72/92 comparisons (78%) and the identified discrepancies were due to the presence of multiple aneuploidy clones, subclonality <40% or subchromosomal, centromere-near copy number changes.

Based on whole chromosome gains and losses detected by MLPA data, a theoretical DI was calculated[63] and correlated to the flow cytometric DI for 305 patients. Between both measures we observed a high correlation (R^2=0.898) and agreement (median difference=0.00; p=0.474) (Supplementary Fig S1). Discrepant cases were revised and could be attributed to methodical limitations of either method. First, MLPA was able to detect copy number changes of single chromosomes while the DI method requires a certain amount of excess/lost DNA to detect an aneuploidy[63] and may therefore miss single copy number changes of smaller chromosomes such as a single gain of chromosome 21. Hence, in the near-diploid range of aneuploidy, MLPA was more sensitive than the flow cytometric DI (Supplementary Fig S1). Second, the theoretical MLPA-based DI was consistently lower than the DI measured by flow cytometry. This, however, was a result of our definition of an MLPA "gain" irrespective of whether the gain was due to a tri/tetra or pentasomy. Therefore, in cases with several tetrasomies, the theoretical MLPA-based DI underestimates the actual DI measured by flow cytometry. This effect becomes most pronounced in the upper range of aneuploidy where tetrasomies occur more frequently.

Combined approach of DNA Index and clustering of MLPA P181 data to identify distinct aneuploidy groups in relapsed BCP-ALL

MLPA P181 analysis was successfully performed in leukemic samples from 413 patients (Fig 1A). Of these, 122 relapses (29%) were diploid, i.e. MLPA P181 detected no copy number alterations and the DI was 1.0 for 83 patients with available data. Hypodiploidy was observed in eight relapses by MLPA and/or DI (Fig 1A) and could be confirmed by FISH or karyotyping data available for five cases (Table

IA). Of note, in four of the eight hypodiploidy cases, MLPA failed to determine the correct normal-diploid level due to the massive chromosome losses. Here, DI and FISH were essential to guide MLPA interpretation and confirm hypodiploidy (Table IA, cases 347, 385, 463, 581). Two hypodiploid cases showed near-haploidy and six were low hypodiploid. All six patients with low hypodiploidy harbored *TP53* mutations (Table I). By DI, half of the relapse cases with hypodiploidy harbored a second hyperdiploid cell population with approximately twice the DNA content of the hypodiploid clone (Table IA).

The remaining 283 patients had a variable number of MLPA P181 copy number alterations and the DI ranged widely from 1.0 to 1.44 (Supplementary Fig S2A). The established DI>=1.16 cut-off, which is commonly used to identify high hyperdiploidy by DI, was not able to discriminate a prognostically favorable group in our relapse cohort as the pEFS of patients with low hyperdiploid relapses (DI>1.0<1.16) was similar to patients with high hyperdiploid relapses (DI≥1.16) (p=0.551) (Supplementary Fig S2B). Therefore, we aimed to define high hyperdiploidy by patterns of MLPA gains and losses. Based on the observations that chromosome gains in high hyperdiploidy occur in a non-random fashion and display distinct patterns depending on the modal chromosome number[67], we used unsupervised clustering of MLPA data to identify hyperdiploidy subgroups in the group of 283 patients.

Identifying hyperdiploidy subgroups by MLPA P181/clustering

Cluster analysis of MLPA data identified six clusters with distinct patterns of chromosome gains (one 'minor aneuploidy' cluster, five hyperdiploidy clusters termed 'HD1' - 'HD5'). The cluster with minor aneuploidy (n=166) was genetically and clinically most similar to diploid cases (Fig 2B, 2C; table II and Fig 4). Interestingly, a small cluster of seven patients with a core pattern of +21+5+16 was associated with *ETV6-RUNX1* (Figs 2B, 2C; cluster 'HD5'). Patients had favorable clinical characteristics such as late first relapses and all patients achieved complete remission (Table II). The pEFS for these patients was similar to *ETV6-RUNX1* positive relapses without hyperdiploidy (Supplementary Fig S4).

Low hyperdiploidy and high hyperdiploidy were recognized in clusters HD1 (n=42) and HD2/HD3 (n=64), respectively. Patients with low hyperdiploidy (HD1) had a median of four chromosome gains with a core pattern of +21+X+14 and, except for one patient with DI=1.16, the DI ranged between 1.0 and <1.16. The chromosome gains of the two high hyperdiploidy clusters (HD2/HD3) largely involved the chromosomes of the classical pattern +4+6+8+10+14+17+18+21+X. However, subclusters HD2 (termed 'High hyperdiploidy, classical subtype') and HD3 (termed 'High hyperdiploidy, extended subtype') slightly differed in their number of chromosome gains (median 8 vs 10 in HD2 vs HD3). In particular, chromosome 5 was gained more often in relapses from HD3 compared to HD2 (Fig 2B). The

distribution of additional genetic alterations was similar across relapses from the low hyperdiploidy (HD1) and the two high hyperdiploidy clusters (HD2/HD3): recurrent fusion genes and leukemia-associated deletions of *IKZF1, CDKN2A/2B, PAX5, ETV6* and *BTG1* were rare, while mutations of genes from the RAS pathway were enriched (Fig 2C). In contrast to these similarities, the clinical characteristics and outcome were highly different between low and high hyperdiploid relapses. Relapse cases with high hyperdiploidy had a longer time to first relapse, excellent remission rates after relapse treatment and favorable pEFS rates of about 70% (Table II and Fig 4B). In contrast, low hyperdiploid relapse was associated with a shorter time to first relapse, a lower rate of second remission and a worse pEFS of 40% (Table II and 4B). In multivariate analysis, high hyperdiploidy (combined clusters HD2 and HD3) was an independent predictor for superior pEFS (Table IV).

Identifying masked hypodiploidy in patients with relapsed B-cell precursor ALL

One of the hyperdiploidy cluster, i.e. cluster 'HD4' comprised only three cases characterized by the highest number of chromosome gains in this study (median 11) and a pattern that was distinct to the other two high hyperdiploid clusters HD2 and HD3 (Fig 2A and 2B). Most of the classical chromosomes were involved, however, gains of chromosomes 4 and 17 were absent or less frequent and, instead, non-classical gains of chromosomes 5, 11, 19, and 22 were present in all three cases (Fig 2B). Furthermore, all patients harbored a *TP53* mutation (Fig 2C). Due to the unusual chromosome pattern and the presence of *TP53* mutations we suspected that these relapses may have descended from a hypodiploid leukemia. Subsequent CytoScan HD array analysis of these cases showed copy-neutral LOH in nearly all disomic chromosomes, thus confirming the hypodiploid origin. As no hypodiploid founder clone could be detected in any of the three cases by DI or FISH (Table 1B), this cluster was termed

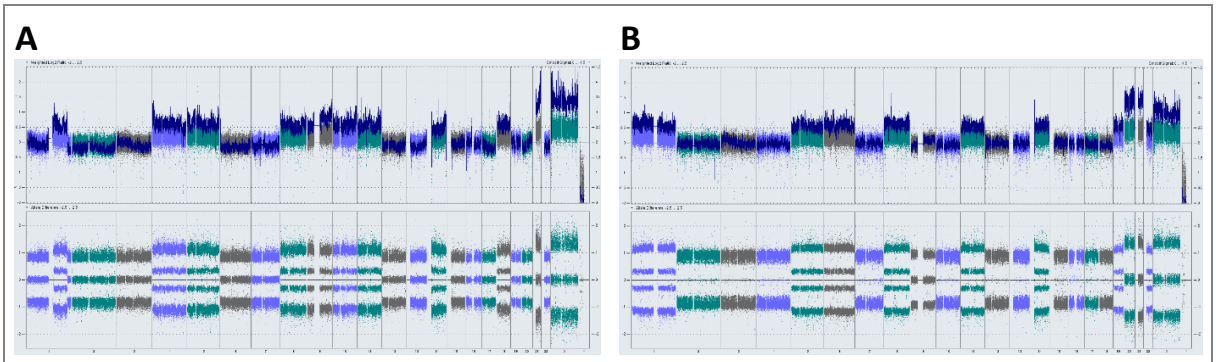

Figure 6. Whole genome view of CytoScan HD array analyses from two B-cell precursor ALL relapse cases. Genome-wide SNP array analyses (CytoScan HD array, Affymetrix) were performed from DNA of leukemic cells at relapse diagnosis. Chromosomes 1-22, X, and Y are displayed from the left to the right in alternating colors. The copy number of chromosomes is shown in the upper panel (log2 ratio), the allelotype in the lower panel (allele peaks). (A) High hyperdiploid relapse case with trisomies of chromosomes 1q, 4, 5, 8, 9, 10, 11, 14, 18 and tetrasomies of 21 and X (upper row). All disomic chromosomes are heterozygous (shown by an allele peak pattern of three tracks) (lower row). (B) Relapse case showing trisomies of chromosomes 1, 5, 6, 8, 11, 14, 19, 22 and tetrasomies of chromosomes 20, 21, X. Except for chromosome 9, all other disomic chromosomes show chromosome-wide copy-neutral LOH (allele peak pattern with only two tracks). This hyperdiploid case most probably descended from a hypodiploid clone that underwent chromosome doubling and subsequently lost the hypodiploid founder clone (masked hypodiploidy).

'masked hypodiploidy'. Figure 6 above shows representative CytoScan HD array analyses of two cases with typical high hyperdiploidy and masked hypodiploidy.

Clinical characteristics of evident and masked hypodiploidy in relapsed B-cell precursor ALL

Taken together, 11 hypodiploid relapse cases were identified: eight patients with evident hypodiploidy by MLPA/DI and three cases with masked hypodiploidy. When compared to the other aneuploidy clusters, hypodiploid relapses were associated with unfavorable clinical characteristics (Table II). Patients had a shorter time to first relapse (median 1.9 years), were more frequently allocated to the high-risk treatment arm and showed poor second remission rates (Table II). Interestingly, those patients with masked hypodiploidy were not identified as patients with high-risk of treatment failure by current risk-stratification due to their rather favorable clinical presentation (Table IB). Overall, nine of 11 patients with hypodiploid relapses suffered a second event (Table I and II) resulting in a very poor outcome of only 9% pEFS (Fig 4A). In multivariate analysis, hypodiploidy remained an independent prognostic marker for worse pEFS and pOS after first relapse (Table IV).

Discussion and clinical consequences

We investigated aneuploidy in 413 pediatric patients with relapsed BCP-ALL representing almost 75% of all patients with a first bone marrow relapse, who were treated by the German ALL-REZ BFM 2002 relapse protocol within a period of about 10 years (2002-2012). This dataset represents one of the largest studies worldwide on aneuploidies in relapsed BCP-ALL.

Failed karyotyping is a major drawback when studying aneuploidy in relapsed leukemia. Therefore, we used an approach that combined ploidy analysis by DNA indexing with MLPA centromere screening to assess chromosomal patterns of aneuploidy. Compared to conventional cytogenetic methods, the MLPA P181 method is a fast, simple and cost-effective method to determine ploidy changes. In contrast to FISH, multiplexing by MLPA allows the assessment of all chromosomes simultaneously and, unlike to karyotyping, it is not dependent on fresh samples and good quality metaphases. Furthermore, even if successful, karyotyping can miss high hyperdiploidy due to clonal selection during metaphase cell preparation.[68] Our validation experiments showed that the copy number status of chromosomes by MLPA was highly concordant with FISH data. Furthermore, the total ploidy change by MLPA agreed well with flow cytometric DI data, which was also observed in another study[69]. However, MLPA P181 showed a relatively high detection limit of 40% in our study and analysis can be challenging in cases with massive chromosomal aberrations where MLPA may not identify the correct normal-diploid level, or in the presence of multiple aneuploidy clones. In these cases, additional DI data and/or FISH analyses of selected chromosomes are vital to guide MLPA data interpretation. Taken together, the

combination of both DI and MLPA can be easily applied in the clinical routine as complimentary methods to detect chromosome changes, especially when karyotyping is not informative or, for example, in laboratories with limited resources.

As described in the introductory paragraph (including Table 2 from introduction), the definitions of high hyperdiploidy or specific prognostic subgroups therein were inconsistent across different clinical studies. Various thresholds for modal chromosome numbers, DI values and diverse specific trisomies (and combinations thereof) have been used to define high hyperdiploidy for identification of the most favourable prognostic group within hyperdiploid ALL. Nevertheless, the studies do not completely disagree and some key features appeared multiple times as marker for excellent outcome within the cytogenetically defined high hyperdiploidy group, such as gains of chromosomes 4, 10, 17, and 18 [22,23,34,35,38,40,41]. Furthermore, three of these studies[22,34,35] pointed out that specific trisomies and a higher MCN and/or DI might identify the same group of patients as both features were strongly associated with each other. This observation was further substantiated by a cluster analysis of 2339 karyotypes with high hyperdiploidy[67], where the authors showed a predictive pattern of extra chromosomes to occur with increasing MCN. Here, extra chromosomes of 21, X, 14, 6, 18, 17, 10 (group I) were present already at low MCN (≤54) and retained their high frequency throughout all MCN levels. Gains of chromosomes 8, 5, 12, 11 (group II) occurred most frequently from MCN>57 onwards, and extra copies of chromosomes 2, 3, 9, 16, 22 (group III) or 1, 7, 13, 15, 19, 20 (group IV) appeared largely at very high MCN or in even near-triploid cases only.[67] In our cohort of relapsed BCP-ALL, cluster analysis identified a similar chromosome pattern of high hyperdiploidy at relapse (clusters HD2/HD3). A core pattern of 21, X, 14, 6, 18, 17, 10 was present in both clusters HD2 and HD3, while gains of non-classical chromosomes, such as 8, 5 or 11, were more frequent in cluster HD3, that was also characterized by a slightly higher number of total gains. Overall, 16% of patients had high hyperdiploid relapses by MLPA P181/cluster analysis. This lower frequency of high hyperdiploidy at relapse compared to initial diagnosis of ALL reflects the generally favorable outcome of this patient group after primary treatment. At relapse diagnosis, patients with high hyperdiploidy present with favorable clinical features (late relapses, high remission rates) and achieve a remarkably high EFS rate of approximately 70% after second-line treatment. In contrast, patients with low hyperdiploid relapses achieved only 40% pEFS. Given the increased mutation rate of RAS pathway genes in this group, patients with low hyperdiploid relapses might be eligible for alternative treatment approaches using MEK inhibitors.[66]

With a pEFS of merely 9%, the group of patients with hypodiploid relapses had the worst prognosis among all ploidy subgroups in our study. The increased frequency of hypodiploidy at relapse (3%) compared to initial diagnosis (1.2%)[43,45,46] agrees well with the high rate of relapse after frontline

treatment.[20,23,44] Of note, three of 11 hypodiploid relapses in our study were not evident by FISH or DI (masked hypodiploidy) due to chromosome doubling and subsequent loss of the hypodiploid founder clone. This characteristic feature of hypodiploidy may lead to a misinterpretation of these cases as high hyperdiploidy, which is a well-known challenge at initial diagnosis of ALL. Carroll et.al[70] estimated that about 15-25% of their hypodiploid cases are missed for that reason and Safavi et.al[50] reported two masked hypodiploidy cases that were initially treated as non-risk, high hyperdiploid patients and both of them suffered a relapse. The relevance of masked hypodiploidy at relapsed disease has been scarcely investigated. Based on a few cases from two studies[44,71], it was assumed that the hypodiploid founder clone will generally dominate at relapse[72]. In contrast, we showed that predominance or exclusive presence of the doubled clone occurred in nearly half of our hypodiploid relapse cases. Hence, the risk of misdiagnosing masked hypodiploidy not only exists at initial diagnosis but also at relapsed ALL. In our study, masked low hypodiploidy was distinguished from "normal" high hyperdiploid cases by (i) a very high number of total chromosome gains, (ii) lack of the classical +4 but, instead, at gains of at least two of the unusual chromosomes 1, 11, 19, and 22. This pattern in combination with *TP53* mutation pointed to a hypodiploid origin that was eventually confirmed by LOH analysis from SNP arrays in these patients. Hence, in the clinical routine, MLPA P181 could serve as a cost-effective pre-screening method to detect potentially masked hypodiploid cases prior to verification by LOH analysis/SNP array.

Interestingly, clinical heterogeneity was observed within the hypodiploidy relapse group depending on whether the hypodiploid or the doubled-hypodiploid clone was predominant at relapse diagnosis. The predominance of the doubled-hypodiploid clone (including masked hypodiploidy) was associated with a favorable clinical presentation and, subsequently, patients were more often allocated to the intermediate-risk arm of the ALL-REZ BFM 2002 protocol. However, four of five patients suffered a second event. Although patient numbers are small, this suggest that patients may benefit from re-allocation to high-risk treatment. In turn, patients with a predominant hypodiploid clone at relapse diagnosis were already treated on the ALL-REZ BFM 2002 high-risk arm due to their poor clinical presentation (very early/early isolated bone marrow relapses) and, yet, all patients suffered a second event. These patients should be eligible for alternative treatment approaches such as immunotherapy or signal transduction inhibitors.

To summarize, high hyperdiploidy represents a favorable cytogenetic marker not only in primary but also in relapsed ALL. The majority of patients with high hyperdiploid relapses can be salvaged by the current treatment strategy. In contrast, patients with hypodiploid relapses have a very poor outcome with current therapy approaches. In multivariate analysis, hypodiploidy was an independent prognostic factor for treatment failure, supporting stratification of hypodiploid relapses into high-risk

arms in future trials or allocation to alternative therapies. With this study on aneuploidy at relapsed ALL, SNP arrays have now been implemented in the standard diagnostic procedures for relapsed ALL, which will facilitate the diagnosis of (masked) hypodiploidy. Furthermore, hypodiploidy and *TP53* mutations will be included as markers for stratification of patients into a very-high-risk group in the upcoming international relapse trial.

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Statutory Declaration

"I, Stefanie Groeneveld-Krentz (born Krentz), by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "*The clinical relevance of aneuploidy in relapses of pediatric B cell precursor acute lymphoblastic leukemia*", independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Anteilserklärung an der erfolgten Publikation

Publikation:

Groeneveld-Krentz S, Schroeder MP, Reiter M, Pogodzinski MJ, Pimentel-Gutierrez HJ, Vagkopoulou R, Hof J, Chen-Santel C, Nebral K, Bradtke J, Turkmen S, Baldus CD, Gattenlohner S, Haas OA, von Stackelberg A, Karawajew L, Eckert C, Kirschner-Schwabe R. **Aneuploidy in children with relapsed B-cell precursor acute lymphoblastic leukaemia: clinical importance of detecting a hypodiploid origin of relapse.** *British Journal of Haematology.* 2019;185(2):266-83. doi:10.1111/bjh.15770. Epub 2019 Feb3.

IMPACT FACTOR: 5.128

Beitrag im Einzelnen: Zusammenstellung eines Untersuchungskollektivs aus der Gesamtheit der Patienten mit ALL-Rezidiv, die innerhalb der klinischen Rezidivstudie ALL-REZ BFM 2002 behandelt wurden (dargestellt in Abbildung 1A); Entnahme der entsprechenden DNA-Proben aus der Biobank sowie deren Qualitätskontrolle; Etablierung und Durchführung der MLPA Methode sowie Auswertung und Interpretation der MLPA Ergebnisse (die Daten liegen der Abbildung 1B zu Grunde); Validierung der MLPA Daten mit anderen Methoden zur Ploidy-Bestimmung (FISH – Tabelle S1 und Tabelle 1, DNA Index – Abbildung S1 und Tabelle 1); Auswertung und Interpretation von SNP-Array Daten (zusammengefasst in Tabelle 1, Teil B); Korrelation der identifizierten aneuploiden Gruppen mit klinischen (Tabelle 2 und 3) und genetischen Parametern (Abbildung 2); Überlebensanalysen nach Kaplan-Meier (Abbildungen 4, S4 und S5) und mittels Cox Regression (Tabelle 4); Erstellen sämtlicher Diagramme und Tabellen für das Manuskript, Entwurf des Manuskripts sowie Mitarbeit (mindestens 50%) an der Überarbeitung/Fertigstellung und bei der Einreichung und Revision.

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Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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Unterschrift des Doktoranden/der Doktorandin

Journal Summary List

Bristish Journal of Haematology: rank #12/71 category „Haematology“

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **“HEMATOLOGY”** Selected Category Scheme: WoS
Gesamtanzahl: 71 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	CIRCULATION RESEARCH	52,753	15.211	0.082820
2	BLOOD	167,858	15.132	0.278040
3	Lancet Haematology	1,307	10.698	0.007350
4	LEUKEMIA	25,265	10.023	0.059580
5	HAEMATOLOGICA	16,138	9.090	0.038930
6	Journal of Hematology & Oncology	4,098	7.333	0.009750
7	BLOOD REVIEWS	2,810	6.600	0.006010
8	ARTERIOSCLEROSIS THROMBOSIS AND VASCULAR BIOLOGY	34,074	6.086	0.044820
9	JOURNAL OF CEREBRAL BLOOD FLOW AND METABOLISM	19,450	6.045	0.028280
10	STEM CELLS	21,694	5.587	0.035680
11	AMERICAN JOURNAL OF HEMATOLOGY	9,458	5.303	0.022620
12	BRITISH JOURNAL OF HAEMATOLOGY	23,861	5.128	0.039010
13	THROMBOSIS AND HAEMOSTASIS	16,701	4.952	0.025770
14	JOURNAL OF THROMBOSIS AND HAEMOSTASIS	17,663	4.899	0.034380
15	BONE MARROW TRANSPLANTATION	12,506	4.497	0.020810
16	CRITICAL REVIEWS IN ONCOLOGY HEMATOLOGY	6,956	4.495	0.012190
17	BIOLOGY OF BLOOD AND MARROW TRANSPLANTATION	10,583	4.484	0.026940
18	JOURNAL OF LEUKOCYTE BIOLOGY	17,244	4.224	0.021200
19	TRANSFUSION MEDICINE REVIEWS	1,292	4.111	0.002350
20	CYTOTHERAPY	5,589	3.993	0.009020
21	SEMINARS IN HEMATOLOGY	2,305	3.926	0.003780
22	TRANSFUSION	13,045	3.423	0.022050
23	SEMINARS IN THROMBOSIS AND HEMOSTASIS	3,876	3.345	0.006270
24	STEM CELLS AND DEVELOPMENT	7,589	3.315	0.016440

Selected publication (incl. Supplementary information)

Groeneveld-Krentz S, Schroeder MP, Reiter M, Pogodzinski MJ, Pimentel-Gutierrez HJ, Vagkopoulou R, Hof J, Chen-Santel C, Nebral K, Bradtke J, Turkmen S, Baldus CD, Gattenlohner S, Haas OA, von Stackelberg A, Karawajew L, Eckert C, Kirschner-Schwabe R. **Aneuploidy in children with relapsed B-cell precursor acute lymphoblastic leukaemia: clinical importance of detecting a hypodiploid origin of relapse.** *British Journal of Haematology.* 2019;185(2):266-83. doi:10.1111/bjh.15770. Epub 2019 Feb3.

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- Journal article, pages 37-54
- Supporting information, pages 55-67
- The supplementary data table Table SII can be accessed online https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fbjh.15770&file=bjh15770-sup-0002-Table.xlsx

Curriculum Vitae

Stefanie Groeneveld-Krentz

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

List of publications

Groeneveld-Krentz S, Schroeder MP, Reiter M, Pogodzinski MJ, Pimentel-Gutierrez HJ, Vagkopoulou R, Hof J, Chen-Santel C, Nebral K, Bradtke J, Turkmen S, Baldus CD, Gattenlohner S, Haas OA, von Stackelberg A, Karawajew L, Eckert C, Kirschner-Schwabe R. **Aneuploidy in children with relapsed B-cell precursor acute lymphoblastic leukaemia: clinical importance of detecting a hypodiploid origin of relapse**. *British journal of haematology*. 2019;185(2):266-83. Impact Factor 5.128

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