Childhood Acute Lymphoblastic Leukemia

Ajay Vora
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Editor
Ajay Vora
Department of Paediatric Haemotology
Sheffield Children's Hospital NHS Trust
University of Sheffield
Sheffield
South Yorkshire
UK

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Contributors

Persis Amrolia Department of Bone Marrow Transplant, Great Ormond St Children's Hospital, London, UK

Molecular and Cellular Immunology Section, UCL Institute of Child Health, London, UK

Deepa Bhojwani Children's Center for Cancer and Blood Diseases, Children's Hospital Los Angeles, Los Angeles, USA

Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

Denise Bonney Paediatric Haematology, Royal Manchester Children's Hospital, Central Manchester University Hospitals NHS Trust, Manchester, UK

Children's Cancer Group, Paterson Building, Institute of Cancer, University of Manchester, Manchester, UK

M. Brüggemann Department of Hematology, University Hospital Schleswig Holstein, Campus Kiel (UNIKIEL), Kiel, DE, Germany

Christiane Chen-Santel Department of Pediatric Oncology/Hematology/BMT, Charité University Medicine Berlin, Berlin, Germany

Rachel Dommett Bristol Royal Hospital for Children, Bristol, UK

Sara Ghorashian Molecular and Cellular Immunology Section, UCL Great Ormond Street Institute of Child Health, London, UK

Christine Harrison Leukaemia Research Cytogenetics Group, Newcastle University, Newcastle upon Tyne, UK

Stephen P. Hunger Department of Pediatrics and the Center for Childhood Cancer Research, Children's Hospital of Philadelphia and the Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

Ilaria Iacobucci St Jude's Hospital, Memphis, USA

Julie Irving Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, UK

Su Han Lum Paediatric Haematology, Royal Manchester Children's Hospital, Central Manchester University Hospitals NHS Trust, Manchester, UK

Anthony Moorman Leukaemia Research Cytogenetics Group, Newcastle University, Newcastle upon Tyne, UK

John Moppett Bristol Royal Hospital for Children, Bristol, UK

Charles Mullighan St Jude's Hospital, Memphis, USA

A. Orfao Department of Medicine, Cancer Research Center (IBMCC-CSIC-USAL) and Cytometry Service (NUCLEUS), University of Salamanca (USAL) and IBSAL, Salamanca, ES, Spain

Etan Orgel Children's Center for Cancer and Blood Diseases, Children's Hospital Los Angeles, Los Angeles, USA

Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

Vaskar Saha Paediatric Haematology, Royal Manchester Children's Hospital, Central Manchester University Hospitals NHS Trust, Manchester, UK

Children's Cancer Group, Paterson Building, Institute of Cancer, University of Manchester, Manchester, UK

Tata Translational Cancer Research Centre, Tata Medical Center, Kolkata, India

Kjeld Schmiegelow, MD, DrMedSci Department of Pediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Copenhagen, Denmark

Institute of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark

Claire Schwab Leukaemia Research Cytogenetics Group, Newcastle University, Newcastle upon Tyne, UK

Ulrik Kristoffer Stoltze Department of Pediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Copenhagen, Denmark

Sarah K. Tasian Department of Pediatrics and the Center for Childhood Cancer Research, Children's Hospital of Philadelphia and the Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

Morten Tulstrup Department of Pediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Copenhagen, Denmark

Jun J. Yang, PhD Department of Pharmaceutical Sciences, Hematological Malignancies Program, St. Jude Children's Research Hospital, Memphis, TN, USA

Inge van der Sluis, MD, PhD Department of Pediatric Hematology-Oncology, Erasmus Medical Center – Sophia Children's Hospital, Rotterdam, The Netherlands

V.H.J. van der Velden Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, Netherlands

J.J.M. van Dongen, MD, PhD Department of Immunohematology and Blood Transfusion, Room E-03-50, Leiden University Medical Center (LUMC), ZA, Leiden, The Netherlands

Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Ajay Vora University College London, London, UK

Allen Yeoh Viva-University Children's Medical Institute, Singapore, Singapore

Khoo Teck Puat-National University Children's Medical Centre, National University Hospital, Singapore, Singapore

Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, Singapore, Singapore

Chapter 1
Epidemiology and Etiology of Childhood ALL

Morten Tulstrup, Ulrik Kristoffer Stoltze, Kjeld Schmiegelow, and Jun J. Yang

1.1 Introduction

Despite extensive research, the etiology of childhood acute lymphoblastic leukemia (ALL) remains largely unknown. There is growing evidence that this cancer may arise from in utero chromosomal abnormalities that can lead to clonal expansion of pre-leukemic precursor cells. The risk factors for ALL in children are multiple, most notably common germline polymorphisms and rare genetic syndromes that directly influence hematopoiesis and cell cycling, as well as possibly infection-related aberrant DNA editing.

1.2 General Epidemiology

The incidence of ALL varies by age, ethnicity, geographic region, and also differs by immunologic and molecular subtypes. In both the United States and the Nordic countries, the overall incidence rate is 3.9 per 100,000/year before the age of

M. Tulstrup • U.K. Stoltze
Department of Pediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Copenhagen, Denmark

K. Schmiegelow
Institute of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark

Department of Pediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Copenhagen, Denmark

J.J. Yang (✉)
Hematological Malignancies Program, St. Jude Children's Research Hospital, Memphis, TN, USA
e-mail: jun.yang@stjude.org

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Fig. 1.1 Age distribution of childhood ALL cases by immunologic and molecular subtypes. Numbers represented are all children diagnosed with ALL in Denmark, Sweden, Norway, Finland, and Iceland between 1992–2007. Upper panel: bar heights represent the number of cases in each age group relative to the total number of cases between 0 and 14 years. Lower panel: relative distribution of subtypes within each age group. The testing for t(12;21) [*ETV6-RUNX1*] by *fluoresence in situ hybridization* was gradually introduced during this period, and accordingly some amp(21) patients have been missed. *Ph+*, Philadelphia chromosome-positive; *HeH*, high-hyperdiploid

15 years [1, 2]. The incidence is higher in Hispanic Americans (4.1 per 100,000/year), and is lower in African American children (2.1 per 100,000/year) [3, 4]. In general, low-income countries have lower incidences of ALL than high-income countries, with a few exceptions such as Costa Rica (4.6 per 100,000/year), however these differences may be the result of incomplete registration [4–7]. The incidence of ALL shows a characteristic peak between 2 and 5 years after birth [2, 7], but age-related ALL risk differs substantially by cytogenetic subtype (Fig. 1.1). ALL in infants (<1 year) is in most cases characterized by *MLL* gene rearrangements (*rMLL*), which are rare in older children [8–10]. Between 2 and 5-year olds, ALL is dominated by high-hyperdiploid (HeH, modal chromosome number >50) and t(12;21)[*ETV6-RUNX1*] karyotypes, while T-cell ALL has a less pronounced peak around 4–9 years [2, 10, 11]. In low-income countries, the 2–5 year age peak is much less obvious, with a higher proportion of T-ALL [5–7, 12–15]. Interestingly, some studies noted incremental increase in ALL incidences specifically in this age range as a function of economic growth and improving living conditions [16–19].

Taken together, these observations (1) suggest that different ALL subtypes may have distinctive etiological mechanisms and (2) point toward possible effects of economic development-related environmental factors on ALL risk.

1.3 Natural History

Monozygotic twins have a 10–20% concordance rate for ALL, and concordant cases have been shown to harbor identical and clonotypic molecular signatures (e.g. *ETV6-RUNX1* fusion sequence, or T-cell receptor (*TCR*), immunoglobuline (*IGH*) gene and *MLL* rearrangements) possibly because a leukemic or preleukemic clone arose prenatally in one twin and spread to the other through placental vascular anastomoses [20–25]. Further evidence for a prenatal initiation is provided by studies backtracking disease-specific molecular markers in both twin and singleton leukemias in dried blood spot samples (DBSS) from birth (Table 1.1).

For infant r*MLL* ALL, rearrangement has been identified in DBSS in the vast majority of cases, suggesting that this disease almost always arises prenatally. Older patients with r*MLL* are usually DBSS negative, but the translocation has been successfully backtracked in one case diagnosed at 6 years. Similar findings have been reported for *ETV6-RUNX1* ALL. This translocation causes a fusion of the *ETV6* and *RUNX1* genes, and the resulting chimeric protein has been shown to promote cell survival in mice and human cells [44–46]. Three studies on concordant (monozygotic) twins revealed identical *ETV6-RUNX1* fusion sequences in both twins and clonal expansion of fusion-positive precursors at a minimum level of 10^{-4} preleukemic cells at birth. Prenatally initiated *ETV6-RUNX1*$^{+}$ cases have had a latency of up to 14 years before overt leukemia occurred [47]. Furthermore, two ALL-discordant twin pairs have been described in which the healthy twin also harbored an *ETV6-RUNX1*$^{+}$ clone at birth [30] or at age 3 [44], suggesting that the translocation in itself is insufficient for leukemia development. Leukemic *ETV6-RUNX1*$^{+}$ cells harbor a variable number of additional mutations; often a deletion of the wildtype *ETV6* allele or other genes involved in B-lymphocyte development and differentiation [48–51]. Molecular studies of concordant monozygotic twins showed that these mutations are unique to each twin and thus occur as secondary postnatal events [51, 52]. An often cited study found that 1% of all healthy newborns harbored *ETV6-RUNX1* at birth (i.e. 100-fold of the incidence of *ETV6-RUNX1* ALL) [53], but subsequent validation studies have raised questions about the reliability of the initial finding [54–58]. Thus, while healthy children in general may harbor *ETV6-RUNX1*$^{+}$ cells without developing ALL, the exact prevalence of such an event has yet to be determined.

HeH ALL cases frequently have detectable clonotypic *IGH* rearrangements in neonatal blood spots (17 of 29) and the hyperdiploidy in itself can also arise prenatally [38]. Importantly, a hyperdiploid clone has been found in a healthy twin sibling of a child with HeH ALL [59]. Recently, a whole-genome sequencing approach has further supported the notion that gross chromosomal gains occur early

Table 1.1 Backtracking studies and their findings

Paper	Subtype	Marker	N	N pos.	Comments
Gale *PNAS* 1997 [26]	r*MLL*	*MLL-AF4*	3	3	
Wiemels *Lancet* 1999 [27]	*ETV6-RUNX1*	*ETV6-RUNX1*	11	8	One twin pair, both positive
Fasching *Blood* 2000 [28]		*IGH*	1	1	
	r*MLL*	*IGH* + *MLL-AF4/AF4-MLL*	2	2	
	T-ALL	*TCR*	2	2	
Yagi *Blood* 2000 [29]	B-ALL	*IGH* + *TCRD*	4	1	IGH and TCRD positive
	HeH	*IGH* + *TCRD*	1	1	IGH and TCRD positive
	r*MLL*	*IGH* + *TCRD*	2	2	Both IGH positive, only one TCRD positive
Maia *Blood* 2001 [30]	*ETV6-RUNX1*	*ETV6-RUNX1*	3	3	Triplets, one healthy
Panzer-Grümayer *Blood* 2002 [31]	HeH[a]	*IGH*	1	1	
Taub *Blood* 2002 [32]	B-ALL	*IGH*	10	5	1 Down syndrome, *TCF3-PBX1*, 1 r*MLL*
	HeH	*IGH*	6	6	
	ETV6-RUNX1	*IGH*	1	1	
Hjalgrim *Br J Cancer* 2002 [33]	*ETV6-RUNX1*	*ETV6-RUNX1*	9	3	
Wiemels *PNAS* 2002 [34]	*TCF3-PBX1*	*IGH* and *TCF3-PBX1*	15	0	
McHale *Genes Chrom Cancer* 2003 [35]	*ETV6-RUNX1*	*ETV6-RUNX1*	14	7	
Teuffel *Leukemia* 2004 [36]	*ETV6-RUNX1*	*ETV6-RUNX1* + *IGH*	2	2	Twins. Shared *ETV6-RUNX1* sequence, different IGH
Maia *Genes Chrom Cancer* 2004 [37]	r*MLL*	*MLL-AF4*	4	1	Specifically chose children with higher age a diagnosis
	ETV6-RUNX1	*ETV6-RUNX1*	7	3	
Maia *Genes Chrom Cancer* 2004 [2] [38]	HeH	*IGH*	11	1	DBSS
	HeH	*Trisomy 15 + 17*	1	1	Cord blood – this patient was DBSS-IGH negative
Fischer *Blood* 2007 [39]	T-ALL	*TCR*	16	1	Other markers: *TAL1, Notch1*, and *TCRD-LMO*.
Gruhn *Leukemia* 2008 [40]	B-ALL	*IGH*	17	11	
	HeH	*IGH*	6	5	
	ETV6-RUNX1	*IGH*	6	3	
	BCR-ABL	*IGH*	2	0	
	r*MLL*	*IGH*	1	0	

Table 1.1 (continued)

Paper	Subtype	Marker	N	N pos.	Comments
Wiemels *Blood Cell Mol Dis* 2010 [41]	HeH	*RAS mutations*	14	0	
	HeH	*IGH*	4	3	All four were also tested for *RAS* mutations
Eguchi-Ishimae *Blood* 2011 [42]	T-ALL	*NOTCH1*	3	1	
Mansur *Br J Haematol* 2015 [43]	T-ALL	*PTEN*	4	3	Infant T-ALL

High-hyperdiploidy (HeH): >50 chromosomes
[a]Chromosome number or DNA index not specified

in life as the sentinel event and that additional postnatal events are also necessary for leukemia development [60].

Clonal development of T-ALL is much less understood and leukemic genomic aberration is rarely detected at birth in children with this ALL subtype, suggesting an entirely different etiology compared to B-ALL.

In summary, r*MLL*, *ETV6-RUNX1*+, and HeH ALL show the most convincing evidence of prenatal initiation, while other subtypes such as T-ALL, *BCR-ABL* and *TCF3-PBX1* are less frequently or never prenatally initiated.

1.4 Environmental Risk Factors

1.4.1 Infectious Disease and Immune Stimulation

It has long been hypothesized that infectious disease plays a role in the development of ALL. In 1988, Leo Kinlen postulated that mixing of previously isolated populations could cause epidemics of an unidentified pathogen to which leukemia was a rare response [61]. This hypothesis was based on observed spatial and temporal clustering of ALL cases, which occurs at an exceedingly rare frequency [62]. The same year, Mel Greaves suggested that children with little early-life immune stimulation can develop leukemia as an aberrant response to a delayed exposure to common infections [63]. This 'delayed-infection hypothesis', which in many ways is similar to the 'hygiene hypothesis' concerning allergies and atopic disease, is particularly relevant to ALL risk in the 2–5 year age peak [64–67]. In these cases, the prenatal formation of a preleukemic clone may constitute a commonly occurring 'first hit', and an aberrant immune response due to delayed immune maturation and subsequent uncontrolled proliferative stress on exposure to a common childhood infection occur subsequently will in rare cases cause a second hit and initiate malignant transformation [64, 65].

A substantial body of evidence has been gathered in support of an association between infections and ALL risk. Since the actual number of childhood infections is difficult to measure, proxy measures such as daycare-attendance (children in

daycare are more exposed to common infections early in life) are typically examined [68]. A meta-analysis from 2010 by Urayama et al. included 14 studies and a total of 6108 cases and found a significantly reduced risk of ALL among children in daycare (OR = 0.76; 95% CI: 0.67–0.87) [69]. A recent study confirmed this finding and furthermore indicated that the protective effect of daycare is even stronger with earlier start of attendance [70]. Another measure of early immune stimulation is breastfeeding, for which two meta-analyses consistently found an association with a reduced risk of ALL; subsequently a large case-control study with 7,399 ALL cases and 11,181 controls also reported an OR of 0.86 (95% CI: 0.79–0.94) for children breastfed for 6 months or more [70–72]. Other proxies for immune stimulation include birth order and vaccinations, but epidemiological findings on these exposures are inconsistent [70, 73–79]. More direct attempts at measuring actual number of infections during early childhood have included patient registries [80–84], questionnaires [70, 85, 86], and interviews [87–89]. Generally, studies using parentally reported measures found an inverse or no association between infections and ALL risk, while the patient registry-based methods, which have the strength of eliminating recall bias, found either positive or null associations. Interpreting data from these studies is difficult for a number of reasons, most notably the heterogeneity of exposure definitions and the timing of infections in relation to ALL diagnosis. According to the delayed infection hypothesis, children prone to ALL-development should have fewer infections in early life and subsequently start developing aberrant responses to common infections, most likely resulting in symptomatic infectious disease. However, in the months leading up to ALL diagnosis the disease itself also becomes a risk factor for infections, and thus the expected direction of causality between infection and leukemia becomes difficult to identify in such epidemiologic studies [84].

Recent molecular studies have shed new lights on the role of infection in ALL development. Whole-genome sequencing of *ETV6-RUNX1* ALL cells revealed that most of the somatic deletions commonly seen in this subtype are mediated by the RAG enzymes, the main function of which is V(D)J recombination in normal pre-B cells [90], potentially as a result of infection-related hyperactivation of RAG. Subsequently, Swaminathan et al. showed that premature activation of the AID enzyme (which normally mediates somatic hypermutation and class-switch recombination in mature B-cells) resulting in inappropriate, synchronous activation of AID and RAG increases genetic instability in pre-B cells, especially those with the *ETV6-RUNX1* fusion [91]. The authors furthermore showed that while infectious stimuli (mimicked by lipopolysaccharide) could induce leukemic transformation of *ETV6-RUNX1*$^{+}$ cells, this development was delayed or prevented in mice without functional *AID* or *RAG*, respectively. Another example highlighting a molecular mechanism involved in infection-mediated ALL development is *PAX5*, a gene commonly mutated in B-ALL. A recent study showed that *PAX5* heterozygous mice were prone to develop ALL, but only if they were exposed to common infections [92]. It is important to note that these molecular studies show that infections are likely involved in ALL development, but provide no direct evidence of how early vs. late infection alters the risk of ALL during childhood.

Table 1.2 Non-infectious environmental risk factors

Risk factor	Certainty[a]	Comments
In utero diagnostic radiation [94, 95]	Inconclusive	
Background ionizing radiation [96–98]	Inconclusive	Uncertain association, but if true may account for 8–30% of all cases
Radon [99–101]	Inconclusive	
Extremely low-frequency electromagnetic fields [102–104]	Inconclusive	If true, this could account for 2–3% of cases
Radio frequency electromagnetic fields [105, 106]	Unlikely	
Birth weight [93]	Confirmed	OR = 1.26 (95% CI: 1.17–1.37) for children ≥4000 g
Maternal age [107, 108]	Inconclusive	
Alcohol [108–111]	Unlikely	
Maternal smoking [108, 109]	Inconclusive	OR = 1.10 (95% CI: 1.02–1.19)
Paternal smoking [112]	Inconclusive	OR = 1.11 (95% CI: 1.05–1.18)
Prenatal folic acid [113–115]	Inconclusive	If anything, folate intake during pregnancy is protective
Postnatal vitamin K [116, 117]	Unlikely	
Pesticides [118–120]	Inconclusive	
Dietary topoisomerase II-inhibitors [121, 122]	Unlikely	

[a]"Confirmed" indicates factors with consistent association in meta-analyses, "inconclusive" denotes factors with some evidence of association but also inconsistent results from different studies, whereas "unlikely" is for those with no reliable evidence of association

1.4.2 Other Risk Factors

Despite a large number of epidemiological studies and meta-analyses, most findings regarding proposed environmental risk factors remain inconclusive. The only confirmed association is high birth weight, although the underlying mechanism is unknown [93]. Other factors such as ionizing radiation, electromagnetic fields and maternal smoking during pregnancy remain uncertain (Table 1.2). A common limitation is that the vast majority of these studies address ALL as a single disease entity and thus may have missed associations with specific ALL subtypes.

1.5 Heritability of ALL

Studies addressing the risk of leukemia among offspring of childhood leukemia survivors have been hampered by small sample sizes [123–126]. More reliable estimates of ALL heritability come from studies on risk in siblings of affected children. These studies have two important limitations: first, because of preleukemic cells' ability to spread *in utero*, twins with leukemia need to be excluded

before estimating disease heritability, and secondly it is difficult to distinguish genetic effects from shared environmental risk factors between siblings. A recent Nordic population-based study reported a standardized incidence ratio (SIR) of 3.2 for ALL risk among siblings [127]. Furthermore, one study investigating 54 sibships with two or more cases of ALL found an unexpectedly high subtype concordance, pointing to a genetic basis of ALL etiology [128]. On the basis of genome-wide SNP data, it was estimated that inherited genetic polymorphisms account for at least 24% (95% CI: 6–42%) of variation in ALL risk [129]. In conclusion, these reports provide evidence for a genetic component in disease susceptibility, although reliable quantitative estimates of genetic contribution to ALL risk are not available.

1.6 High-Penetrance Genetic Predisposition

Out of more than 125 known cancer predisposition genes (CPGs), only 27 genes (associated with 9 rare syndromes and two non-heritable congenital disorders) are convincingly linked to childhood ALL (Table 1.3 and Fig. 1.2) [130, 131].

In a 2015 a registry study of 4939 childhood ALL cases, only 29 subjects were diagnosed with non-Down syndrome (DS) predisposition syndromes (0.6%) [161]. However, a recent comprehensive study of whole genome or whole exome sequencing in 588 non-DS childhood leukemia cases found germline mutations in known CPGs in 26 cases (4.4%) [161, 162]. This suggests that high-penetrance Mendelian genetics, discussed in detail below, may play a larger role in ALL etiology than previously appreciated.

1.6.1 Syndromes Where ALL Is a Dominant Cancer Phenotype

DS is one of the most common congenital abnormalities (1 in 691 live births) and also the most recognizable ALL-predisposition syndrome [140, 163]. ALL and AML risk is significantly increased, with SIR before 30 years of 24.4 and 20.3, respectively. Interestingly, individuals with DS have significantly lower incidence of solid cancers than the background population [142, 164]. DS-associated ALL is more likely to have somatic rearrangements involving the CRLF2 gene and almost always has B-cell immunophenotype. DS patients represent the only known group where ALL is the most common malignancy at any age. Taken together DS-ALL constitutes 2–3% of ALL [131, 165].

While the driver of leukemogenesis remains uncertain for DS it is likely that chromosome 21 is involved, as an acquired extra copy of chromosome 21 is also seen in hyperdiploid ALL and the intracromosomal amplification of chromosome 21 seen in the iAMP21-ALL subtype [139].

Table 1.3 ALL syndromes

Syndrome	Heritability and involved genes	Association study/studies (ages at ALL diagnosis)	Approx. ALL relative risk	Common clinical features	Assoc. ALL Subtype	Commonly associated cancers	Estimated birth incidence[b]
Ataxia-telangiectasia (A-T)	AR, *ATM*	14 leukemias (all ages, presumably ALL) in 263 A-T patients [132]	OR = ~71 [132, 133]	Cerebellar ataxia, oculom. apraxia, telangiectasia	T-ALL	NHL, BC, OV, GC, STS	1 in 40,000–100,000 [134]
Bloom syndrome (BSyn)	AR, *BLM*	12 ALLs (mean age 19.4 (range: 5–40)) in 272 BSyn patients [135]	OR = ~92 [135][a]	Short stature, skin lesions, infections, male infertility		Epithelial, AML, NHL	Unknown, 272 cases documented [135]
Constitutional Mismatch Repair Deficiency (CMMRD)	AR, *PMS2* (58%) *MLH1*/ *MSH2* (22%) *MSH6* (20%) [136]	9 ALLs (median age 6 (range: 2–21)) in 146 CMMRD patients [136]	OR = ~131 [136][a]	CALM, hyperpigmentation, NF1 symptoms,		Gliomas, NHL, GI, PNET, MB	1 in 775,000 [137]
Constitutional Robertsonian Translocation rob(15;21) (q10;q10)c	rob(15;21)c	None	OR (iAMP21-ALL only) = 2700 [138]	None	iAMP21-ALL	None	1 in 100,000–200,000 [139]

(continued)

Table 1.3 (continued)

Syndrome	Heritability and involved genes	Association study/studies (ages at ALL diagnosis)	Approx. ALL relative risk	Common clinical features	Assoc. ALL Subtype	Commonly associated cancers	Estimated birth incidence[b]
Down Syndrome (DS)	Trisomy 21 (95%) [140] Rob. or other (4%) mosaic DS (1%) [141]	20 ALLs (ages 0–29) in 2814 DS patients [142]	SIR (95% CI) = 24.4 (14.9–37.6) [142]	Extensive; flat facial profile, CHD, hearing/vision loss,	HeH	AML, TC	1 in 691 [140]
ETV6 deficiency syndrome	AD, *ETV6*	16 ALLs in >35 *ETV6* mutation carriers [143–146]	Unknown, likely very high [144]	Thrombocytopenia	B-ALL	Likely ST	Unknown, 9 families documented [143–146]
Fanconi Anemia (FA)	AR, *FANCA*(60–70%) *FANCC* (14%) *FANCG* (10%) *BRCA2* (3%) +13 more (3–13%) [147]	7 childhood ALLs in 1300 FA patients [148]	OR = ~11 [148] [a]	Short stature, CALM. None in 25–40% [149]		AML, MDS, GU, head/neck ST	1 in 100,000–360,000 [149, 150]
Li-Fraumeni Syndrome (LFS)	AD, *TP53*	12 leukemias (mean age 11 (range: 2–17), presumably ALL) in 415 LFS patients [151]	OR = ~60 [151] [a]	None	Hypodiploid ALL	OS, ACC, CNS, STS	1 in 5000 [152]-20,000 [153]

Neurofibromatosis 1 (NF1)	AD, *NF1*	12 NF1 patients (ages 1.25–14.5) in 5725 ALL cases [154]	OR(95% CI) = 5.4 (2.8–9.4) [154]	CALM, freckling, neurofibromas, learning disabilities		MPNST, JMML, MDS, GIST	1 in 2712 [155]- 3000 [156]
Nijmegen Breakage Syndrome (NBS)	AR, *NBN*	6 vs. 0 NBS cases in a ALL cases (389) vs. controls (7653) study [157]	OR(95%CI) = 1325 (860–2168) [157]	Microcephaly, short stature, pulm. Infections	T-ALL	NHL	Unknown, 170 cases documented [158]
PAX5 deficiency syndrome	AD, *PAX5*	11 B-ALLs in 21 PAX5 mutation carriers [159, 160]	Unknown, likely very high [159]	None	B-ALL	None	Unknown, 3 families documented [159, 160]

AD autosomal dominant, *AR* autosomal recessive, *OR* odds ratio, *SIR* standardized incidence ratio, *iAMP21* intrachromosomal amplification of chromosome 21, *DS* Down Syndrome, CI95%, 95% confidence interval, *CALM* café-au-lait macules, *NHL* non-Hodgkin lymphoma, *BC* breast cancer, *OV* ovarian cancer, *STS* soft tissue sarcoma, *AML* acute myeloid leukemia, *GI* gastrointestinal (cancers), *PNET* primitive neuroectodermal tumor, *MB* medullablastoma, *TC* testicular cancer, *MDS* myelodysplastic syndrome, genitourinary (cancers), *ST* solid tumors, *OS* osteosarcoma, *ACC* adrenocortical carcinoma, *CNS* central nervous system (tumors), *MPNST* malignant peripheral nerve sheath tumors, *JMML* juvenile myelomonocytic leukemia, *GIST* gastrointestinal stromal tumors

[a]OR was estimated as the ratio of the reported ALL incidence in individuals with genetic syndrome of interest vs. incidence in the general population (i.e., 1 in 2000 between ages 0 and 15). For example, the frequency of ALL is ~5 per 1000 individuals with FA (7 in 1300 subjects) and thus ~11-fold higher than the expected ALL incidence in general populations

[b]Incidences shown for ARs are for homozygote/biallelic status

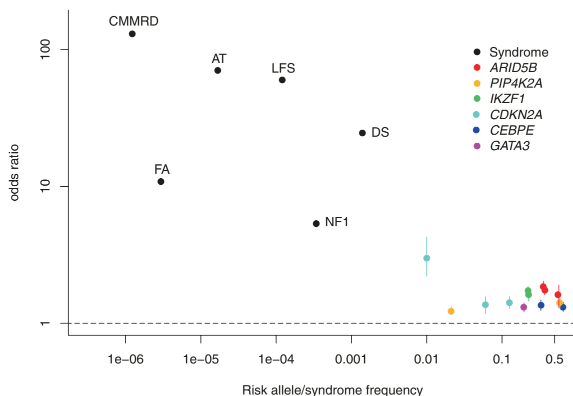

Fig. 1.2 Effect sizes and frequencies for known ALL genetic risk factors. Syndrome risks and frequencies are based on best available evidence as described in Table 1.3; SNP odds ratios are based on references in Table 1.4, and SNP risk allele frequencies are based on worldwide populations from the 1000 Genomes Project. CMMRD, constitutional mismatch repair-deficiency; *AT* ataxia-telangiectasia, *LFS* Li-Fraumeni syndrome, *DS* Down syndrome, *FA* Fanconi anemia, *NF* neurofibromatosis type 1

In fact, iAMP21-ALL has recently been found to be more frequent in individuals with the germline translocation rob(15;21)(q10;q10)c, a rare constitutional genetic abnormality. Amplification of the genes involved in the translocation duplicates the entire abnormal chromosome and confers an estimated 2,700-fold increased risk of iAMP21-ALL [138]. However, considering the rarity of both iAMP21-ALL and rob(15;21)c, <1 in 1,500 ALL cases are likely to be related to rob(15;21)c associated.

PAX5 is known to be somatically mutated or deleted in approximately 30% of B-ALL cases [166]. In 2013, one germline *PAX5* mutation was found in three kindreds of familial ALL [159, 160]. The 3 families had 18 documented and 3 obligate mutation carriers with 11 cases of B-ALL, with another 2 ALLs in untested children. These *PAX5* mutations may be exclusively related to ALL risk, but further study is warranted.

ETV6, like *PAX5,* is known to be recurrently mutated or translocated in leukemic cells [166, 167]. In 2015, three studies independently reported nine families with *ETV6* germline mutations, all having a dominantly heritable thrombocytopenia and high incidence of ALL among mutation carriers [143, 144, 146]. Collectively, 35 documented and 4 obligate carriers have developed a total of 14 leukemias (mostly ALL), with another 2 occurring in untested children. One systematic sequencing study targeting germline *ETV6* in 4,405 ALL cases, identified 31 *ETV6* variants potentially related to 35 ALL cases, with carriers found to be significantly older than non-carriers (mean age 10.2 vs. 4.7) [144]. Thus, *ETV6* mutations may be present in nearly 1% of all ALL cases, and perhaps higher in patients over 5 years of age.

1.6.2 Syndromes Where ALL Is Part of a Mixed Cancer Phenotype

Li-Fraumeni Syndrome (LFS) is a rare cancer predisposition syndrome, in which germline *TP53* mutation confers a ~90% lifetime risk of developing cancer in a spectrum of tissues with one third being diagnosed before 18 years of age. The increased ALL risk is largely restricted to cases with low hypodiploid leukemia karyotype (underlying *TP53* mutation present in 43.3% of low hypodiploid ALL) [168].

Ataxia-telangiectasia (A-T) is a rare syndrome caused by recessive mutations in the *ATM* gene and typically presents with progressive cerebellar ataxia before 4 years of age [134]. A-T patients have a high risk of leukemias (especially T-cell ALL) and lymphomas, as well as hypersensitivity to ionizing radiation and chemotherapy related to the role of ATM in DNA repair [132, 134].

Bloom Syndrome is characterized by pre- and postnatal growth deficiency (stature typically <1.5 m), skin lesions and high risk of ALL, AML, lymphoma, and epithelial carcinomas [135]. Twelve ALLs were found in less than the 300 cases registered world-wide and in at least two cases ALL preceded Bloom Syndrome diagnosis [169, 170].

Nijmegen Breakage Syndrome (NBS) is another very rare recessive syndrome, which mainly occurs in Slavic populations [157] (a Slavic founder deletion of five bases in the *NBN* gene is found in >90% of NBS cases), yet NBS has also been described in >8 other countries with private mutations [157, 158, 171, 172]. Patients display microcephaly, intrauterine growth retardation with short stature, recurrent sinopulmonary infections and increased risk of cancers, especially lymphoma and leukemia [157, 173].

Fanconi Anemia (FA) is a rare recessive syndrome with a high risk of AML, MDS and other hematological diseases set at ~10%/year [147, 174]. In a registry with 1300 FA patients only 7 ALLs were reported and FA-leukemias are predominantly myeloid (96%) [147, 148]. While skeletal deformations and classic hematological findings often lead to diagnosis early in life, malignancies including ALL can be the presenting feature [175, 176].

There is a long string of genetic syndromes for which sporadic reports described ALL as a possible cancer manifestation, although the matter has not been systematically examined. The most common are RASopathies (e.g. NF1) [177–179], where 6 ALLs were seen among 1176 mutation carriers in 1 study [180]. Others include: Bruton’s Agammaglobulinemia [181], Familial Platelet Disorder with Associated Myeloid Malignancies [182, 183], Weaver syndrome [184], Sotos syndrome [185], Rubinstein-Taybi syndrome [186], Börjeson-Forssman-Lehmann Syndrome [187] and SH2B3 deficiency [188].

It should be noted that ALL predisposition syndrome may not be symptomatic prior to leukemia diagnosis with only non-specific clinical features such as growth failure and microcephaly. Family history needs to be carefully examined to identify possible underlying genetic causes in a pediatric oncology setting.

1.7 Low-Penetrance Genetic Predisposition

Emerging from the 'common disease—common variant' hypothesis, the past two decades have seen the application of first candidate gene-driven and later genome-wide association studies in ALL etiology research [189, 190].

Single nucleotide polymorphism (SNP)-based candidate gene studies (CGSs) have explored ALL etiology by focusing on genes involved in carcinogen metabolism, folate metabolism, and DNA repair pathways. A 2010 systematic review identified 47 CGSs on 25 variations in 16 genes all tested for association with ALL, showing pooled significance ($P < 0.05$) in only 8 variants (OR range; 0.73–1.78) with an apparent false positive report probabilities of at least 20% [191]. Other studies have focused on human leukocyte antigen (HLA) genes, particularly class II loci *HLA-DR* and *HLA-DP*, with the latter showing evidence of significantly different associations between ALL subtypes as well as interactions with proxies for immune stimulation [192, 193]. However, a larger study has cast doubt on the validity of these findings [194].

2009 saw the first two genome-wide association studies (GWAS) independently demonstrating associations between ALL susceptibility and SNPs in *ARID5, IKZF1 and CEBPE* [195, 196]. Subsequently, SNPs in four other genes have been found to be associated with either overall ALL risk or subtype-specific risk, with a total of 13 SNPs in 6 genes having been widely validated thus far (Table 1.4) [197–201].

Table 1.4 GWAS results

Gene	rsid(reference)	OR(95% CI)	Associated subtype, OR(95% CI)	RAF
ARID5B	rs7089424 [202]	1.65 (1.54–1.76)	HeH, 2.17 (1.5–3.1)	0.37
	rs10821936 [196]	1.91 (1.6–2.2)		0.36
	rs10994982 [197]	1.86 (1.71–2.03)		0.57
CDKN2A	rs17756311 [197]	1.36 (1.18–1.56)		0.06
	rs3731217 [203]	0.71 (0.64–0.78)		0.13
	rs3731249 [204]	2.99 (2.21–4.26)		0.01
CEBPE	rs2239633 [202]	1.31 (1.22–1.42)		0.64
	rs4982731 [197]	1.36 (1.24–1.48)		0.33
GATA3	rs3824662 [198]	1.31 (1.21–1.41)	Ph-like, 3.85 (2.7–5.4)	0.20
IKZF1	rs4132601 [202]	1.71 (1.58–1.85)		0.22
	rs11978267 [197]	1.59 (1.45–1.74)		0.23
PIP4K2A	rs7088318 [197]	1.40 (1.28–1.53)	HeH	0.59
	rs10828317 [198]	1.23 (1.15–1.32)		0.02

Selected SNPs associated with ALL risk. Risk allele frequencies are global frequencies from the 1000 Genomes Project. *SNP* single nucleotide polymorphism, *HeH* high-hyperdiploid, *OR* odds ratio, 95% CI, 95% confidence interval, *RAF* risk allele frequency, *Ph-like* Philadelphia-like ALL

The heterogeneity of ALL is reflected in the GWAS findings, with some SNPs showing a stronger association with specific subtypes. *ARID5B*, for instance, is most strongly associated with HeH ALL. SNPs in *TP63* and *GATA3,* on the other hand, show isolated associations with *ETV6-RUNX1* ALL and Ph-like ALL, respectively [196, 199, 201, 204, 205].

While there is little doubt that the GWAS findings identified genuine inherited risk factors for ALL, there is a paucity of studies describing the molecular mechanisms underlying these associations. Somatic deletions in both *CDKN2A* and *IKZF* are frequent in ALL, and these two genes play important roles in tumor suppression and lymphocyte development, respectively [200, 206]. In one recent study, 35 tumors from *CDKN2A* risk variant rs3731217 carriers preferentially retained the risk allele, suggesting that the SNP is advantageous during tumor growth [204]. *ARID5B* is also involved in lymphocyte differentiation, but its mechanism in ALL development is poorly understood.

Within the validated risk variants, no significant gene–gene interactions have been reported [195, 196, 203]. The effects of these risk alleles are relatively stable across ethnicities, and risk allele frequencies correlate well with population differences in ALL incidence [197]. One pathway-based GWAS on ALL risk was recently described but these results have yet to be reproduced [207]. Inspired by the observations that ALL subtypes differ across both environmental and genetic risk factors, other researchers have attempted to identify interactions between the two by combining genotypes with data on various environmental exposures [208–211]. These studies, however, have so far failed to reliably identify gene-environment interactions.

Studies on childhood ALL etiology will improve knowledge of the pathogenesis, predict disease risk, and provide new targets for treatment.

The low-penetrance genetic predispositions discussed above (e.g., risk alleles identified by GWAS) constitute a minor increase in the absolute risk of developing ALL, e.g. from 1 in 2,000 to 1 in 1,500. While the effects of these variants individually are modest with limited clinical implication, their cumulative impact can be comparable to those of the highly penetrant genetic predisposition syndromes. However, it is debatable whether early diagnosis of an aggressive cancer like ALL can lead to improved outcome [212]. Hence, clinical surveillance aimed at early diagnosis of ALL may not necessarily benefit at-risk subjects and may in fact lead to uncertainty and anxiety for the families [213].

Still, many of the genetic syndromes discussed above may modify health conditions other than the risk of developing ALL. Preemptive surveillance for non-ALL cancers (e.g. *TP53* carriers) and/or treatment modification (e.g. avoidance of radiation therapy in cases with A-T) can lead to lower mortality and morbidity for the children and their at-risk family members [214–218]. For this reason, recognition and diagnosis of predisposition syndromes in pediatric oncology is crucial. In fact, it has been suggested that pediatric cancer patients under the age of five should be evaluated for A-T before starting chemotherapy and/or radiotherapy because of potentially fatal adverse effects of conventional doses due to defective DNA repair in these cases [134, 219].

1.8 Future Directions

While substantial progress has been made in identifying risk factors for ALL (especially the role of inherited genetic variants), our understanding of ALL disease etiology is far from complete. An important field of research in the coming years will be to identify gene-gene and gene-environment interactions that contribute to ALL leukemogenesis, and whether approaches can be developed to target these processes and reduce disease risk and burden in genetically predisposal children.

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Chapter 2
Clinical Presentation and Prognostic Factors

John Moppett and Rachel Dommett

2.1 Clinical Presentation

2.1.1 Introduction

The clinical presentation of ALL results from the interaction of two biological variables: the organs and tissues that are the site of the developing lymphoid system (or are those to which lymphoid blasts readily or feasibly spread) and the degree to which such organ and tissue involvement leads to clinical symptomatology. There is such diversity in both of these factors that the protean presentations of ALL can sometimes be a challenge to the most experienced of clinicians. Fortunately, the commonest presentation is with the classic signs and symptoms of bone marrow and lymphoid organ involvement. However, it is common to have other signs and symptoms overlaid on this classic set of symptoms, and, more challengingly, sometimes none of the classic of symptoms are present at all. Finally, it should be noted that the clinical presentation of ALL is influenced significantly by the healthcare and socioeconomic systems within which the child resides (Fig. 2.1).

2.1.2 Symptoms and Signs Related to Bone Marrow Involvement

On review of the diagnostic bone marrow aspirate in ALL, it is typical, though not universal, to find greater than 90% of the bone marrow is effaced by lymphoblasts. This has some very clear symptomatic implications.

J. Moppett (✉) • R. Dommett
Bristol Royal Hospital for Children, Upper Maudlin Street, Bristol BS2 8BJ, UK
e-mail: john.moppett@uhbristol.nhs.uk

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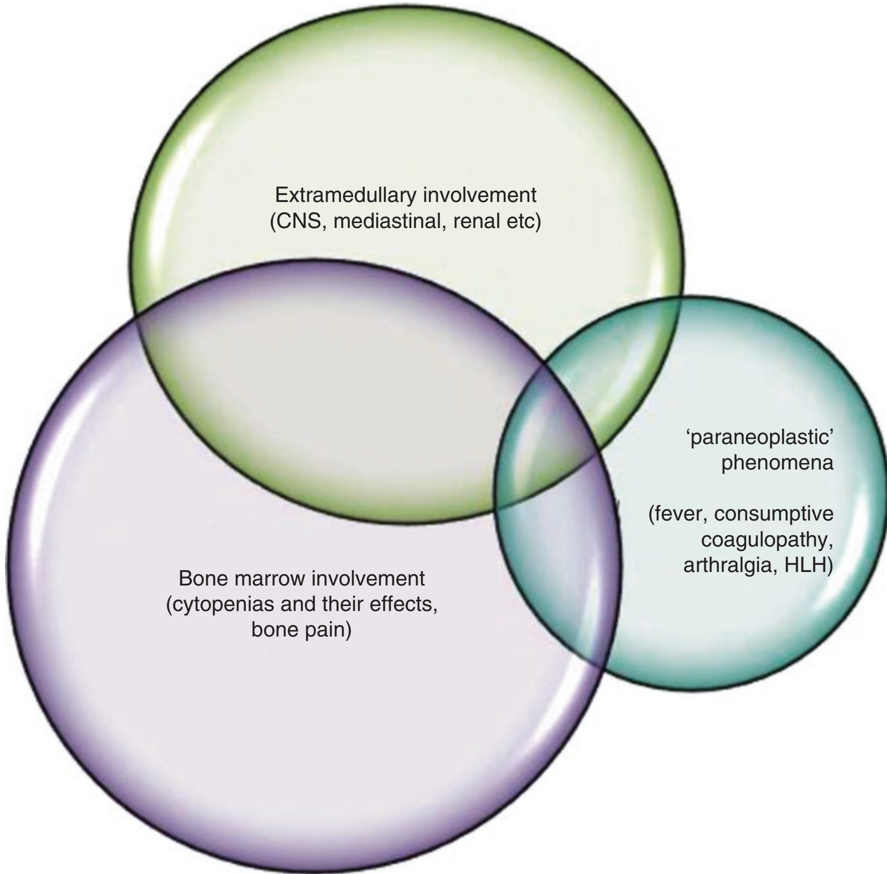


Fig. 2.1 The factors affecting the presentation of ALL

The bone marrow is the primary organ of haematopoiesis, and this capacity is reduced as the bulk of medullary leukaemia increases. Thus the symptoms and signs of anaemia (pallor, lethargy), thrombocytopenia (bruising, petechiae and mucosal bleeding) and functional leucopenia (fever and infection), the classic cardinal features of ALL at presentation, are the most common [1]. These have usually been present for several weeks prior to clinical referral, no doubt due to the many more common benign causes of these symptoms individually. Hepato-splenomegaly and lymphadenopathy, the other classic features of lympho-reticular involvement are commonly found at presentation but are less likely to be a presenting feature highlighted by the parents. It is, of course, the combination of these classical symptoms and signs that alerts the physician to the likely diagnosis of ALL. However, only 23% of patients have all three of the classic triad of fever, lethargy and petechiae/easy bruising at presentation (Table 2.1) [1].

The next most frequent symptom is bone pain, seen to some degree in 40–50% of patients [1, 2]. This is due to medullary expansion or peri-osteal infiltration. Interestingly, significant bone pain is associated with more normal blood counts

Table 2.1 Prevalence of symptoms and signs at diagnosis

	ALL, % (n = 100)	pre-B ALL, % (n = 89)	T ALL, % (n = 11)	Fisher's test, 2p < 0.10
Musculoskeletal pains	49	54	9	0.008
Leg pain (av.dur.)	34 (7 weeks)	37	9	0.09
Back pain (av.dur.)	15 (9 weeks)	17	0	–
Limp (av.dur.)	16 (9 weeks)	18	0	–
Joint pain (av.dur.)	17 (10 weeks)	19	0	–
Constitutional Symptoms	83	83	83	–
Fatigue (av.dur.)	53 (5 weeks)	53	55	–
Anorexia (av.dur.)	43 (3 weeks)	44	36	–
Abdominal pain (av.dur.)	24 (3 weeks)	22	36	–
Headache (av.dur.)	10 (2 weeks)	8	27	–
Weight loss	11	10	18	–
Bone marrow insufficiency symptoms	77	80	55	–
Fever (av.dur.)	66 (2 weeks)	69	45	–
Bleeding (av.dur.)	35 (1 Week)	37	18	–
Visible organomegaly	19	12	73	0.00004
Lymph gland enlargement (av.dur.)	16 (5 weeks)	11	54	0.002
Abdominal distension	5	3	18	0.09
Bone marrow insufficiency signs	90	92	73	0.08
Pallor	84	88	55	0.014
Fever: temp. >37.5°C	45	47	27	–
Purpura	44	46	27	–
All three findings	23	25	9	–
Organomegaly on examination	71	67	100	0.03
Hepatomegaly	51	51	55	–
Spelnomegaly	42	37	82	0.007
Lymphadenopathy	26	22	55	0.03
Mediastinal tumor	1	0	9	–
Sign of joint or skeletal lesion	10	11	0	–
Arthritis	6	7	0	–
Vertebral lesion	4	5	0	–
Abnormal blood count	96	97	90	–
Hb <6 mmol Fe/l	80	84	45	0.008
Platelet count $<100 \times 10^9$/l	67	72	27	0.005
Leukocyte count $>20 \times 10^9$/l	28	24	64	0.010
Leukocyte count $<4.0 \times 10^9$/l	35	36	27	–
2 or 3 cell lines affected	78	80	64	–
Neutrophil count $<1.0 \times 10^9$/l	68	73	27	0.004
Lymphoblasts in blood smear	85	84	91	–
Biochemical marker elevation	78	75	100	–
Lactate dehydrogenase conc. >500 IU/l	68	64	100	0.015
Urate conc. >0.35 mmol/l	24	24	27	–

Adapted from [1]

ALL acute lymphoblastic leukaemia, *av.dur.* average duration, *conc.* concentration, *pre-B* precursor B-cell, *T* T cell

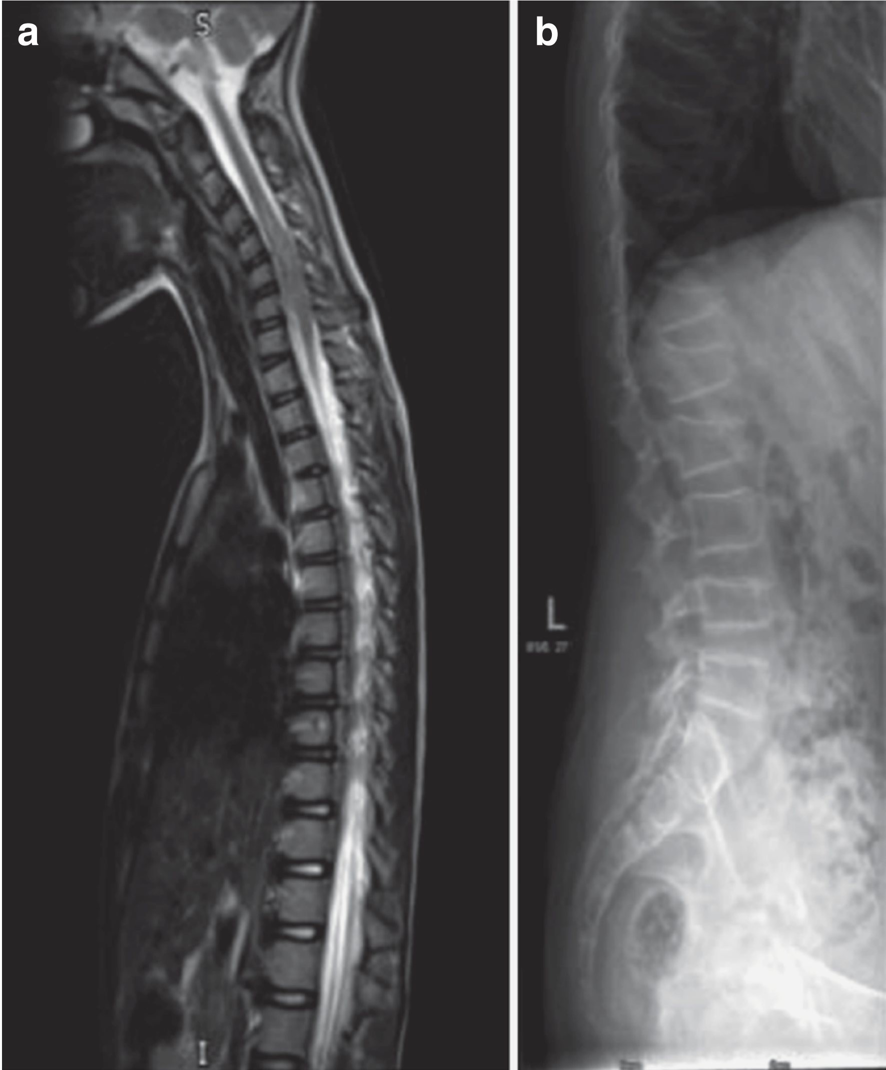


Fig. 2.2 (**a**) Intraspinal mass at presentation (**b**) multiple spinal fractures at diagnosis

than in patients without bone pain [2, 3] and therefore often occurs prior to development of marrow failure. Joint pain and arthritis are also common, seen in 18% and 9% of cases respectively, and so cases may be initially misdiagnosed as reactive arthritis, osteomyelitis or Juvenile Idiopathic Arthritis (JIA) [3, 4]. Back pain is often part of such painful presentations and should alert the clinician to the possibility of intradural masses. A full neurological examination to exclude signs of cord compression should always be performed (Fig. 2.2). Back pain should alert

the physician to the possibility of vertebral fractures. Sixteen percent of children have vertebral fractures at diagnosis; 55% of these have back pain and 35% of patients presenting with back pain will have a vertebral fracture [5].

2.1.3 Symptoms and Signs Related to Extensive Bulk Disease

Hyperleucocytosis (WCC > 200) is associated with an increased risk of complications, particularly intra-cerebral haemorrhage (Fig. 2.2), neurological, renal and pulmonary infiltration [6]. The risk of symptomatic hyperleucocytosis correlates with white count (CNS haemorrhage seen in 17% of those with WCC >400 compared to 3.6% for WCC 200–400 (Fig. 2.3) [6–8].

Large mediastinal masses in association with ALL can obstruct venous return from the upper venous system leading to raised venous pressure and causing the Superior Vena Cava Obstruction Syndrome (SVCO). Clinically, facial (and sometimes upper limb) swelling associated with difficulty breathing, nasal stuffiness, visual difficulties and headache are seen. On examination plethora, distended upper venous vessels, conjunctival induration, papilloedema, mental changes due to cerebral oedema and even coma can be seen (Fig. 2.4). SVCO can be associated with Superior Mediastinal Syndrome (SMS) where in addition to raised upper system venous pressure, impairment to venous return to the heart and pericardial effusions may lead to hypotension and tamponade. Tracheal compression can cause significant respiratory compromise and patients may have orthopnoea. ALL is the second most common cause of this complication after T-lymphoblastic lymphoma in children [9].

2.1.4 Central Nervous System

Using standard morphological techniques, 8–10% of patients have detectable blasts in the cerebrospinal fluid at diagnosis [10, 11], but less than 5% present with neurological symptoms [12]. Symptoms include manifestations of raised intracranial pressure (headache, vomiting, papilloedema), seizures and meningism secondary to leptomeningeal involvement. Local infiltration can cause cranial nerve palsies, most commonly the facial nerve which can be misdiagnosed as Bell's palsy or hypothalamic and cerebellar symptoms [13]. Leukaemic infiltrate around the spinal cord can result in symptoms of acute cord compression requiring emergency management to prevent permanent paralysis. Neurological symptoms at diagnosis may also be secondary to leucostasis or an intracranial bleed.

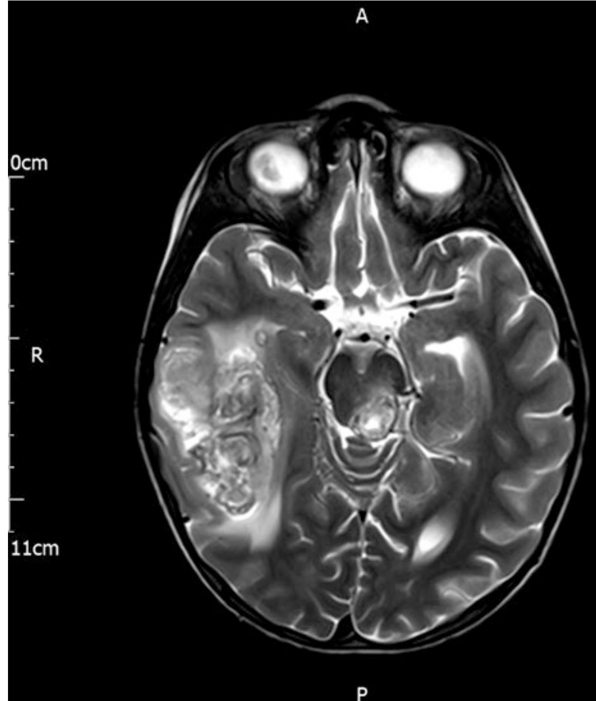
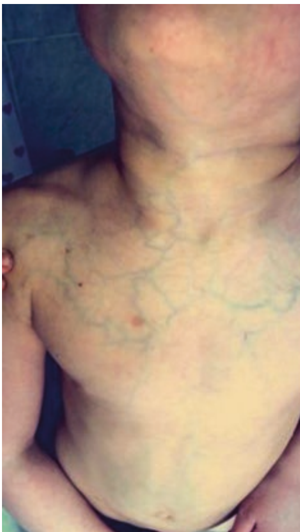


Fig. 2.3 Large R Temporal and midbrain haemorrhage at presentation in T-ALL (white cell count 750)



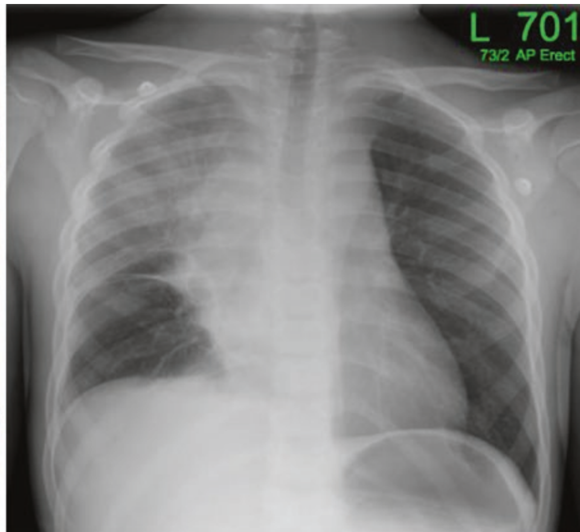


Fig. 2.4 Distention of upper venous vessels and widened mediastinum in T-ALL

2.1.5 The Eye

Leukaemia can involve nearly all ocular structures and is either due to leukaemic infiltration or complications of leucostasis or bleeding [14]. Retinal involvement is commonly secondary to haemorrhage and probably underestimated unless formal ophthalmic assessment is undertaken. Infiltration of the optic nerve may manifest as reduced visual acuity or blindness with the need for early intervention with radiotherapy to potentially salvage vision. Involvement of other ocular structures including the orbit, iris, cornea, conjunctiva and anterior chamber in the form of a hypopyon is more common at relapse. Hence patients presenting with photophobia, pain, blurred vision or conjunctival irritation should be assessed by an experienced ophthalmologist for evidence of disease.

2.1.6 Genitourinary System

Testicular disease usually manifests as painless enlargement of the scrotum. The differential diagnosis is that of a hydrocele secondary to lymphatic obstruction. Studies suggest microscopic evidence of disease is present in approximately one fifth of patients at diagnosis however clinically detectable disease is uncommon, seen in approximately 2% of cases [15] and associated with T-cell disease [16]. A biopsy is indicated only if the testes remain persistently enlarged despite treatment or to confirm an isolated relapse.

Renal enlargement at diagnosis can be secondary to leukaemic infiltration or the complications of hyperuricaemia, haemorrhage or infection [17]. Priapism is a recognized but rare presentation. It may reflect infiltration of vessels, leucostasis or sacral nerve root involvement.

The female genital tract is also reported as a site of extramedullary disease but rarely in isolation. Sites of disease include the ovaries, fallopian tubes, and uterus.

2.1.7 Cardiovascular System

Pericardial infiltration and effusion can occur at diagnosis with the potential risk of cardiac tamponade [18]. Symptomatic cardiac involvement is otherwise rare [19, 20]. Hypereosinophilic syndrome associated with pre B-ALL can present with cardiomegaly and congestive heart failure without evidence of circulating blasts or other cytopenias. The presence of the t(5;14)(q31;q32) translocation resulting in activation of the interleukin-3 gene on chromosome 5 by the @IgH locus on chromosome 14 is the pathogenetic mechanism of eosinophilia and leukemogenesis in these patients [21].

2.1.8 The Skin

Leukaemic infiltration of the skin can present as subcutaneous nodules [22]. It is more in AML but can occur in patients with infant and T cell ALL. Pyoderma gangrenosum in association with ALL is also reported but more commonly seen in myeloid leukaemia.

2.1.9 Head and Neck

Tonsillar, adenoidal or adnexal involvement may present with the typical symptoms of enlargement and obstruction. Salivary and parotid gland enlargement are also reported presentations of ALL.

2.1.10 Gastrointestinal System

In the abdomen, infiltration of the appendix or mesenteric nodes may result in symptoms of an acute abdomen requiring surgical intervention. Children with ALL frequently present with hepatomegaly and mild liver functional impairment. Severe jaundice is rarely seen as a presenting feature and can reflect infiltration of hepatic sinusoids [23]. In this scenario chemotherapy dose modifications should be considered.

2.1.11 Hypercalcaemia

Hypercalcaemia (symptomatic or identified by routine biochemistry), either in isolation or in combination with osteolytic lesions can be the only presenting symptom in children with ALL. This is either due to localised bone destruction by leukaemic infiltration and local release of a range of cytokines and other osteoclast-stimulating factors directly onto the surface of bone or release of parathyroid hormone (PTH) like protein from lymphoblasts [24]. In PTH-independent hypercalcaemia of unknown origin, a bone marrow aspirate/trephine should be a necessary part of the diagnostic work up. The extremely rare and very poor prognostic lesion t(17;19)(q22;p13) is associated with hypercalcaemia in 70% of cases [25].

2.1.12 Haemophagocytic Lymphohistiocytosis (HLH)

HLH has some overlapping symptomatology with acute leukaemia, and can rarely be present at the diagnosis of ALL [26]. Hyperferritinaemia, coagulopathy and liver dysfunction are usually much more severe in HLH compared to

ALL. Underlying triggers for HLH (infection or primary genetic HLH (Munc, Perforin) in the context of developing ALL) should be looked for in this situation.

2.1.13 Aplastic Presentation

Pancytopenia followed by a period of spontaneous haemopoietic recovery is recognized in a small percentage of cases prior to evolution of frank leukaemia [27]. The pancytopenic phase is commonly accompanied by high fevers and infection and precede the diagnosis of ALL by weeks or, rarely, 18 months. The differential diagnosis in this scenario is aplastic anaemia and bone marrow examination is essential. Clinically, lymphadenopathy and hepatosplenomegaly are uncommon in aplastic anaemia. Often the diagnosis is apparent during the aplastic phase by the presence of small clones with karyotypic abnormalities or re-arranged antigen receptor genes.

2.1.14 Rare Presentations

There is a wide variety of reported rare presentations of ALL. These include; hyperthyroidism, thyroid nodules, central hypoventilation, isolated soft tissue masses and torticollis.

2.1.15 Asymptomatic Pancytopenia

A diagnosis of ALL is occasionally made on an incidental blood count in children with non-specific symptoms.

2.1.16 The Diagnostic Interval and Pathway

The interval from first presenting to a medical practitioner to diagnosis is short in the majority of patients (median 2 days) who have with typical symptoms [28]. Most children are seen in primary care before diagnosis, so the family practitioner plays a key role in facilitating a timely diagnosis and onward referral. Earlier consultations with non-specific 'low-risk but not no risk' symptoms such as fever, pallor and lethargy are commonly reported and may or may not prompt a request for blood tests [29]. More alarming symptoms such as bleeding or bruising generally prompt rapid onward referral irrespective of the clinical wellness of the patient. A longer diagnostic interval is reported in patients initially consulting a family practitioner as opposed to a paediatrician for whom ALL may be lower on the differential

diagnosis [28]. National referral guidance for family practitioners in the UK supports emergency referral for patients presenting with symptoms of acute leukaemia including unexplained petechiae and recommends blood tests within 48 h for more non-specific symptoms [30]. Diagnostic delay may be a greater problem in the developing world, due to both parental (education/poverty) and physician (incorrect initial diagnosis) delays [31].

Older age is associated with longer intervals and may reflect patients more likely to present with fatigue or bone pain. The initial referral pathway in patients presenting with arthritis or arthralgia symptoms may be via Orthopaedics or Rheumatology, which can prolong the diagnostic interval [4].

There is no impact of diagnostic interval on outcome in childhood ALL. Disease biology is presumed to be the main factor as patients with high risk disease often experience shorter diagnostic intervals.

2.1.17 Differential Diagnosis

For patients with the classic triad of features, the only significant differential is other bone marrow infiltrative disorders (for example Acute Myeloid Leukaemia, Myelodysplasia, Neuroblastoma and Aplastic Anaemia). Careful review of the blood film in ALL usually reveals peripheral lymphoid blasts but bone marrow morphology, flow cytometry and genetics are critical components of confirming the diagnosis of ALL in this context.

Immune Thrombocytopenic Purpura (ITP) is a much more common cause of isolated thrombocytopenia than ALL. It can usually be readily distinguished from ALL by the short history of bruising, petechiae and mucosal bleeding in a well child, the absence of splenomegaly or pathological lymphadenopathy and normal haematological indices other than platelets. Review of the blood film by an experienced morphologist obviates the need for a diagnostic bone marrow in almost all cases.

As mentioned above the presenting features of ALL can mimic those of JIA. Thus, although blood film review and LDH can help distinguish ALL and JIA in many cases, it is recommended that a diagnostic bone marrow is performed in all cases of JIA prior to starting treatment with steroids [4, 32, 33].

2.2 Laboratory Features

2.2.1 Haematology

Variable degrees of cytopenia are the hallmark of ALL. Full tri-lineage cytopenia is, however, only seen in one third of cases, whilst rarely (<5% of cases) the blood count may be entirely normal. Median white cell count at presentation is 12, with nearly half of cases presenting with a WCC <10, 22% over 50 and 12% over 100 [34]. Blasts are usually but not always morphologically detectable in the peripheral blood.

Coagulopathy is usually mild, and rarely (except in association with hyperleucocytosis) associated with haemorrhage. Hyperfibrinolysis and acquired fXIII deficiency can rarely be seen at presentation requiring coagulation factor support [35]. Patients with t(17;19) ALL can present with disseminated intravascular coagulopathy (DIC).

2.2.2 Biochemistry

Mild hypokalaemia is the commonest electrolyte disturbance seen at diagnosis in acute leukaemia [36]. Hyperuricaemia is common, seen in 50% of cases at presentation [37], but severe renal impairment at diagnosis is rare and usually associated with bulky disease and renal infiltration. Likewise, active tumour lysis syndrome prior to any treatment, with associated hyperphosphataemia, hypocalcaemia and hyperkalaemia can occasionally be seen at presentation. As noted above hypercalcemia at presentation is associated with the t(17;19) gene translocation. Mildly raised liver enzymes are seen in 10–20% of cases and have no prognostic significance. Significant hyperbilirubinaemia can occasionally be seen if there is significant hepatic sinusoidal involvement by ALL.

2.2.3 Bone Marrow Aspirate

The bone marrow aspirate is usually effaced by lymphoblasts. The WHO morphological classification into L1/L2/L3 was initially shown to have prognostic significance, as were the classic hand-mirror cells [38, 39], but neither has prognostic or clinical utility on contemporary protocols. L3 morphology is helpful in differentiating ALL from the rare leukaemia presentation of Burkitt lymphoma. Haemodilute aspirates are seen in the well-recognised 'inaspirable' cases but the bone marrow trephine is universally packed in this scenario. Rarely the aspirate may be hypocellular with few lymphoblasts (see 2.11 above) or may show bone marrow necrosis.

2.2.4 Lumbar Puncture

Examination of the CSF is a routine part of the diagnostic workup. B lymphocytes are not normally found in the CNS and only small numbers of T-lymphocytes. CNS leukaemia is classically categorised on cytospin analysis as CNS1 no blasts, CNS2 < 5 blasts/uL and CNS3 ≥ 5 cells/uL. More recently the term traumatic lumbar puncture has been added, either with or without blasts, based on evidence that the presence of contaminating blasts from peripheral blood adversely affects prognosis [40].

Immunophenotyping and genetic analysis are critical to the diagnostic workup of ALL. These are discussed in details in Chaps. 3 and 4.

Table 2.2 Routine investigations in suspected ALL

Blood tests	Comments
Full blood count and film	
Coagulation screen including fibrinogen	
Group and Save	
Electrolytes, creatinine, urea	
Uric acid	
LDH	
Liver function	
TPMT genotype	Affects 6MP dosing (often started 5th week of treatment)
Immunoglobulins	To detect underlying immune deficiency states
Viral serology (VZV, EBV, CMV, hepatitis B, HSV)	Pre-transfused CMV state important for patients proceeding to SCT. VZV status affects VZV prophylaxis strategy in many supportive care guidelines. Prior HSV exposure alerts to risk of HSV stomatitis
Pregnancy test	All females of childbearing age
Bone Marrow tests	
Morphology	
Flow cytometry	
Cytogenetics and molecular genetics	
Minimal residual disease	Should be 1st aspirate from a second site to avoid haemodilution
Other	
CSF cell count, cytospin and flow cytometry	Should have therapeutic intrathecal chemotherapy given at the same procedure
Chest X-ray	Critical investigation before transferring any? ALL between hospitals

2.3 Diagnostic Workup

The routine investigations required in ALL are shown in Table 2.2. It is important that other investigations such as spinal, chest, CNS or renal imaging are considered depending on presenting symptomatology.

2.4 Prognostic Factors

The significance of individual prognostic factors has varied over time, partly as other factors are shown to be more relevant, and importantly as effective therapy is developed in response to known prognostic factors they lose their significance in contemporary trials (which should therefore be seen as a mark of successful treatment adaptation). Thus prognostic factors are never independent of the treatment protocol within which they are described.

2.5 Individual Prognostic Factors

2.5.1 Age

Increasing age as a continuous variable is associated with a worse prognosis, and has remained so throughout the history of development in leukaemia treatment [41]. For ease it is typically studied as a categorical variable. The best prognosis is seen in children aged <10, an intermediate prognosis in those age 10–15 with the worst prognosis for those age 16+ [42, 43]. In part increasing age is a surrogate marker for immunophenotype and genetics, with T-ALL under-represented and the good risk genetic lesions seen much more commonly in children aged <10 [44]. However, increasing age is associated with an increased risk of toxicity and attendant increased treatment related mortality and morbidity which limits delivery of intensive treatment [43]. This is of particular concern for teenagers and young adults.

2.5.2 White Cell Count

White cell count was one of the earliest prognostic factors to be identified [41], and despite risk adapted treatment remains a significant prognostic factor in multivariate analysis on contemporary protocols [34]. It's also a continuous variable but often applied in categorical thresholds as in the NCI risk score.

2.5.3 NCI Risk Score

In 1993 the National Cancer Institute in the USA sponsored a conference with the intention of improving uniformity of risk-stratification practice across study groups, so that results of the various groups protocols could be better compared [45]. This conference, after reviewing outcome data at the time, came up with the a prognostic score that defined patients as NCI standard risk (age 1–9.99 and WCC <50) or high risk (age $\geq$ 10 or WCC $\geq$ 50). This has subsequently been applied by many treating groups to stratify patients for treatments of different intensity and but has also found use in comparison of outcomes between trials. It continues to have prognostic value, even in contemporary trials that use it as a risk-stratifying factor [34], thus confirming that treatment modifications based on age and WCC have only been partially successful.

2.5.4 Gender

Male sex was identified early as a marker of adverse prognosis [46]. It was subsequently included in risk scores (e.g. Oxford Hazard Score) [47–49]. In many studies since it has remained of prognostic significance though loses significance

in multivariate analysis on contemporary protocols [34]. The reasons behind the prognostic value of gender in ALL include higher prevalence of T-ALL and reduced sensitivity to standard doses of oral maintenance chemotherapy in males [50, 51].

2.5.5 CNS Disease

The presence of CNS disease has for many years been recognised as an adverse prognostic factor, and treatment modification based on the presence or absence of CNS disease is almost universal in contemporary protocols. Additionally CNS2 and TLP+ were identified as markers of poor prognosis in some protocols [40, 52]. CNS3 status retains an adverse outcome but CNS2 and TLP+ are no longer prognostic in trials with early CNS directed therapy [53]. The presence of CNS disease at diagnosis tends to lose prognostic significance in multivariate analysis as it is associated with other high risk features such as white cell count and T-cell phenotype. The increasing evidence of sub-microscopic CNS disease at presentation using more sensitive methods such as flow cytometry, quantitative PCR and next generation sequencing, whilst of considerable scientific interest, have yet to be shown of prognostic signficance on which treatment should be modified [54]?

2.5.6 Disease Bulk

Early studies showed a poor prognostic value of markers of disease bulk such as hepatomegaly, splenomegaly and mediastinal mass, and these were incorporated into multifactorial hazard scores by some co-operative groups [55, 56]. However, they have lost prognostic significance in the modern era and are rarely used as risk-stratifying factors any longer.

2.5.7 Immunophenotype

T-ALL historically has a significantly worse prognosis than B-precursor disease [45], though this correlates with other high risk features (age, white cell count) and its significance has been significantly ameliorated in contemporary protocols. High CD45 expression has recently been shown to be a marker of poor prognosis in BFM protocols that remains significant in multivariate analysis. This requires further validation in other treatment strategies [57].

2.5.8 Genetics

As discussed in much greater detail in Chap. 4, ALL is a genetically heterogeneous disease. Several of these genetic subgroups have clear prognostic implications. Good risk genetic subtypes include ETV6-RUNX1 (t(12;21)) and high hyperdiploidy, whilst poor risk genetic lesions include the Philadelphia Chromosome (t(9;22)), MLL gene rearrangements, hypodiploidy, near haploidy and intrachromosomal amplification of chromosome 21 (iamp21). All of these are used as stratifying risk factors in some co-operative group trials.

More recently a variety of copy number alterations (CNA, for example ERG, CRLF-2, IKZF-1) and cryptic translocations (e.g. EBF1-PDGFR-B) have been identified in ALL cells that have prognostic value in some protocols [58–62].

Host genetics also has prognostic (as well as therapeutic) value, the best known of which is Thiopurine Methyl transferase (TPMT) status, which has prognostic value in some but not all protocols [63, 64]. More recently genomic profiling has shown that germline single nucleotide polymorphisms (SNPs) are associated with relapse risk [65].

2.5.9 Response to Treatment

The previously mentioned risk factors can be considered static risk factors; that is, they are not modifiable and many can in principle be identified at diagnosis. However, dynamic risk factors, namely response to treatment have gradually come to be the most important prognostic variables in clinical use today.

Prolonged time to complete remission was identified very early in the evolution of leukaemia therapy as having a poor prognosis [66]. Early response to treatment (defined as response assessment prior to the end of induction treatment) was first identified as having prognostic significance by the CCG [67] and has repeatedly been shown to be of major prognostic significance since then [68]. In a landmark trial CCG-1882 it was subsequently shown that intervention on the basis of unfavourable early response could improve prognosis [69].

Absolute lymphocyte count (ALC) at the end of induction (EOI) is also of prognostic value, but higher lymphocyte counts at EOI are associated with other favourable features so EOI ALC loses prognostic significance in multivariate analysis [70].

The development of minimal residual disease analysis by molecular and flowcytometric methods during the 1990s enabled much better assessment of response to treatment and this was immediately noted to be of major prognostic significance [71, 72]. MRD analysis has become a routine component of all contemporary treatment protocols and is the most powerful prognostic tools in use today. This is discussed in detail in Chap. 6.

It is clear, however, that the above mentioned prognostic variables interact. MRD responses vary depending on genetic subtype of disease [73], and despite the routine risk stratification of treatment by age, WCC, MRD and genetics, these four variables continue to have prognostic value in multivariate analysis and are used as risk-stratifying factors in most contemporary protocols.

The integration of such variables enables the identification of groups of patients with very low risk and very high risk of relapse. However, many relapses now occur in those considered intermediate risk and further evolution of prognostic algorithms is required [53, 55]. Novel genetic features noted above and/or further developments in MRD technology may enable more specific prediction of risk in the future.

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Chapter 3
Diagnostic Flow Cytometry and Immunophenotypic Classification

Julie Irving

3.1 Introduction

Haemopoiesis begins with a quiescent stem cell that gives rise to daughter cells capable of differentiation along multiple lineages. Differentiation progresses in a series of stages to produce functional, mature cells of all lineages and is orchestrated by sequential gene expression [1]. CD antigens are cell surface proteins which have diverse functional roles in haemopoiesis including signal transduction, enzymes, growth factor receptors and adhesion molecules and can be widely expressed or restricted to a specific stage of maturation/activation of a defined lineage. Thus, patterns of CD antigen expression can identify the lineage, maturation and functional stages of cells and are invaluable for evaluating normal haemopoiesis and the malignant state, including ALL.

While the immunophenotypic diagnosis of ALL was initially performed using fluorescence microscopy and immunocytochemistry, because of the increasing requirement for more extensive antigen expression, these methods have been supplanted by flow cytometry. This powerful methodology allows objective analysis of large numbers of cells in a short time and is multi parameter, with most routine cytometers having the capacity to measure expression levels of at least six antigens in each cell simultaneously. As such, it has become preferred method for the immunophenotyping for acute leukaemias (AL) and other haematological malignancies. Figure 3.1 shows the maturation cascade of B cells in a normal bone marrow compared to that of a patient with B-LL/Lymphoma, where the cells are arrested at an early stage of differentiation.

J. Irving
Wolfson Childhood Cancer Research Centre, Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne NE1 7RU, UK
e-mail: julie.irving@newcastle.ac.uk

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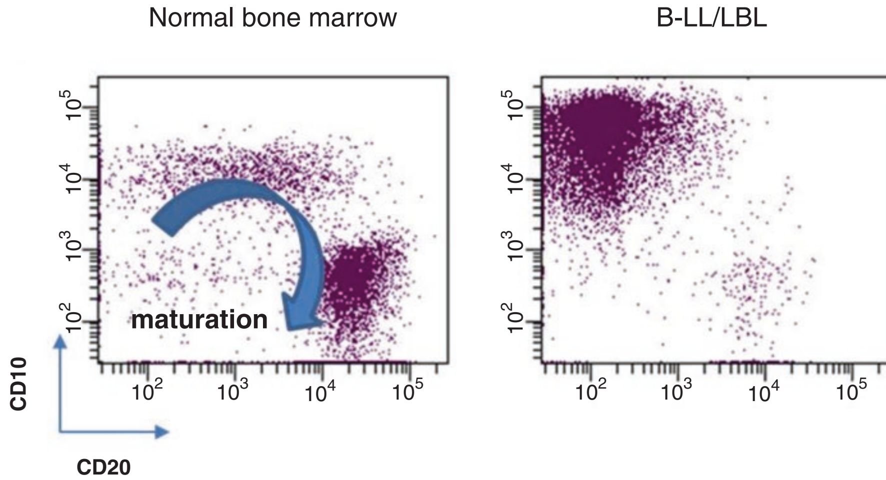

Fig. 3.1 Normal and arrested B cell maturation. Normal bone marrow and a newly diagnosed B-LL samples were labelled with antibodies to CD19, CD10 and CD20 and analysed by flow cytometry. *Dot plots* of CD10 and CD20 are shown of cells already gated for lymphoid and CD19 positivity

3.2 Immunophenotype of T-Lymphoblastic Leukemia/ Lymphoma

The WHO classification defines T Lymphoblastic Leukemia/Lymphoma as a neoplasm of lymphoblasts committed to the T cell lineage involving BM, blood or presenting as a tissue-based mass involving thymus, lymph nodes or extranodal sites. By convention, a T-LBL diagnosis is made when there is no or minimal blood or BM involvement (<20% infiltrate), while a diagnosis of T-ALL is made when there is extensive blood and BM disease. T-ALL is less common that B lineage ALL, accounting for around 15% of all cases and is associated with older adolescents and a male predominance. In contrast, almost 90% of LBL are of T cell lineage.

While the earliest marker of T cell lineage is CD7, the most specific marker is CD3, thus CD7 and CD3 expression are required for the diagnosis of T-lineage leukaemia/(Table 3.1) [2]. Cytoplasmic CD3 precedes surface CD3 during normal lymphoid development and in two-thirds of T-ALL is solely cytoplasmic, with about half of these cases co-expressing cytoplasmic TCR proteins [3, 4]. In the remainder, CD3 is expressed on the cell surface along with TCR proteins. Other CD antigens usually expressed include CD2, CD5 and TdT and commonly expressed are CD1a, CD4 and/or CD8, CD10 and/or CD21. CD34, CD10 and myeloid antigens, including CD13 and/or CD33 can be expressed too [5]. CD79a is weakly expressed in about one third of cases. The antigen expression can define intrathymic

Table 3.1 Immunophenotypic subgroups of childhood ALL

Antigen expression -% of cases positive											
Subtype	CD19	CD22[a]	CD79	CD10	CD7	CD5	CD3	Cytoplasmic IgM	Surface IgM	Surface Ig κ or λ	Prevalence (%)
Early Pre-B	100	>95	>95	95	5	0	0	0	0	0	60–65
Pre-B	100	100	100	>95	0	<2	0	100[b]	0	0	20–25
B	100	100	100	50	0	0	0	>95	>95	>95	2–3
T	<5	0	30	45	100	95	100[a]	0	0	0	15–18

Modified with permission from Pui et al. [2]
[a]May be only present in the cytoplasm
[b]IgM heavy chains only

differentiation stages including early T-ALL (surface CD3−, CD4− and CD8−), mid or common (surface CD3−, CD4+, CD8+, and CD1a +) and late (surface CD3+, CD1a− and either CD4 + or CD8+). T-ALL usually have a more immature immunophenotype compared to T-LBL but there is overlap [6].

3.3 Early T-Cell Precursor ALL

In 2009, Coustan-Smith et al. used gene expression profiling to identify a distinct subtype of T ALL, known as early T-cell precursor (ETP) ALL [7]. ETP-ALL constitutes up to 16% of all T ALL and is characterised by the immunophenotype, CD1a and CD8 negative, CD5 weak positive or negative (<75% blasts positive) and positivity (>25% blasts) for at least one stem-cell or myeloid marker antigen including CD34, CD117, HLADR, CD13, CD33, CD11b and CD65. They also typically express CD2 and cytoplasmic CD3 and may express CD4, but these are not part of the definition. Although the gene expression profile of ETP-ALL is similar to that of the murine ETP, there is overlap with normal and myeloid leukaemia haematopoietic stem cell profiles and the mutational spectrum is more typical of myeloid malignancies (discussed further in Chap. 4) [8]. In early studies, ETP-ALL patients were shown to be high risk, with increased rates of remission failure, relapse and a poorer overall survival [7, 9, 10]. The high risk nature has also been observed in adolescent/adult studies, suggesting the need for development of a more effective clinical management strategy for this subgroup [11]. However, recent data from larger patient cohorts treated on more contemporary regimens show a non-significant (UKALL 2003) or no difference (COG AALL0434) in outcome for ETP versus non ETP T-ALL, thus at present there are insufficient grounds to alter risk stratification of children with an ETP-ALL immunophenotype [12, 13]. However, ETP-ALL has been added as a provisional new entity in the revised WHO classification of myeloid neoplasms and acute leukaemia [14].

3.4 Immunophenotype of B-Lymphoblastic Leukemia/ Lymphoma

B-ALL/LBL cells are characterized by the expression of the B-cell markers CD19, cCD22, and cCD79a and lack expression of cytoplasmic (or surface) CD3 and of myeloperoxidase. Most are positive for CD10, CD24, surface CD22, CD34 and Tdt [5], while CD20 is variable [15]. CD45 is often absent and is a useful for tracking disease during treatment [16] (see Chap. 6). There are three recognised stages, early pre-B ALL, pre-B ALL, and B-cell ALL. The first two are often grouped and referred to as precursor B ALL. Early pre-B ALL is characterized by absent immunoglobulin synthesis, thus surface immunoglobulins and cytoplasmic IgM heavy chains are undetectable and are the major group, representing 60–65% of the total. The next maturation step, pre-B ALL, is defined by the presence of cytoplasmic IgM heavy chains but no detectable surface immunoglobulins. The more mature, B-cell ALL, has expression of complete surface immunoglobulins and represents ~3% of childhood ALL cases. CD20 and CD10 are often expressed and CD34 is negative. Morphologically, they have a FAB L3 appearance and are associated with c-MYC gene rearrangements (see Chap. 4). B-cell ALL and the leukaemic phase of Burkitt's lymphoma are often indistinguishable. Mature B-ALL is treated as stage IV Burkitt's lymphoma.

3.5 Acute Leukaemia of Ambiguous Lineage

While most acute leukaemias (AL) can be classified as derived from the myeloid or lymphoid lineage, in up to 5%, blasts have immunophenotypic features of both or neither lineage. They may have blasts which co express myeloid and lymphoid markers (biphenotypic or BAL) or there are two different distinct populations of blasts (bilineal) (Fig. 3.2). Two diagnostic algorithms have been used to define this entity. The first of these was published in 1995 by the European Group for Immunological Characterization of Acute Leukemias (EGIL) who described a point system to score biphenotypic ALL [17, 18] (Table 3.2). The second, published in 2008 by the World Health Organization (WHO) defined acute leukaemia of ambiguous lineage as *showing no clear evidence of*

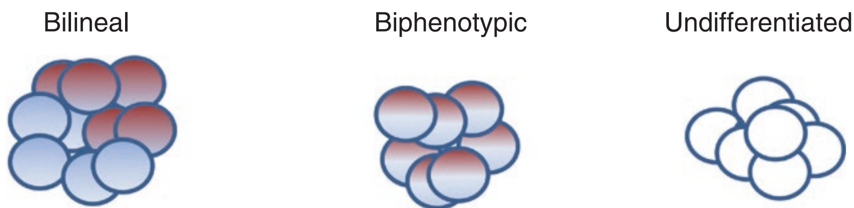

Fig. 3.2 Diagrammatic representation of bilineage, biphenotypic and undifferentiated acute leukaemia. *Red* and *blue* colouring representing myeloid and lymphoid CD antigen expression

Table 3.2 EGIL scoring matrix for lineage assignment

Points	T cell lineage	B cell lineage	Myeloid lineage
2 points	CD79	CD3	MPO
		TCR	
1 points	cCd22	CD2	cd13
	CD10	CD5	CD33
	CD19	CD8	CDw65
	CD20	CD10	CD117
0.5 points	Tdt	Tdt	CD15
	CD24	Cd17	CD15
		cd1A	CD24

Modified with permission from Bene et al. [17]
A score of 2 or more is necessary to assign a lineage

Table 3.3 2016 WHO criteria for lineage assignment for a diagnosis of mixed phenotype acute leukaemia

Myeloid lineage
[a]Myeloperoxidase (detected by flow cytometry, immunohistochemistry or cytochemistry)
or
Monocytic differentiation -at least 2 of the following: nonspecific esterase, CD11c, CD14, CD64, lysozyme
T lineage
Strong[b] cytoplasmic CD3 (detected by flow cytometry with antibodies to CD3 epsilon chain)
or
Surface CD3 (rare in mixed phenotype acute leukemia)
B lineage (multiple antigens required)
Strong[b] CD19 with at least *1* of the following strongly expressed: CD79a, cytoplasmic CD22, CD10
or
Weak CD19 with at least *2* of the following strongly expressed: CD79a, cytoplasmic CD22, CD10

Modified from Arber et al. [14]
[a]There are caveats related to weaker antigen expression, or to expression by IHC only
[b]Strong defined as equal or brighter than the normal B or T cells in the sample

differentiation along a single lineage and included acute undifferentiated leukaemia, where leukaemic cells have no lineage specific antigens and grouped bilineal and biphenotypic acute leukaemias under a new heading of Mixed Phenotype Acute Leukaemia (MPAL) [19] (Table 3.3). This new algorithm relies on fewer, more lineage-specific markers, with myeloid lineage designation requiring the presence of myeloperoxidase (detected by flow cytometry, immunohistochemistry, or cytochemistry) or at least two monocytic parameters including nonspecific esterase, CD11c, CD14, or CD64; T lineage, cytoplasmic or surface CD3 and B lineage, at least two antigens, including CD19, CD79a, CD22, and CD10. MPALs that are Ph+ or MLL rearranged are considered a distinct subgroup.

All possible combinations of MPAL are observed, with B/myeloid and T/myeloid representing ~90% of all cases, while and B/T and B/T/myeloid are more rare [20, 21]. Because of the more limited set of lineage markers and cytogenetic exclusions, several studies show fewer patients classified as WHO-MPAL, compared to the EGIL-BAL [22, 23]. However, whichever classification is used, they appear to have an inferior survival compared to standard ALL and are more in line with AML [24, 25]. While there is no consensus policy on the best treatment for MPAL, the iBFM AMBI2012 Study/Registry [26] aims to retrospectively analyse immunophenotype, molecular genetics, therapy and outcome of MPAL as a first step towards standardizing therapy and to better understand the biology of this AL subtype.

3.6 Antigen Expression: Correlation to Prognosis and Cytogenetics

While the above text describes the key antigens necessary to diagnose and classify ALL, the increasing availability of antibodies to a wide range of CD antigens has revealed substantial phenotypic heterogeneity. This is simply depicted in Fig. 3.3 showing the variability in expression levels of CD19, CD34 and CD10 in four diagnostic B lineage ALL. Clearly this heterogeneity is not fully represented by simply stating the percentage of positive cells and while mean fluorescent intensity is a gauge

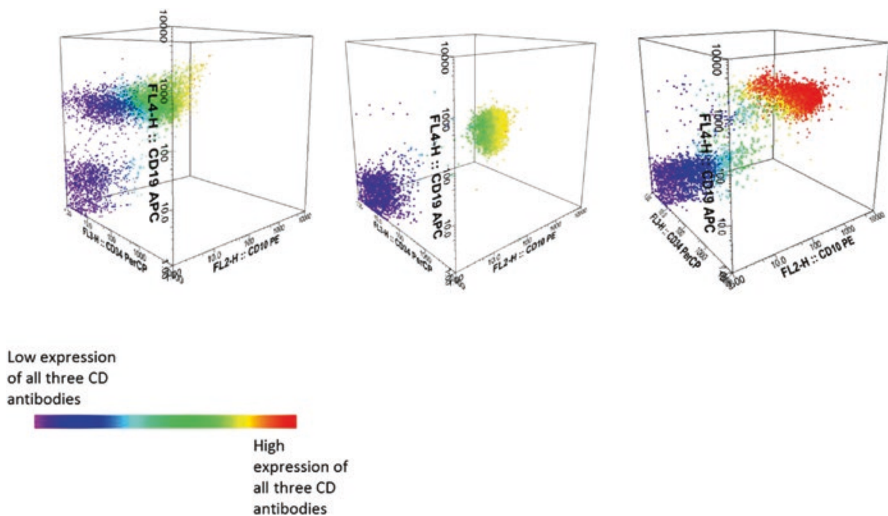

Fig. 3.3 Heterogeneity of CD antigen expression in ALL. Expression of CD34, CD10 and CD19 was assessed in three newly diagnosed precursor B ALL cells by flow cytometry and analysed using FlowJo software and depicted in three dimensions. Non leukaemic cells, negative for all three antigens, are *purple*, while leukaemic cells show a spectrum of colour depending on the intensity of all three antigens

of antigen levels, it is relative and can vary over time due to cytometer age, antibody batches and with instrument service and/or laser replacement. Absolute fluorescence can be measured using beads with varying amounts of known levels of fluorochromes which are used to create a standard curve from which fluorescence of an antigen in ALL cells can be expressed in units termed mean equivalents of soluble fluorescein (MESF). Using these approaches, several studies have investigated whether more extensive immunophenotyping can offer further prognostic relevance.

In a large American study (POG 1991) of more than 1200 children with B lineage ALL, fluorescent intensity as measured in absolute terms using the MESF approach found that two CD antigens, CD45 and CD20 were highly prognostic [27]. Patients with the brightest expression of CD45 (>75th percentile) or relatively bright CD20 (>25th percentile) on their ALL blasts had an increased risk of treatment failure which was independent of traditional risk factors including age, white blood cell count, DNA ploidy or poor risk chromosomal translocations. More recent data from a European clinical trial (IBFM 2000) confirmed these observations in both Pre B and T lineage ALL [28]. In this case, CD45 levels in ALL cells were expressed relative to that of normal mature lymphocytes in the same sample. Similar to the American trial, children with high CD45 expression were associated with a lower event-free survival (EFS) rate; for PreB this was 72% compared to 86% and for T ALL, 60% compared to 78%. The difference in EFS was mainly attributable to a higher cumulative relapse rate and again, CD45 expression maintained its significance in multivariate analyses. These findings may be explained by the functional role of CD45 as an integral membrane protein tyrosine phosphatase which regulates antigen receptor and cytokine signalling by dephosphorylating SRC and JAK family kinases, key pathways which regulate cell growth and survival and are known to be aberrant in ALL. Interestingly, the gene encoding CD45 (*PTPRC*) has recently found to be inactivated by gene deletion in T ALL [29], thus appropriate expression levels of CD45 appear critical for normal lymphocyte cell function.

While the CD45 correlative data are mirrored in two large independent clinical trials, for CD20, two separate reports (three consecutive St Jude Total Therapy trials and NILG-ALL 09/00) concluded that CD20 expression was not associated with inferior outcome [15, 30]. Whether this is due to differences in relative rather than absolute quantitation methodologies or loss of prognostic relevance in more contemporary regimens, is not clear. Other antigens of interest are those associated with the myeloid lineage but data are not consistent and their independence as prognostic markers is less clear. Another study showed that expression of CD38 was highly variable and low expression on leukaemic blasts relative to that of normal B cell progenitors was significantly associated with MRD positivity [31]. Since MRD positivity is a surrogate marker of outcome, it suggests that levels of this antigen too may have prognostic relevance.

One difficulty with correlating antigen levels with prognosis is the interdependence of immunophenotype with cytogenetic abnormalities which have well defined prognostic relevance and thus has prompted investigations into whether immunophenotyping can accurately classify key cytogenetics subtypes [32]. For example, those with the good prognostic *ETV6-RUNX1* ALL gene fusion, de Zen et al. showed

a higher intensity of CD10 and HLADR and lower levels of CD20, CD45, CD135 and CD34, compared to *ETV6-RUNX1* negative cases [26]. This 'characteristic' immunophenotype classified *ETV6-RUNX1* ALL with a sensitivity of 86% (i.e. the true positive rate) and a specificity of 100% (i.e. true negative rate). Another study prospectively evaluated an immunophenotypic signature for *ETV6-RUNX1* ALL which consisted of dual CD9 and CD20 negative/weak positivity in more than 200 children and found it to have an 88% sensitivity and 71% specificity for the presence of the gene fusion [33]. While these data are good, the general consensus is that they are not sufficiently robust to replace standard cytogenetic analyses to classify *ETV6-RUNX1* ALL, particularly since the fluorescence in situ hybridisation methodology used as standard to detect this gene fusion, will also identify high risk cases with iAMP21 (see Chap. 4) [34].

3.7 Extended Leukaemia Immunophenotyping

The EuroFlow Consortium [35] are international experts in the fields of flow cytometry and molecular diagnostics and aim to develop and standardize fast, accurate and highly sensitive flow cytometric tests for the diagnosis, prognosis and treatment effectiveness in haematological malignancies. The ALL immunophenotyping panel designed by the Euroflow consortium enables the diagnosis and subclassification of ALL according to the WHO classification but also goes on to provide more extensive immunophenotypic characterization [23]. This additional information allows discrimination of the leukaemia from normal and regenerating precursor B-cells, termed a leukaemia-associated immunophenotype, that can subsequently be used to track minimal residual disease during ALL therapy [36] (see Chap. 6) and also includes markers associated with genetic aberrations, such as the chondroitin sulphate proteoglycan, NG2, associated with 11q2 3 rearrangements [33]. In addition, they have developed a simple flow cytometric immunobead assay to detect ALL related fusion proteins in cell lysates which utilises a bead-bound catching antibody to detect one half of the fusion protein and a fluorochrome-conjugated antibody to bind the other half [37, 38]. Such assays have been developed for a number of fusion proteins relevant to ALL and have high specificity and sensitivity. For example, fully concordant results were obtained between the immunobead assay and reverse transcriptase PCR of fusion gene transcripts for BCR-ABL [38].

3.8 Conclusions and Perspective

Immunophenotyping has played an important role in the impressive improvement in survival rates for ALL over the last few decades and will continue to be essential in diagnosis and patient stratification. The recent impressive activity of novel antibody therapies such as the bi-specific T-cell engaging antiCD19/CD3 antibody,

Blinatumomab [39] and targeted immunotherapy using patient-specific chimeric antigen receptor T cells [40, 41], further emphasises the importance of immunophenotyping, since expression levels of the target antigen predict response and emerging resistance to these new agents. In one of the largest trials for relapsed ALL (IntReALL) [42], standard risk patients are being randomized to receive the targeted anti-CD22 drug, Epratuzumab during consolidation. CD22 levels have been shown to vary 100-fold [43], thus IntReALL flow laboratories have developed a standardised methodology to quantify both the levels of CD22 and amount of Epratuzumab binding in ALL cells relative to that of mature B cells in a fixed preparation of peripheral blood (Mejstrikova et al. unpublished observations). Subsequent correlation of these parameters with response will determine their value as predictive biomarkers of Epratuzumab response and optimise the use of this drug in future trials. Following the impressive clinical benefit of the tyrosine kinase inhibitor (TKI), imatinib, for children with Ph+ ALL [44], this other class of targeted drug is also likely to be increasingly used in ALL therapy. In this respect too, immunophenotyping may have an emerging role. The development of robust antibodies that are highly specific to phosphorylated antigens and suitable for flow cytometry allows key signalling pathways to be monitored for hyper-activation in ALL subgroups such as Ph-like (e.g. phosphorylated CRKL) or Ras pathway mutated ALL (e.g. phosphorylated ERK) [45, 46]. Patients with these high risk ALL types may be candidates for TKI therapies, such as dasatinib and MEK inhibitors, respectively.

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Chapter 4
Cytogenetics and Molecular Genetics

Christine J. Harrison, Anthony V. Moorman, Claire Schwab, Ilaria Iacobucci, and Charles Mullighan

4.1 Introduction

Childhood acute lymphoblastic leukaemia (ALL) is characterised by genetic aberrations, which drive the initiation and progression of the leukaemic clone [1–3]. These genetic abnormalities arise as primary or secondary events. Primary abnormalities are usually present in all leukaemic cells and initiate transformation of the pre-leukemic clone, which upon acquisition of additional cooperating (secondary) genetic changes manifests as overt ALL. Primary abnormalities are often chromosomal translocations, resulting in chimeric fusion genes, oncogene deregulation or aneuploidy (gain or loss of whole chromosomes); whereas secondary abnormalities are usually copy number alterations (CNA) (frequently micro-deletions) and point mutations, which may be present in only a subset of the leukaemic cells, giving rise to a complex branching sub-clonal architecture [4]. In ALL, there are strong correlations between the primary chromosomal abnormality and the spectrum of secondary or cooperating mutations observed in each subtype [5]. Comprehensive genetic testing can confirm the diagnosis of ALL and identify important prognostic and predictive biomarkers. Key primary chromosomal abnormalities are used to stratify patients into different risk groups used to tailor therapy.

C.J. Harrison (✉) • A.V. Moorman • C. Schwab
Leukaemia Research Cytogenetics Group, Newcastle University, Newcastle upon Tyne, UK
e-mail: christine.harrison@newcastle.ac.uk; anthony.moorman@newcastle.ac.uk; claire.schwab@newcastle.ac.uk

I. Iacobucci • C. Mulligan
St Jude Children's Research Hospital, Memphis, TN, USA
e-mail: Ilaria.Iacobucci@stjude.org; Charles.Mullighan@stjude.org

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4.2 Abnormality Detection Methodologies

A bone marrow or peripheral blood sample taken at initial diagnosis provides the material for genetic diagnostic tests (Table 4.1). A combination of cytogenetic analysis, fluorescence in situ hybridisation (FISH) and reverse transcription followed by polymerase chain reaction (RT-PCR) are the gold-standard methods, but more modern techniques, such as DNA copy number arrays, Multiplex Ligation-dependent Probe Amplification (MLPA), targeted gene sequencing and exome, transcriptome or genome-wide sequencing approaches are increasingly being used to identify genetic abnormalities. Metaphase analysis and karyotyping allow chromosomal abnormalities to be accurately described according to the International System for Human Cytogenetic Nomenclature [6]. By making use of gene specific probes, either alone or in combination, FISH provides an accurate high throughput screening method for a range of chromosomal abnormalities in interphase [7].

More recently, array-based comparative genomic hybridization and single nucleotide polymorphism (SNP) arrays allow genome-wide copy number changes to be identified at increasingly high resolution. MLPA is a rapid multiplex PCR method for detection of abnormal copy numbers of selected DNA or RNA loci. MLPA is able to identify the frequent, single gene aberrations which are too small to be detected by FISH, but do not comprehensively identify all alterations in each gene interrogated. Increasingly, next generation sequencing approaches are being used to detect mutations, structural variations, copy number and zygosity changes, which are revolutionising genetic screening in routine practice [8].

4.3 Chromosomal Abnormalities in BCP-ALL

In B-cell precursor ALL (BCP-ALL), genetic changes play an important role in diagnosis, whilst providing important clinical information. In about 75% of these cases, significant specific chromosomal rearrangements occur including, high hyperdiploidy (51–65 chromosomes), the translocation t(12;21)(p13;q22) (encoding *ETV6-RUNX1*), t(1;19)(p13;q22) (*TCF3-PBX1*), hypodiploidy (≤44 chromosomes), rearrangements of *KMT2A* (*MLL*) at 11q23, t(9;22)(q34;q11.1)/*BCR-ABL1*, rearrangements of the immunoglobulin heavy chain (*IGH*) and intrachromosomal amplification of chromosome 21 (iAMP21) (Table 4.2). These alterations are correlated with age (Fig. 4.1). For example, *KMT2A* rearrangements, particularly the t(4;11)(q21;q23) translocation, are most frequent in infants less than 1 year of age. The dramatic decrease in high hyperdiploidy and *ETV6-RUNX1* fusion after the age of 10 years is mirrored by an increase in the proportion of patients with *BCR-ABL1* fusion and *IGH* translocations in teenagers and young adults. iAMP21 specifically occurs in older children. In view of the association with prognosis, based on detailed analysis over a number of clinical treatment trials, genetic abnormalities can be grouped together according to their known risk group to produce simplified survival curves indicating good, intermediate and poor outcomes. Table 4.2 provides details of some of the principal chromosomal abnormalities that have been associated with favourable or poor outcome. The majority of clinical trials

Table 4.1 Overview of the principal genetic techniques used for the diagnosis and classification of acute lymphoblastic leukaemia

Technique(s)	Scope/target of test	Abnormality resolution	Sensitivity	Detectable types of abnormality
G banded cytogenetics	Evaluation of chromosome number and morphology	Low	Low/medium	Translocations (gene fusions), deletions, amplifications, aneuploidy
Locus specific FISH	Enumeration and localisation of specific DNA target sequences	Medium	Medium/high	Translocations (gene fusions), deletions, amplifications, aneuploidy
DNA index	Measurement of DNA content	N/A	High	Aneuploidy
Reverse Transcription (RT) Polymerase Chain Reaction (PCR)	Qualitative and quantitative assessment of fusion transcripts. can be multiplexed	N/A	High	Gene fusions (translocations)
Multiplex Ligation-Dependent Probe Amplification (MLPA)	Enumeration of multiple specific DNA target sequences	High	Medium	Copy number alterations – deletions and amplifications
Single Nucleotide Polymorphism (SNP) arrays	Simultaneous evaluation of tens of thousands SNPs across the genome	Very high (depends on distribution of SNPs)	Medium	Copy number alterations (deletions and amplifications), aneuploidy, and copy number neutral (CNN) loss of heterozygosity (LOH)[a]
Array Comparative Genome Hybridisation (aCGH)	Simultaneous enumeration of tens of thousands DNA probes across the genome	Very high (depends on distribution of probes)	Medium	Copy number alterations – deletions and amplifications – and aneuploidy
Targeted gene sequencing	Next generation sequencing of genomic regions using a customised library	Very high	Very high	Originally developed to detect sequence mutations but with appropriate library definition, sophisticated bioinformatic pipelines and long read lengths all types of abnormality can be accurately detected

[a]Also referred to as acquired uniparental disomy (aUPD)

Table 4.2 Overview of the key prognostic chromosomal abnormalities in paediatric B-cell precursor acute lymphoblastic leukaemia

Genetic subtype	Chromosomal abnormality	Molecular genetic features	Frequency in paediatric/TYA ALL
Good risk abnormalities			
ETV6-RUNX1	t(12;21)(p13;q22)	*ETV6-RUNX1* fusion	25%
		ETV6 deletion	
High hyperdiploidy	51–65 chromosomes	Whole chromosome gains,	25–30%
		FLT3, NRAS, KRAS, PTPN11, PAX5 mutations,	
t(1;19)/*TCF3*-PBX1	t(1;19)(q23;p13)	*TCF3-PBX1*	3–5%
High risk abnormalities			
Philadelphia chromosome	t(9;22)(q34;q11)	*BCR-ABL1* fusion	2–3%
		IKZF1 deletion	
KMT2A (MLL) rearrangements	t(4;11)(q21;q23)	*KMT2A-AFF1* fusion	2% overall but ~75–80% among infants
	t(6;11)(q27;q23)	*KMT2A-MLLT4* fusion	
	t(9;11)(p21;q23)	*KMT2A-MLLT3* fusion	
	t(10;11)(p12;q23)	*KMT2A-MLLT10* fusion	
	t(11;19)(q23;p13.3)	*KMT2A-MLLT1* fusion	
t(17;19)/*TCF3-HLF*	t(17;19)(q22;p13)	*TCF3-HLF* fusion	<1%
Near haploidy	<30 chromosomes	Whole chromosome losses and polyploidisation	1%
		RAS pathway mutations	
Low hypodiploidy	30–39 chromosomes	Whole chromosome losses and polyploidisation	1%
		TP53 mutations	
iAMP21	Grossly abnormal chromosome 21	Deregulation of genes on chromosome 21	2–3% overall. More common in older children
		CRLF2 deregulation, RAS pathway mutations	
Ph-like	DUX4/ERG	MEF2D	ZNF384

stratify patients to different treatment regimens according to the presence of genetic abnormalities. Figure 4.2 illustrates the benefit of such risk stratification in two consecutive UK clinical trials of paediatric ALL.

4.3.1 Favourable Risk Abnormalities

4.3.1.1 High Hyperdiploidy

High hyperdiploidy (51–65 chromosomes) is characterized by non-random gain of chromosomes, most commonly X, 4, 6, 8, 10, 14, 17, 18 and 21 [9]. It is most frequently observed in childhood BCP-ALL, found in approximately 30% of cases,

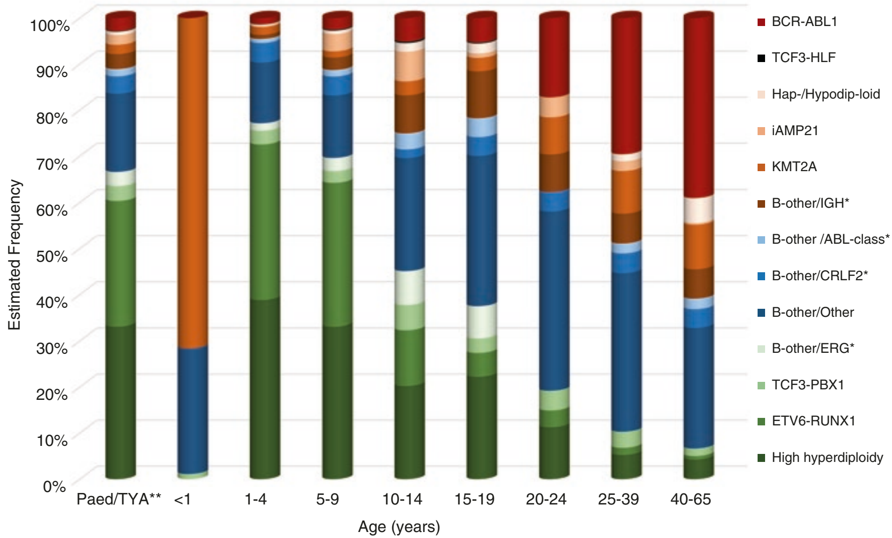

Fig. 4.1 Estimated age-specific frequencies of key genetic abnormalities in acute lymphoblastic leukaemia. * Estimates for *IGH*, *ABL*-class, *CRLF2* and *ERG* abnormalities are based on data from screening B-other ALL patients only. Adults (25–65 years) were not screened for *ERG*-altered ALL. ** Overall estimates for patients aged 1–24 years old at diagnosis

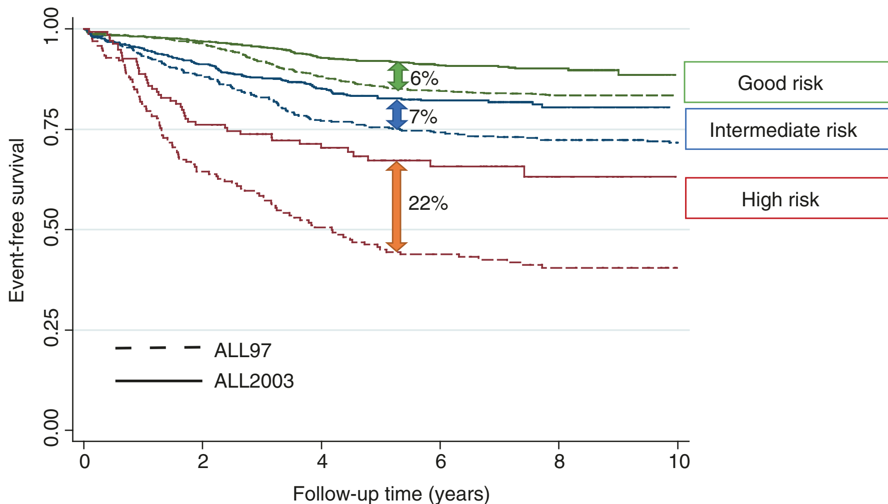

Fig. 4.2 Improvement in outcome for patients two consecutive clinical trial stratified by genetic risk group. Patients with *ETV6-RUNX1* or high hyperdiploidy were classified as good risk while those patients with *BCR-ABL1*, *KMT2A* translocations, near haploidy, low hypodiploidy, iAMP21 or *TCF3-HLF* were classified as high risk. All other patients were classified as intermediate risk

with maximal prevalence at 2–4 years of age. It is associated with a favourable prognosis but up to 10% patients relapse [10]. About 50% of cases contain structural chromosomal abnormalities in addition to their chromosomal gains, most commonly these changes are unbalanced [11]. Importantly, cells from high hyperdiploid cases are difficult to culture with the normal cells outgrowing the leukemic cells, thus cytogenetic analysis often fails. In such cases flow cytometric analysis of DNA index and/or FISH analyses are useful to accurately detect high hyperdiploidy [12]. These limitations are also being circumvented by the use of digital karyotyping from whole genome sequencing.

Little is known about the mechanisms responsible for, and the molecular consequences of high hyperdiploidy. Genome-wide cytosine methylation profiling of ALL has shown that many of the genes on triploid chromosomes that do not show increased expression are subject to methylation-induced silencing [13].

4.3.1.2 t(12;21)(p13;q22)/*ETV6-RUNX1*

The t(12;21)(p13;q22)/*ETV6-RUNX1* fusion is present in about 25% of childhood BCP-ALL. The translocation is usually cryptic on cytogenetic analysis, requiring FISH or RT-PCR detection. The presence of *ETV6-RUNX1* at diagnosis correlates with a good prognosis and recent data indicate that an overall survival of 99% is achieved on contemporary risk-directed therapy [14]. The rearrangement commonly arises in utero but the prolonged latency to overt leukaemia and twin studies, together with screening of normal cord blood, has shown that the *ETV6-RUNX1* fusion gene is present at a 100-fold higher incidence than the corresponding risk of the leukaemia, indicating that additional genetic events are needed for the development of this disease [15–18]. This suggestion is supported by the description of multiple recurring submicroscopic genetic changes targeting multiple cellular pathways in this type of ALL [19].

4.3.1.3 t(1;19)(q23;p13)/*TCF3-PBX1*

The translocation t(1;19)(q23;p13), results in the *TCF3-PBX1* fusion, present in about 6% of childhood BCP-ALL [20]. It correlates with a pre-B immunophenotype with the leukaemic cells expressing cytoplasmic immunoglobulin heavy chain. It is readily identified by cytogenetics, FISH and RT-PCR. *TCF3-PBX1*-ALL was originally considered to be a high risk leukaemia that often presented with central nervous system involvement and an increased risk of relapse [21]. On modern intensive protocols, patients with *TCF3-PBX1* fusion are classified as good or intermediate risk [22], although some studies have reported it has an independent risk factor for central nervous system (CNS) relapse [23, 24]. At the cytogenetic level, the translocation occurs either as a balanced t(1;19) or, more commonly as an unbalanced der(19)t(1;19) with duplication of the long arm of chromosome 1 distal to *PBX1*.

4.3.2 Poor-Risk Chromosomal Abnormalities

4.3.2.1 t(9;22)(q34;q11.1)/*BCR-ABL1*

The t(9;22)(q34;q11.1)/*BCR-ABL1* results in the formation of the Philadelphia chromosome (Ph), which is the hallmark of chronic myeloid leukaemia (CML). It is found in about 3% of paediatric ALL, increasing in incidence with age (Fig. 4.1) [25]. At the chromosomal level, about 60% of Philadelphia positive ALL have additional secondary aberrations present at diagnosis including gain of a second copy of the Ph and a hyperdiploid karyotype [26, 27]. In these cases with a hyperdiploid karyotype, it is important to identify *BCR-ABL1* so that the patient is treated appropriately. In a minority of cases, the t(9;22) is cryptic by cytogenetic analysis, although the *BCR-ABL1* fusion can be detected by FISH and PCR [28]. The *BCR-ABL1* fusion is associated with a poor prognosis in all age groups, a high incidence of CNS involvement at diagnosis, a high white cell count and early development of multidrug resistance [29, 30]. *BCR-ABL1* is a predictive biomarker for targeted therapy with a tyrosine kinase inhibitor (TKI) of ABL1, such as imatinib or dasatinib [31]. TKIs directly inhibit the leukaemogenic effect of the BCR-ABL1 oncoprotein and in combination with standard chemotherapy the historically poor outcome of *BCR-ABL1* positive ALL has improved considerably [32, 33].

4.3.2.2 t(17;19)(q22;p13)/*TCF3-HLF*

A variant of the t(1;19) translocation results in fusion of the *HLF* gene, located at 17q22, to *TCF3* as a result of the t(17;19)(q23;p13) translocation [34]. At the molecular level two types of rearrangements give rise to chimeric oncoproteins, which comprise either exons 1–13 (Type 1) or exons 1–12 (Type 2) of *TCF3* and exon 4 of *HLF*. These two molecular subgroups strongly correlate with specific clinical features: type 1 with disseminated intravascular coagulation and type 2 with hypercalcemia [35]. This translocation is very rare with an estimated incidence of 0.1% in BCP-ALL [1]. Patients are older with a median age of 13 years and a low white cell count. However, the majority of known patients have relapsed and died within 2 years of diagnosis [1, 21]. Thus despite the rarity of this translocation, it is important that these patients are accurately identified.

4.3.2.3 11q23/*KMT2A* Gene Rearrangements

Childhood leukaemia with rearrangements of the *KMT2A* (formerly *MLL*) gene represents a unique entity with both lymphoid and myeloid features and a poor outcome. It is characterized by an early leukemic initiation (likely in utero for most childhood cases) as indicated by the high prevalent of *KMT2A* translocations in infant ALL (<1 year), where they account for approximately 80% patients [36],

compared to their presence in only about 3% of childhood ALL [22]. The *KMT2A* gene, located at 11q23, undergoes rearrangements, usually translocations, with a plethora of partner genes; with *AFF1* (*AF4*), *MLLT1* (*ENL*), *MLLT4* (*AF6*), *MLLT3* (*AF9*) and *MLLT10* (*AF10*) accounting for >85% of ALL cases [37]. In view of the wide range of partners, FISH with a dual colour break-apart probe directed to the 11q23 locus provides the most reliable method of detection. The prognosis of *KMT2A* rearranged infant leukaemia is very poor with an event free survival of approximately 37% [36, 38]. Among older children with ALL, the event free survival is about 45%, with the t(4;11)(q21;q23) being associated with a worse prognosis [39]. Targeted therapies for *KMT2A* transformed leukaemia is attractive due to the aggressiveness of this disease. Studies have highlighted the importance of epigenetic dysregulation in this subgroup and, in particular, the requirement for the histone methyltransferase, DOT1L, which interacts directly or indirectly with several of the *KMT2A* fusion partners [40]. Selective killing of cells with *KMT2A* rearrangements upon exposure to EPZ004777, a potent inhibitor towards DOT1L, has been shown [41]. These studies raise the possibilities of developing a targeted therapy for these high-risk patients using such DOT1L inhibitors [42]. In addition, inhibitors directed towards the protein-protein interaction between *KMT2A* fusion proteins and Menin have shown promising results with reversal of the oncogenic activity of *KMT2A*-rearranged leukaemia [43].

4.3.2.4 Near-Haploidy and Low Hypodiploidy

Near-haploidy (23–31 chromosomes) and low hypodiploidy (32–39 chromosomes) are defined by extensive whole chromosomal loss and a dismal outcome [44, 45]. The leukaemic cells of both subgroups commonly undergo doubling of their chromosome number by endoreduplication, so that hypodiploid and hyperdiploid/triploid cell populations coexist, a phenomenon known as "masked" hypodiploidy. Consequently, the hypodiploid clone may not always be readily evident at diagnosis if subclonal. Thus interphase FISH and/or flow cytometric analysis of DNA index should be used in combination with cytogenetic analysis at diagnosis to ensure accurate detection of hypodiploid clones, as this influences risk stratification and treatment. This doubling can create a diagnostic dilemma if only the masked sub-clone is detected, which can masquerade as high hyperdiploidy [44]. However, the pattern of chromosomal loss/gain is distinctive and these two subgroups are usually distinguishable from one another. Patients with near-haploidy (23–29 chromosomes) tend to be younger, with a median age of 7 years. The prognosis is poor, with a 3-year event free survival of only 29%. The most common diploid chromosomes are chromosomes 21, 14, 18 and the sex chromosomes: indeed, loss of chromosome 21 is not observed in ALL. Structural rearrangements are rare and chimaeric fusions have not been identified by whole genome or RNA-sequencing [46]. Low hypodiploid (30–39 chromosomes) patients tend to be older than those with near-haploidy, the majority being 10 years or older with a median age of 15 years. Only chromosomes 7 and 17 have been shown to always be monosomic. The prognosis of this ALL subtype is equally poor to that of near-haploid ALL, but is

strongly influenced by response to initial remission-induction chemotherapy, with patients achieving minimal residual disease (MRD) negativity at the end of induction having a more favourable outcome [47]. Near haploid and low hypodiploid ALL have distinct constellations of genetic alterations [46]. Near haploid cases have a high incidence of mutations activating Ras signalling and inactivating deletions and mutations of the IKAROS family gene, *IKZF3* (AIOLOS). The majority of low hypodiploid cases have mutations of the tumour suppressor gene, *TP53*. These *TP53* sequence mutations are commonly present in matched non-tumour DNA, suggesting germline inheritance. This has been confirmed in a limited number of family studies, indicating that low hypodiploid ALL is a manifestation of Li-Fraumeni syndrome [46, 48]. Hypodiploid cells from both near-haploid and low hypodiploid cases exhibit activation of Ras-Raf-MEK-ERK and phosphatidylinositol-3-OH kinase (PI3K) signalling that is sensitive to PI3K and PI3K/mTOR inhibitors, suggesting that PI3K inhibition may provide an alternative therapeutic approach.

4.3.2.5 Intrachromosomal Amplification of Chromosome 21 (iAMP21)

iAMP21 manifests as a grossly abnormal chromosome 21 generated via breakage-fusion-bridge cycles and chromothripsis [49–53]. The result of these rearrangements is the amplification and loss of multiple regions along the length of chromosome 21. It is defined as a primary cytogenetic change, usually observed in patients lacking other key cytogenetic alterations, although rare cases of iAMP21 in association with *ETV6-RUNX1* and *BCR-ABL1* have been described [54]. The consistent feature of iAMP21-ALL is amplification of the chromosome 21 in which *RUNX1* is located, providing the basis for a convenient and reliable FISH detection assay, using probes specific for the *RUNX1* gene. The internationally accepted definition of iAMP21 is three or more extra copies of *RUNX1* on a single abnormal chromosome 21 (5 or more signals per cell) [55]. Patients with iAMP21-ALL are older with a median age of 9 years but a low median white cell count (WCC) [54, 56]. Studies by the UK and the Children's Oncology Group, USA (COG) have demonstrated that iAMP21 patients treated as standard risk have a very high rate of relapse (>80%) but that this is significantly reduced (<20%) when the patients are treated intensively [57, 58]. Thus iAMP21 can be considered both a prognostic and predictive biomarker in paediatric ALL. However, the Associazione Italiana di Ematologia ed Oncologia Pediatrica (AEIOP) and Berlin-Frankfurt-Munster (BFM) study groups reported that MRD can also be used to identify iAMP21-ALL patients at risk of relapse [59, 60].

4.3.3 B-ALL Lacking Sentinel Chromosomal Rearrangements

Approximately 70% paediatric BCP-ALL harbour an established genetic abnormality of diagnostic and/or prognostic relevance (Fig. 4.1) such as those described above. The remaining 30% patients, without one of these abnormalities, are collectively

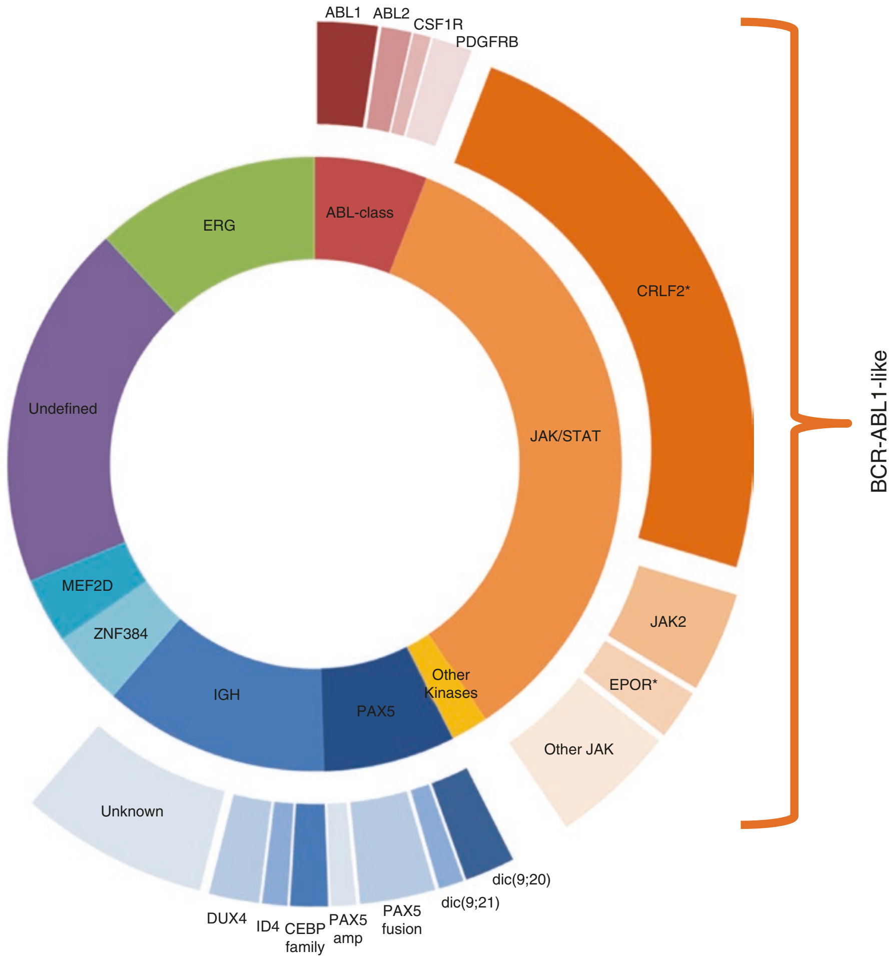

Fig. 4.3 A diagram illustrating the genetic heterogeneity of B-other acute lymphoblastic leukaemia

referred to as "B-other" ALL, as they have historically been considered to lack an established chromosomal rearrangements. However, gene-expression profiling, targeted genetic analysis and genome-wide profiling, including DNA and RNA sequencing of such cases have identified a number of distinct biological subgroups as well as specific genetic abnormalities of clinical relevance (Fig. 4.3) [61, 62].

4.3.3.1 Ph-Like or *BCR-ABL1*-Like ALL

The leukaemic samples from Ph-like patients harbour a gene expression similar to *BCR-ABL1* ALL, but such patients lack the gene fusion [63–67]. The genomic profile of these patients is enriched for *IKZF1* deletions, *CRLF2* deregulation and

JAK2 mutations. Overall Ph-like ALL accounts for ~50% of B-other (~15% of childhood BCP-ALL) and is associated with a poor outcome. Approximately 50% of Ph-like ALL harbour *CLRF2* rearrangements. Transcriptome and whole genome sequencing has shown that non-*CRLF2*-rearranged Ph-like ALL harbour a diverse range of genomic alterations that activate cytokine receptors and tyrosine kinases including: *ABL1, ABL2, EPOR, JAK2* and *PDGFRB,* present in up to 5% of BCP-ALL overall, with a higher prevalence with increasing age [68, 69]. These alterations are most commonly chromosomal rearrangements resulting in chimeric fusion genes, of which *EBF1-PDGFRB* is the most common [70] (Fig. 4.4). A complex network of these kinase-activating aberrations has been revealed with many occurring in few patients [71–73]. In up to 20% of Ph-like cases alternative alterations activating kinase signalling occur, including activating mutations of *FLT3* and *IL7R*, as well as focal deletions of *SH2B3*, which constrain JAK signalling. These diverse genetic alterations activate a limited number of signalling pathways, notably *ABL1, PDGFRB* and JAK-STAT signalling. As primary leukemic cells and xenografts of Ph-like ALL were highly sensitive to TKIs [68, 74], responsiveness of refractory Ph-like ALL patients to appropriate TKI therapy, for example *EBF1-PDGFRB* ALL to imatinib [75, 76], was not unexpected. Thus it is accepted that the majority of Ph-like ALL will show response to therapy with a limited range of TKIs, for *ABL1, ABL2* and *PDGFRB* rearrangements, and JAK inhibitors, such as ruxolitinib, for alterations activating JAK-STAT signalling (*EPOR, IL7R, JAK2* and *SH2B3*) [70, 71, 75, 77]. The optimal detection method is challenging given the number of genes involved and the complex nature of some of the chromosomal rearrangements which give rise to these fusion genes. FISH using probes to target the kinase gene provides a simple and efficient strategy for detection of many of the fusions, especially *ABL1*, *ABL2*, *PDGFRB*, *CSF1R*, and *JAK2*, and can readily be incorporated into current screening algorithms. However, assays based on next-generation sequencing technology are likely to provide a more comprehensive approach.

4.3.3.2 *CRLF2* Rearrangements and Janus Kinase Mutations in ALL

The cytokine receptor, *CRLF2,* is rearranged or mutated in approximately 5% of BCP-ALL but 50% of Down syndrome ALL (DS-ALL) and 50–60% of Ph-like ALL [78–81]. It is located in the pseudoautosomal region of the sex chromosomes (PAR1) at Xp22.3/Yp11.3. It encodes cytokine receptor-like factor 2 (thymic stromal lymphopoietin receptor, TSLPR). CRLF2 forms a heterodimeric receptor with interleukin-7 receptor alpha for the ligand, TSLP (thymic stromal lymphopoietin). *CRLF2* is rearranged by translocation into the immunoglobulin heavy chain locus (*IGH-CRLF2*), or by a focal deletion upstream of *CRLF2,* resulting in expression of *P2RY8-CRLF2* that encodes full-length CRLF2. Both rearrangements result in aberrant overexpression of CRLF2 on the cell surface of leukaemic lymphoblasts that may be detected by flow cytometric immunophenotyping [79]. Less commonly a CRLF2 p.Phe232Cys mutation results in receptor dimerization and overexpression [80]. Approximately half of

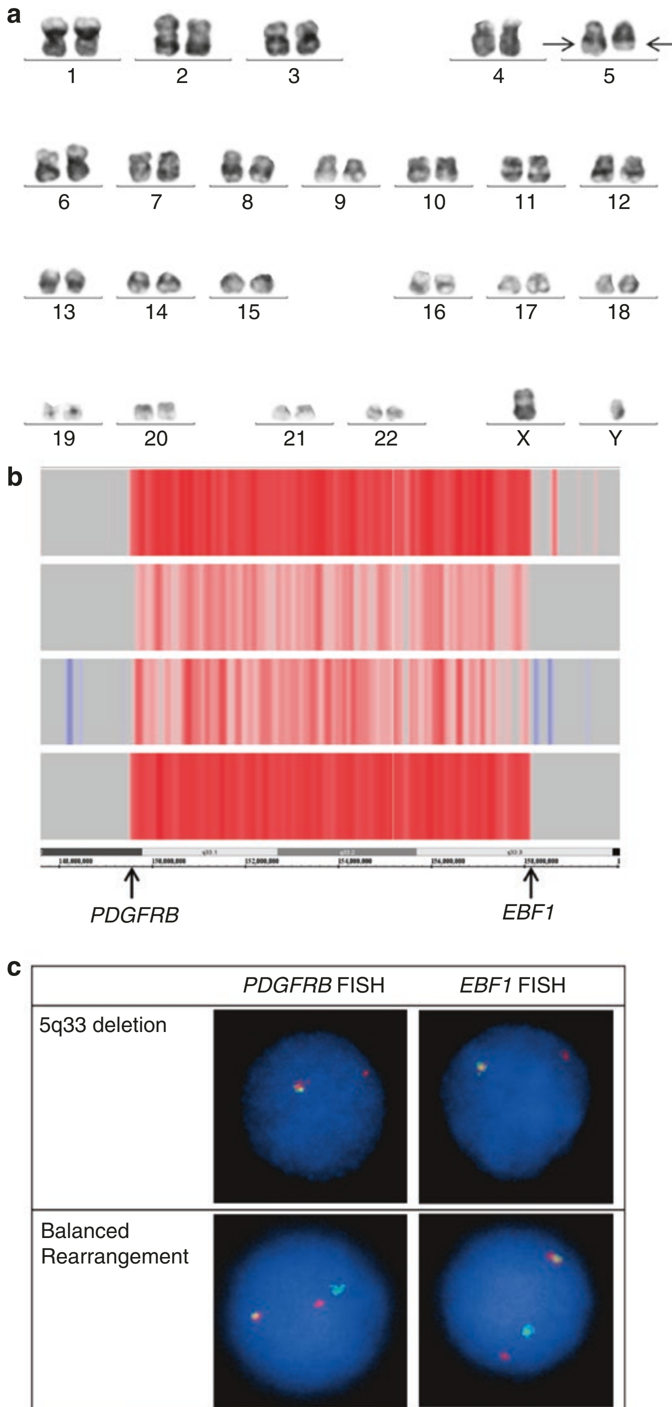

Fig. 4.4 *EBF1-PDGFRB* gene fusion can result from a balanced t(5;5)(q31;q33) translocation (**a**) or an interstitial deletion of chromosome del(5)(q3q33), red = deletion (**b**). Both abnormalities can be readily detected by the application of dual colour break apart probes (**c**)

CRLF2-rearranged ALL harbour activating mutations of the Janus kinase genes, *JAK1* and *JAK2,* otherwise uncommon in BCP-ALL [78, 79, 82]. These JAK mutations are most often missense mutations at or near p.Arg683 in the pseudokinase domain of JAK2, distinct from the JAK2 p.Val617Phe mutations of myeloproliferative disease. Less common are activating mutations in the kinase domain of *JAK1* and *JAK2*. The *JAK1/2* mutant alleles alone and in cooperation with *CRLF2* overexpression are transforming *in vitro,* suggesting that these two lesions are central to lymphoid transformation [83–85]. The prognostic impact of *CRLF2* alterations has been the subject of debate, with some indicating poor outcome [86, 87], while others concluded that they were not [60, 88–90]. However, this is in part due to differences in methodology for measuring *CRLF2* expression or genetic alteration [86, 91, 92]. Importantly, deregulated expression of CRLF2 receptor is only observed in leukaemic cells with a *CRLF2* rearrangement. The type of *CRLF2* alteration is age-associated, with *P2RY8-CRLF2* more common in children and DS-ALL, and *IGH-CRLF2* in older patients. In non-DS ALL, *CRLF2* alterations and *JAK* mutations are associated with *IKZF1* deletion/mutation and poor outcome, particularly in cohorts of high risk BCP-ALL [62, 86–88]. Recent studies performed by COG have confirmed that *CRLF2* and *IKZF1* alterations are associated with inferior outcome in multiple cohorts, and notably, that elevated *CRLF2* expression in the absence of rearrangement is also an adverse prognostic feature [92].

Although *CRLF2* may not be a robust prognostic marker, it is an attractive therapeutic target particularly within the context of DS-ALL, who are prone to the toxic side-effects of chemotherapy. Therefore, inhibition of the JAK and PI3K pathways represent potential therapeutic strategies in these cases [68, 71]. *CRLF2* rearranged leukaemic cells with deregulated CRLF2 exhibit activation of JAK-STAT and PI3K/mTOR pathways and are sensitive to JAK and mTOR inhibitors in vitro and in vivo [74, 93]. An early phase trial of the JAK inhibitor, ruxolitinib (ADVL1011), in relapsed and refractory disease, including cases with *CRLF2* rearrangements and/or JAK mutations, has been initiated (clinicaltrials.gov identifier NCT01164163).

4.3.3.3 *DUX4* and *ERG*-Deregulated ALL

Deregulation of the homeobox transcription factor gene Double Homeobox 4, *DUX4*, and the ETS transcription factor gene *ERG* are hallmarks of a subtype of B-progenitor ALL with a distinct immunophenotype and gene expression profile that comprises up to 7% of BCP-ALL. *DUX4* encodes a double homeobox transcription factor located in a macrosatellite *D4Z4* repeat in the subtelomeric region of the long arm of chromosome 4. Deletion of part of this repeat unit is causative of facioscapulohumeral dystrophy, and *DUX4* rearrangements have been reported in a subset of Ewing-like sarcoma (CIC-DUX4) [94, 95]. In this subtype of BCP-ALL, rearrangements of *DUX4* to *IGH* place *DUX4* under the control of the immunoglobulin heavy chain enhancer, resulting in increased expression of *DUX4,* commonly with an aberrant C terminus [96–99]. Less commonly *ERG-DUX4* fusions have also been described [98]. In addition, *DUX4*-rearranged BCP-ALL cases exhibit transcriptional deregulation and deletion of *ERG*. Multiple prior studies have reported intragenic deletions of the *ERG* gene in about 5% of childhood ALL

[19, 100, 101]. In these studies, *ERG* deletions commonly involved internal exons, resulting in loss of the central inhibitory and pointed domains and expression of an aberrant C-terminal ERG fragment that retains the ETS and transactivation domains. Recent genome sequencing studies have shown that *DUX4*-rearranged cases express an aberrant ERG isoform (ERGalt) [99]. ERGalt utilizes a non-canonical first exon whose transcription is initiated by DUX4 binding. This isoform retains the DNA-binding and transactivating domains of ERG, but inhibits wild-type ERG transcriptional activity and is transforming. This configuration represents a distinct subtype of BCP-ALL in which rearrangement of a transcription factor (*DUX4)* results in deregulation of a second transcription factor (ERG) that cooperate in leukaemogenesis. Notably, *DUX4/ERG* ALL is associated with a favourable outcome, despite the presence of concomitant genetic alterations otherwise associated with a poor outcome, such as *IKZF1* deletions, present in about 40% of cases [62, 101, 102].

4.3.3.4 Translocations Involving the *IGH* Locus

IGH translocations are well recognised in lymphoid malignancies, where the juxtaposition of an oncogene to the *IGH* enhancer drives its over-expression [103]. *IGH* translocations are frequent in lymphomas and mature leukaemias. However, recent studies have revealed an extensive network of *IGH* translocations specific to BCP-ALL, which drive the expression of a variety of oncogenes [80, 104]. The most common *IGH* translocation involves *CRLF2*, accounting for ~25% cases as discussed above [78, 79]. Another cytokine receptor, EPOR has rarely been described as an *IGH* partner, although a range of cryptic *EPOR* abnormalities have now been described [69]. Other recurrent translocation partners include four members of the *CEBP* gene family (*CEPBA*/19q13, *CEBPB*/20q13, *CEBPD*/8q11 and *CEPBE*/14q11) [105] and *ID4*/6p22 [106], accounting for ~10% and 7% cases, respectively. Although many *IGH* partner genes have now been identified, there does not appear to be any functional link between them. Given their wide spectrum and the finding that several, including *IGH-CRLF2*, are cytogenetically cryptic, FISH using a break-apart probe specific for the *IGH* locus provides a reliable detection method. The most notable clinical feature of patients with *IGH* translocations is their age profile. Their frequency is low among children under 10 years old (<3%) but considerably higher (10%) among adolescents and young adults (15–24 years) [104] (Fig. 4.1). Patients with *IGH* translocations have been shown to have an inferior outcome compared to other patients in both the adolescent and young adult groups [104].

4.3.3.5 *PAX5* Rearrangements

A number of recurrent chromosomal abnormalities have been reported, which target the B-cell development gene *PAX5* [19, 107–111]. The consequence of many of these aberrations is whole or partial deletion of the *PAX5* gene, however, a subset result in the expression of in-frame fusion genes encoding chimeric

proteins [110]. The most frequent abnormality is the dic(9;20)(p13;q11) found in ~1–2% of BCP-ALL. It is rarely associated with any of the major cytogenetic subtypes [112, 113]. Due to the similarity in size and banding pattern of 9p and 20q, it is often misidentified as monosomy 20 [114]. Although cases are identical at the cytogenetic level, the breakpoints within *PAX5* and at 20q11 are heterogeneous at the molecular level, suggesting that loss of genetic material rather than expression of a fusion protein is the functional consequence of this aberration [115, 116]. As stated previously the *BCR-ABL1*-like group is enriched for patients with dic(9;20) [64, 117]. A subset of dic(9;20) patients also have the *P2RY8-CRLF2* fusion, possibly accounting for their *BCR-ABL1*-like gene expression signature [88]. The dic(9;12)(p11–12;p11–13) occurs at a lower frequency (1%) than dic(9;20) and is often seen secondary to *ETV6-RUNX1*, where it is associated with loss of the non-translocated copy of *ETV6* and loss of the entire *PAX5* gene [22, 118]. In contrast, when dic(9;12) is present in patients lacking any major cytogenetic abnormalities, it is associated the *PAX5-ETV6* fusion protein [118, 119]. Expression of the *PAX5-ETV6* fusion in BCP-ALL has been shown to alter gene expression with an opposite dominant effect over the wild-type PAX5 and is thought to drive leukaemogenesis in these patients [120]. Many other fusion genes involving *PAX5* have been identified in BCP-ALL [19, 107–109, 111, 121–123]. Collectively they occur in approximately 2% of cases, but many have been reported in few or single cases, therefore complete elucidation of their functional consequences is difficult. Similar to the *PAX5-ETV6* fusion, *PAX5* translocations commonly result in the 5′N-terminal DNA binding domain of *PAX5* fusing to the 3′C-terminal of the partner gene substituting the PAX5 regulatory domains [110]. It has been demonstrated that several of these fusions inhibit the normal transcriptional activation effects of PAX5, although it remains to be directly shown that these fusions promote leukaemogenesis through haploinsufficiency of wild-type *PAX5*, which has been shown for *Pax5* haploinsufficiency in mouse models [124], or whether they are exerting an oncogenic effect.

MEF2D and *ZNF384* Gene Fusions

Myocyte Enhancer Factor 2D (*MEF2D*) and Zinc Finger 384 (*ZNF384*) characterize distinct B-ALL subtypes, accounting for 6.7% and 7.3% adults and 3.4% and 3.9% paediatric patients, respectively [97, 125, 126]. MEF2D is a member of the myocyte-specific enhancer factor 2 (MEF2) family of transcription factors involved in neuronal development and myogenesis and regulated by class II histone deacetylase. The N-terminus of MEF2D can be fused to one of several partners, most commonly BCL9, HNRNPUL1, SS18, FOXJ2, CSF1R and DAZAP1 [73, 98, 104, 125, 126]. MEF2D ALL is associated with older age of onset, an aberrant (CD10 negative, CD38 positive) immunophenotype and poor outcome. Expression of MEF2D fusions can transform NIH3T3 fibroblasts and mouse hematopoietic cells *in vitro* and can give rise to the development of leukaemia with low penetrance in mice [96]. Moreover, the fusions deregulate expression of MEF2D transcriptional targets,

including HDAC9, and human xenografts of MEF2D ALL are exquisitely sensitive to HDAC inhibitors, such as panobinostat [125].

The *ZNF384* gene encodes a putative C2H2 zinc finger transcription factor involved in the regulation of matrix metalloproteinases. *ZNF384* gene translocations result in attachment of 5′ partner gene sequence to almost the entire *ZNF384* gene. Common fusion partners include *EP300*, *CREBBP*, *TAF15*, *SYNRG*, *EWSR1*, *TCF3* and *ARID1B* [96–98, 125, 127, 128]. *EP300-ZNF384* fusion also characterizes a recently established mixed-phenotype acute leukaemia cell line (JIH-5) [129], suggesting that it may have a role in this leukaemia subtype. The *EP300-ZNF384* fusion alone has been demonstrated to promote the rapid development of acute leukaemia in mouse models. *ZNF384* fusions confer an intermediate prognosis and gene set enrichment analysis has shown significant up-regulation of the JAK-STAT pathway, suggesting a potential benefit from treatment with inhibitors of this pathway [96].

ETV6-RUNX1-Like

ETV6-RUNX1-like ALL represents 12% of B-other ALL, characterized by a gene expression profile similar to that of *ETV6-RUNX1*-positive cases but lacking this fusion. Alterations of *ETV6*, either by the generation of alternative gene fusions, or, rarely, *ETV6* deletions, in combination with *IKZF1* lesions, are frequent in this subgroup, suggesting that they may represent an alternative mechanism to recapitulate the same transcriptional perturbation as seen in classical *ETV6-RUNX1* fusion positive cases [98].

4.3.4 Secondary Genetic Alterations in BCP-ALL

DNA CNA such as submicroscopic deletions and amplifications, and sequence mutations are common cooperating genetic events among all cytogenetic subgroups. A range of micro-deletions affecting genes in key pathways have been reported, including lymphoid development (*PAX5, IKZF1, EBF1*), cell cycle regulation and tumour suppression (*CDKN2A/CDKN2B*, *RB1*), putative regulation of apoptosis (*BTG1*), lymphoid signalling, transcriptional regulation and co-activation (*ETV6, ERG*), regulation of chromatin structure and epigenetics [19, 130–132]. These CNA are often secondary aberrations, may be subclonal, and are acquired, lost or enriched between diagnosis and relapse [133–135]. The nature and frequency of secondary genetic lesions is subtype dependent (Fig. 4.5). For example, *KMT2A*-rearranged ALL harbours very few additional structural or sequence alterations, in comparison to other subtypes [19, 136–139]. Cooperating mutations in high hyperdiploid ALL include activating mutations in the receptor tyrosine kinase/RAS pathway in about 30% of cases, suggesting that activation of the RAS pathway or kinase signaling are important cooperating events in this ALL subtype [140]. A study of relapsed high hyperdiploid ALL identified a high

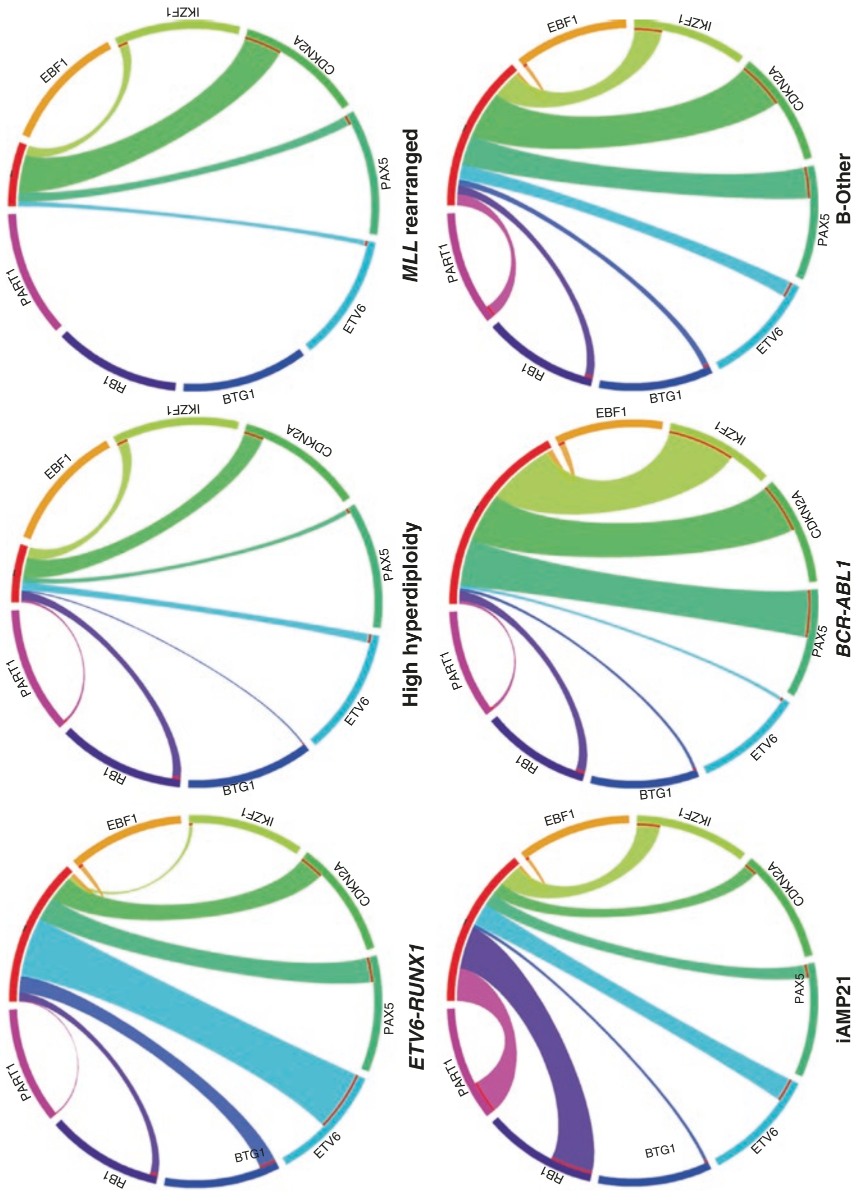

Fig. 4.5 Distribution of common secondary copy number alterations differs markedly according to the primary genetic subgroups in acute lymphoblastic leukaemia

incidence of mutations in the *CREB*-binding protein (*CREBBP*) [141]. *CREBBP* mutations have also frequent in relapsed ALL [142].

Alterations of *PAX5* (~35%), *IKZF1* (~15%) and *EBF1* (~5%) are the most common alterations, with at least two thirds of BCP-ALL harbouring one or more lesions in this pathway [19, 63]. They are usually loss of function or dominant negative lesions resulting in arrested lymphoid maturation, which is characteristic of ALL. Notably, while *PAX5* alterations are the most common genetic alteration in BCP-ALL, they are not associated with outcome [63, 143]. *IKZF1* deletions occur in 15% BCP-ALL [5], although they are more frequent in high risk cases, in particular Ph positive ALL (>70% cases) [139, 144, 145] and *BCR-ABL1*-like ALL (>40%) [63, 64, 68]. In addition, they are associated with other high risk features, such as older age, high WCC, persistent MRD and Down syndrome [5, 90]. *IKZF1* alterations include focal or large deletions that result in loss of expression of IKZF1. Focal deletions of coding exons 4–7 remove the N-terminal DNA-binding zinc fingers, leading to expression of a dominant negative isoform, IK6. Initial reports suggested that all BCP-ALL patients harbouring an *IKZF1* deletion had a significantly inferior outcome, implying that it was a reliable prognostic marker [63]. However, more recent studies based on larger and more representative cohorts have suggested that its effect is variable, with a worse outcome in Ph positive ALL [101, 102, 145–147]. It has been shown that the presence of an *IKZF1* deletion does not abrogate the prognosis associated with other good risk genetic abnormalities, such as *ETV6-RUNX1* and *DUX/ERG* alterations [89, 99, 101, 102, 148]. These findings correlate with results from studies of the interaction of *IKZF1* deletions and MRD, which have reported that *IKZF1* deletions are not prognostic among patients who clear their disease rapidly. Instead the prognostic effect is strongest in patients with higher levels of disease burden after initial chemotherapy [146, 147, 149]. Recurring sequence mutations in BCP-ALL most commonly affect lymphoid development (*PAX5*, *IKZF1*), Ras signalling (*NRAS*, *KRAS* and *NF1*), cytokine receptor signalling (*IL7R, JAK2*) and tumour suppression (*TP53*) [150]. Interestingly, certain genes are involved in multiple types of genetic aberrations, including CNA, translocations and sequence mutation (for example, *PAX5*).

Assessing the prognostic relevance of individual CNA does not consider that many cases harbour more than one deletion. This limitation has been addressed by integration of the CNA profile into existing established cytogenetic risk group classification. The CNA profile, based on the presence or absence of the eight most frequently deleted genes, segregates patients with intermediate risk cytogenetics (mostly B-other) into two new genetic risk groups (Fig. 4.4) [10]. The prognosis of patients with good or high risk cytogenetics was unaffected by their CNA profile. However, intermediate cytogenetic risk patients, separated into two subgroups (good risk versus intermediate/high risk CNA profile) with differential OS rates (98% v 87%) [10]. Thus this approach has identified a group of B-other ALL patients with a good risk CNA profile and a very low risk of relapse who potentially could be considered for treatment de-intensification. The validity of this approach is supported by observations that the prognostic effect of *IKZF1* deletions depends on the presence/absence of other deletions (e.g. *ERG* and *CDKN2A/B* deletions) and MRD levels [62, 101, 102, 149].

4.4 Genetic Rearrangements in T-Lineage ALL

T-ALL accounts for approximately 15% of childhood ALL. It is characterized by an older age of onset and male predominance [151]. Chromosomal abnormalities are evident on cytogenetic analysis in up to 70% of T-ALL cases, and commonly involve one of the T-cell antigen receptor loci, including *TRA* and *TRD* at 14q11, *TRB* at 7q34 and *TRG* at 7p14. The most common rearrangements are listed in Table 4.3. They occur in approximately one-third of T-ALL, but may be cryptic on cytogenetic analysis. These rearrangements may arise from aberrant antigen receptor gene recombination errors in the normal recombination process, leading to the generation of functional antigen receptors [152]. Rearrangements in T-ALL commonly dysregulate transcription factors, including members of the bHLH family (*MYC*, *TAL1*, *TAL2*, *LYL1* and *BHLHB1*), genes encoding the LIM-only domain proteins (*LMO1* and *LMO2*) and homeodomain genes (*TLX1* and *TLX3*). In addition, T-ALL cases frequently harbour cryptic rearrangements of *ABL1,* activation mutations of *NOTCH1*, and a spectrum of submicroscopic genetic alterations) commonly involving *CDKN2A/CDKN2B*, *PTEN* and *MYB* [153]. Essentially, T-ALL can be subdivided into three subtypes based on morphology, immunophenotype and genetics (Fig. 4.6) [154].

4.4.1 TAL1/LMO2 Rearranged T-ALL

T-ALL with rearrangements of *TAL1/LMO2* are classified as mature disease with a characteristic immumophenotype (Fig. 4.6). Alteration of *TAL1* at 1p32 is the most frequent transcription factor rearrangement in T-ALL. It arises from either the translocation, t(1;14)(p32;q11), in 3% of cases, which juxtaposes *TAL1* to the *TRA/TRD* locus, or the more frequent cryptic interstitial deletion at 1p32, present in approximately 15% of cases, resulting in a chimeric *SIL-TAL1* fusion transcript [155, 156]. Additional cases without these rearrangements express high TAL1 mRNA levels [157]. Less commonly, the *TAL2* gene is juxtaposed to the *TRB* locus as a result of the translocation, t(7;9)(q34;q32) [158]. TAL1 and LYL1 are members of the class II family of bHLH proteins. Functional evidence implicates that TAL1 mediates leukaemogenesis through a dominant negative mechanism [159].

The LIM-domain only proteins, LMO1 and LMO2*,* are commonly rearranged in T-ALL, most frequently from the translocations, t(11;14)(p15;q11) and t(11;14)(p13;q11), that juxtapose *LMO1* and *LMO2* into the *TRA* and *TRD* loci. Additional cases harbour cryptic focal deletions proximal to *LMO2* resulting in dysregulation of this locus [19, 160]. Expression of *LMO1* and *LMO2* results in T-cell self-renewal and leukaemia when expressed in thymocytes [161].

Table 4.3 Common genetic aberrations in T-ALL

Type of aberration	Chromosomal abnormality	Genetic rearrangement
Aberrant expression of transcription factors and related genes	t(1;7)(p34;q34)	*TRB-LCK*
	TAL1 deletion	*STIL-TAL1*
	t(6;7)(q23;q34	*TRB-MYB*
	t(7;9)(q34;q32)	*TRB-TAL2*
	t(7;9)(q34;q34.3)	*TRB-NOTCH1*
	t(7;11)(q34;p13)	*TRB-LMO1*
	t(7;11)(q34;p15)	*TRB-LMO2*
	t(7;12)(q34;p13.3)	*TRB-CCND2*
	t(7;19)(q34;p13)	*TRB-LYL1*
	t(8;14)(q24;q11)	*TRA/D-MYC*
	t(11;14)(p13;q11)	*TRA/D-LMO1*
	t(11;14)(p15;q11)	*TRA/D-LMO2*
	t(12;14)(p13;q11)	*TRA-CCND2*
	inv(14)(q11q32)	*TRD-BCL11B*
	t(14;14)(q11;q32)	*TRD-BCL11B*
	NKX2-1 rearrangements	*NKX2-1*
	NKX2-2 rearrangements	*NKX2-2*
	MEF2C rearrangements	*MEF2C*
	t(14;21)(q11;q22)	*TRA-OLIG2*
Abnormalities of homeodomain genes	t(7;10)(q34;q24)	*TRB-TLX1*
	t(10;14)(q24;q11)	*TRA/TRD-TLX1*
	t(5;14)(q35;q32)	*BCL11B-TLX3*
Abnormalities of the *HOXA* cluster	inv(7)(p15q34)	*TRB-HOXA*
	t(7;7)(p15;q34)	*TRB-HOXA*
	t(7;14)(p15;q11)	*TRD-HOXA*
	t(7;14)(p15;q32)	*BCL11B-HOXA*
Fusion transcripts	t(6;11)(q27;q23)	*KMT2A-MLLT4*
	t(9;9)(q34;q34)	*NUP214-ABL1*
	t(9;14)(q34;q32)	*EML1-ABL1*
	t(10;11)(p12;q14)	*MLLT10-PICALM*
Copy number changes	N/A	*MYB* duplication
	del(9p)	*CDKN2A*
	del(18)(p11)	*PTPN2*
Mutations	N/A	*NOTCH1* mutations
	N/A	*FBXW7* mutations
	N/A	*CNOT* mutations
	N/A	*PHF6* mutations

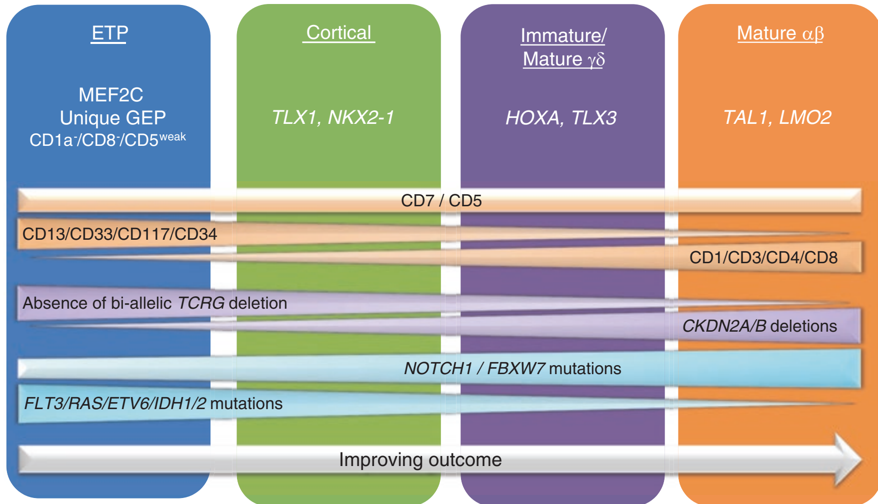

Fig. 4.6 Immunogenetic subtypes of T-ALL

4.4.2 TLX1/TLX3 Rearranged T-ALL

The cortical subtype of T-ALL is characterized by rearrangements of *TLX1* and *TLX3*. More generally, the homeobox family of transcription factors comprises two classes of genes. Class I HOX genes are in four clusters (*HOXA, HOXB, HOXC* and *HOXD*) and class II genes are distributed throughout the genome. The HOX genes exert key roles in regulation of haematopoiesis and leukaemogenesis [162]. The two HOX genes, *TLX1* and *TLX3,* are often rearranged in T-ALL. Approximately 7% of childhood T-ALL cases have ectopic expression of TLX1 arising from the translocation, t(10;14)(q24;q11), and the variant, t(7;10)(q35;q24), that juxtapose *TLX1* to the *TRA* or *TRB* loci [163, 164]. Additional cases lacking *TLX1* rearrangement exhibit overexpression of this gene [157].

Approximately 20% of childhood T-ALL cases exhibit overexpression of *TLX3* [165, 166], most commonly from the cryptic translocation, t(5;14)(q35;q32), that juxtaposes *TLX3* to *BCL11B* [167], a zinc finger protein expressed during T-cell ontogeny, recently identified as a target of deletion and somatic sequence mutation in T-ALL [168]. Several variant translocations have been identified, including *BCL11B* to *NKX2–5* [169, 170] and rearrangement of *CDK6* to *TLX3* [171]. Data regarding the prognostic importance of *TLX1* and *TLX3* in T-ALL is conflicting, which in part may be due to the presence of additional genetic alterations and mutations in these cases [172].

Exome sequencing has identified novel targets of mutation, including *CNOT3*, a member of transcriptional regulatory complex, and ribosomal proteins [173]. To gain further insight into the male sex preponderance of T-ALL, Ferrando and colleagues performed targeted capture and sequencing of X chromosome genes. They

identified sequence mutations and deletions of *PHF6* in 16% of childhood T-ALL [174]. *PHF6* alterations result in loss of *PHF6* expression and are associated with *TLX1/3* and *TAL1* rearranged ALL [174]. Although the role of PHF6 in leukaemogenesis is poorly understood, it may have complex and multifactorial roles as a tumour suppressor gene.

4.4.3 Early T-Cell Precursor ALL

A subtype of immature T-lineage ALL has been described in which the leukaemic cells lack expression of mature/cortical thymic markers, such as CD1a, CD8 and CD5, and exhibit aberrant expression of myeloid and stem cell markers. These cells exhibit a gene expression profile reminiscent of the murine early thymic (double negative 1 stage) T cell precursor that retains myeloid/macrophage differentiation capacity. These early T-cell precursor (ETP)-ALL comprise an aggressive subtype with a poor prognosis [175, 176]. *MEF2C* rearrangements have been identified a proportion of these cases [177], although they show marked diversity in the frequency and nature of genetic alterations [178]. Several cases have shown complex, multi-chromosomal structural alterations with the hallmarks of chromothripsis [179]. However, three pathways were shown to be frequently mutated: hematopoietic development, cytokine receptor and Ras signaling, as well as chromatin modification [180–185]. Loss-of-function alterations in genes encoding regulators of hematopoietic development are present in two-thirds of ETP-ALL and most commonly involve *ETV6, GATA3, IKZF1* and *RUNX1*. It is notable that many of these genes are known targets of mutation and rearrangement in other subtypes of ALL and AML. Activating mutations in cytokine receptor and Ras signaling were also present in the majority of cases, including *NRAS, KRAS, FLT3, JAK1, JAK3* and *IL7R,* similar to those previously described in other ALL subtypes. Activating mutations of IL7R, encoding the alpha chain of the interleukin 7 receptor, have also been reported [182, 186]. The IL7R mutations induce cytokine independent proliferation and activation of JAK-STAT signalling that is abrogated by JAK inhibitors, such as ruxolitinib [178]. Although IL7R mutations are only present in a proportion of ETP ALL cases, evidence of JAK-STAT activation on phosphoflow cytometry or gene expression profiling is present in the majority of cases, suggesting that JAK inhibitors are a rational therapeutic strategy for this T-ALL subtype.

ETP-ALL has a high frequency of mutations of epigenetic regulators. Most common were mutations or deletions of genes encoding components of the polycomb repressor complex 2 (PRC2; *EZH2, SUZ12, EED)*, which normally mediates histone 3 lysine 27 (H3K27) trimethylation. A range of deleterious mutations in the SET domain and elsewhere in EZH2 are observed that are predicted to be loss-of-function. As the mutational spectrum of ETP-ALL is similar to that observed in myeloid leukaemia and the transcriptional profile of ETP-ALL is similar to that of normal and malignant human hematopoietic stem cells and myeloid progenitors, but *not* the normal human early T-cell precursor [178],

"early T-cell precursor" ALL is likely a misnomer, and ETP-ALL may be more appropriately considered to be part of a spectrum of immature leukaemia of variable and often ambiguous lineage.

4.4.4 *Other T-ALL Genetic Subtypes: KMT2A Rearranged and PICALM-MLLT10*

KMT2A is rearranged in about 5% of T-ALL, most commonly to *MLLT1* [187] and more frequently in adolescents, *KMT2A*-rearranged T-ALL represents a distinct biologic entity with a transcriptional profile that differs from other *KMT2A* -rearranged cases [157, 188].

The translocation, t(10;11)(p13;q14), may be cytogenetically cryptic and results in expression of the *PICALM-MLLT10 (CALM-AF10)* fusion [189]. It is observed in up to 10% of T-ALL. Notably, both partner genes are infrequently fused to *KMT2A* and, like *KMT2A*-rearranged ALL, *PICALM-MLLT10* cases exhibit upregulation of *HOX* genes and *MEIS1,* suggesting common oncogenic pathways. This rearrangement is associated with a poor outcome.

4.5 Relapsed ALL

Leukaemias are characterised by heterogeneous subpopulations of cells containing different aberrations, which are constantly reshaping and evolving. This evolution does not proceed in a sequential linear fashion, but follows a complex branched pathway, where multiple subpopulations co-exist in the same sample and compete for ascendency. The mechanisms driving clonal evolution are incompletely understood. Recurrent infections during childhood have been suggested to drive clonal evolution [190, 191]. Recently several studies, including a comprehensive sequence analysis of 1,700 breakpoints of chromosomal rearrangements in human B-cell malignancies, have suggested that genetic lesions driving clonal evolution arise from cooperation between recombination-activating genes (RAG1 and RAG2) and activation-induced cytidine deaminase (AID) [139, 192, 193]. Mutations and copy number alterations occur independently and repeatedly through external or intrinsic factors in some but not all cells without a preferential order [4, 194]. A new clone will grow out when the cells overcome diverse evolutionary bottlenecks by advantages in competitive regenerative capacity, treatment resistance and proliferation in particular stroma or environments, or develop the capability to enter senescence. Increased knowledge of individual gene mutations in ALL has allowed us to investigate the dynamics of clonal evolution and the origin of relapse.

Relapse occurs across the spectrum of ALL subtypes. It has long been recognized that ALL genomes are not static, but exhibit acquisition of chromosomal abnormalities over time [195]. There is thus intense interest in genomic profiling of

matched diagnosis and relapse samples to dissect the genetic basis of clonal heterogeneity in ALL and the relationship of such heterogeneity to risk of relapse. Although the primary chromosomal abnormality is usually retained between diagnosis and relapse, it has been shown that the majority of ALL show changes in the patterns of their secondary genomic alterations from diagnosis to relapse [135, 196] and that many relapse-acquired lesions are present at low levels at diagnosis [133, 135]. The spectrum of chromosomal abnormalities at relapse is similar to that seen at diagnosis, although the frequency of good and poor risk abnormalities shifts in favour of the latter [197, 198]. The prognostic relevance of chromosomal abnormalities is retained at relapse. Thus patients with *ETV6-RUNX1* or high hyperdiploidy have a significantly increased likelihood of achieving a second durable complete remission compared with other patients [198]. In addition, the presence of high risk chromosomal abnormalities denotes an increased risk of refractory disease, second relapse or death irrespective of other clinical risk factors [198]. A number of genes are preferably deleted or mutated at relapse compared to diagnosis. These include *TP53*, *NR3C1*, *CREBPB* and *NT5C2*. Some of these recurring mutations have been identified to influence drug sensitivity and risk of second relapse. Mutations in the transcriptional coactivator and acetyl transferase CREBBP (CREB-binding protein, or CBP) is a relapse-acquired lesion in up to 20% of relapsed ALL samples [141, 142]. CREBBP acetylates both histone and non-histone targets, and has a role in regulating the transcriptional response to glucocorticoid therapy [142, 199, 200]. Recently, two groups independently identified relapse-acquired mutations in the 5′ nucleotidase gene *NT5C2* that confer increased resistance to purine analogues [201, 202]. Thus, mutations that confer resistance to drugs commonly used to treat ALL represent a key mechanism of treatment failure and resistance.

4.6 Inherited Genetic Variation and ALL Risk

In the last decade, data from multiple studies have supported an important role for common inherited variants and rare deleterious mutations in the risk of developing ALL. Genome-wide association studies using microarrays to genotype millions of single-nucleotide polymorphisms in patients and ethnically matched controls associated polymorphisms in genes including *IKZF1* (7p12.2), *CDKN2A/CDKN2B* (9p21), *ARID5B* (10q21.2), *CEBPE* (14q11.2), *PIP4K2A* (10p12.2) and *GATA3* (10p14) with the risk of developing ALL [203–208]. Some of these variants are typically linked to a specific ALL subtype. Examples include the relationship of the *ARID5B* and *PIP4K2A* genotype with hyperdiploid ALL, whereas the risk allele in *GATA3* has been associated with Ph-like ALL [203, 205]. Several genes, such as *IKZF1*, *CEBPE*, and *GATA3,* encode transcription factors that are also targets of somatic genetic alteration in ALL. *CDKN2A/B* encode the INK4/ARF family of tumour suppressors and cell-cycle regulators; this locus is commonly deleted in B- and T-ALL. It is unknown how these variants infer their risk. For some but not all, it has been shown that the variants influence gene expression. Inherited genetic

factors may play a role in determining the natural course of the disease and its response to therapies. *GATA3* variants are associated with Ph-like ALL and other ALL subtypes with poor outcome [203, 205]. Different responses to treatment regimens between ethnic groups may also in part be explained by genetic variation. Hispanic children have a higher incidence of ALL [209] and increased relapse rate relative to Europeans [210]. Racial disparities in the incidence and outcome of childhood ALL have also been linked to *ARID5B* genetic polymorphisms [211].

Deleterious germline mutations have been identified in familial and sporadic ALL. *TP53* alterations occur in 91% of low-hypodiploid ALL in children; 43% of which are found in non-tumour cells [46, 48], suggesting that low-hypodiploid ALL represents a manifestation of Li-Fraumeni syndrome. In addition to the germline TP53 and Ras mutations observed in low-hypodiploid ALL, other hypodiploid cases carry germline mutations involving genes mediating DNA repair that are also likely to be pathogenic [46]. Down syndrome individuals have a 20-fold increased risk of developing AML and ALL, while the rare constitutional Robertsonian translocation, rob(15;21)(q10;q10)c, is associated with an approximately 2,700-fold increased risk of developing iAMP21-ALL compared to the general population [53]. This dicentric Robertsonian chromosome is susceptible to chromothripsis, which is the initiating mechanism of iAMP21-ALL in these individuals [53].

Familial ALL is uncommon, but these rare kindreds are highly informative. Two studies have reported families with autosomal-dominant ALL, in which affected individuals carried a novel germline PAX5 mutation, p.Gly183Ser, that attenuated the transcriptional activity of PAX5 [212]. Somatic *PAX5* sequence mutations are common in BCP-ALL, typically involving the DNA-binding paired domain or the C-terminal transactivating domain. This Ser183 mutation results in partial loss of transcriptional activation, which may act by impeding interaction between PAX5 and cofactors that enhance PAX5 activity. Leukaemic cells exhibited loss of the non-mutated PAX5 allele by deletion of chromosome arm 9p, suggesting that germline heterozygosity of this variant is tolerated but that severe attenuation of PAX5 activity is required for leukaemogenesis [212]. There are recent reports of several families with deleterious inherited mutations in the ETS domain of *ETV6* [213, 214], a common target of mutation and rearrangement. The mutations identified correspond to hotspots of recurrent somatic mutations, affecting DNA binding efficiency and altered intracellular localization of the protein. Moreover, they have a dominant negative effect on the transcriptional repressor function of wild type *ETV6*.

4.7 Future Strategies/Conclusions

The extensive genetic heterogeneity that exists within ALL provides a wealth of potential genetic biomarkers that could be used to assist patient management. Prognostic biomarkers, such as *ETV6-RUNX1* and high hyperdiploidy, define a cohort of patient with a low risk of relapse on standard therapy, whereas patients with high

risk cytogenetics require more intensive or targeted therapy. In the past 10 years, genomic analysis has revolutionised the way researchers and clinicians think about the biology of ALL and new therapeutic options are beginning to emerge. Additional research is required to assess the clinical utility of some of these discoveries, as a number of questions remain unanswered. For example (1) What is the optimal way to use copy number alterations as prognostic biomarkers in B-other ALL within the context of MRD-driven protocol? (2) Which kinase-activating abnormalities are predictive biomarkers for treatment with appropriate inhibitors? (3) What is the role of these new genetic biomarkers in directing therapy after first relapse? In addition to addressing these translational questions, the large-scale application of whole genome, exome and transcriptome sequencing, alongside proteomic and epigenetic studies, will undoubtedly identify new genetic biomarkers, which may add to or replace our current repertoire of prognostic and predictive biomarkers.

Recently we have seen how the outcome of *BCR-ABL1* positive ALL has been dramatically improved by treatment with TKI, reducing the requirement for bone marrow transplantation in Ph positive adults. These studies have clearly shown that application of novel agents in the appropriate biological arena to a suitable target can dramatically improve survival. Evolving studies are revealing other potential candidates with promise for future therapies. However, there remain many challenges ahead before these novel drugs become integrated into routine clinical practice. The discovery of germline mutations has highlighted the role of genetic predisposition to certain subtypes of disease, which are clearly more widespread than previously envisaged. We should continue to search for novel targets, which will surely emerge from the detailed analysis of accumulating data. Total cure for ALL maybe achieved within the not too distant future.

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Chapter 5
Pharmacokinetics, Pharmacodynamics and Pharmacogenetics of Antileukemic Drugs

Kjeld Schmiegelow and Inge van der Sluis

5.1 Introduction

At least 85% of children with acute lymphoblastic leukemia (ALL) can be cured by the best contemporary therapy, but it is uncertain which of the multiple effector mechanisms of the antileukemic agents that are responsible for the efficacy (Fig. 5.1 and Table 5.1). This contrasts the modern era of targeted therapy, where molecular mapping of chemoresistant cancer cells has led to development of drugs that specifically target aberrant pathways (see Chap. 9).

Antileukemic chemotherapy [1, 2] has its roots in the late 1940s, when Sidney Farber and coworkers demonstrated that antifolates could induce remission in childhood ALL [3]. A few years later Joseph Burchenal and coworkers obtained similar results with thiopurines [4]. Soon Vincristine (VCR) and glucocorticosteroids (Steroid) and even adrenocorticotropic hormone were shown to be most effective (and least toxic) for inducing morphologic bone-marrow remission (<5% leukemic blasts), while a combination of daily oral 6-mercaptopurine (6MP) and weekly oral methotrexate (MTX) was superior for remission maintenance. By the late 1960s all the currently used, so-called *traditional*, antileukemic drugs were available, i.e.

K. Schmiegelow, MD, DrMedSci. (✉)
Department of Pediatrics and Adolescent Medicine, University Hospital Rigshospitalet, Copenhagen, Denmark

Institute of Clinical Medicine, University of Copenhagen, København, Denmark

Department of Pediatrics and Adolescent Medicine, JMC-4072, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark
e-mail: kschmiegelow@rh.dk

I. van der Sluis, MD, PhD
Department of Pediatric Hematology-Oncology, Erasmus Medical Center – Sophia Children's Hospital, Rotterdam, The Netherlands

The Princess Maxima Center for Pediatric Oncology, Utrecht, The Netherlands

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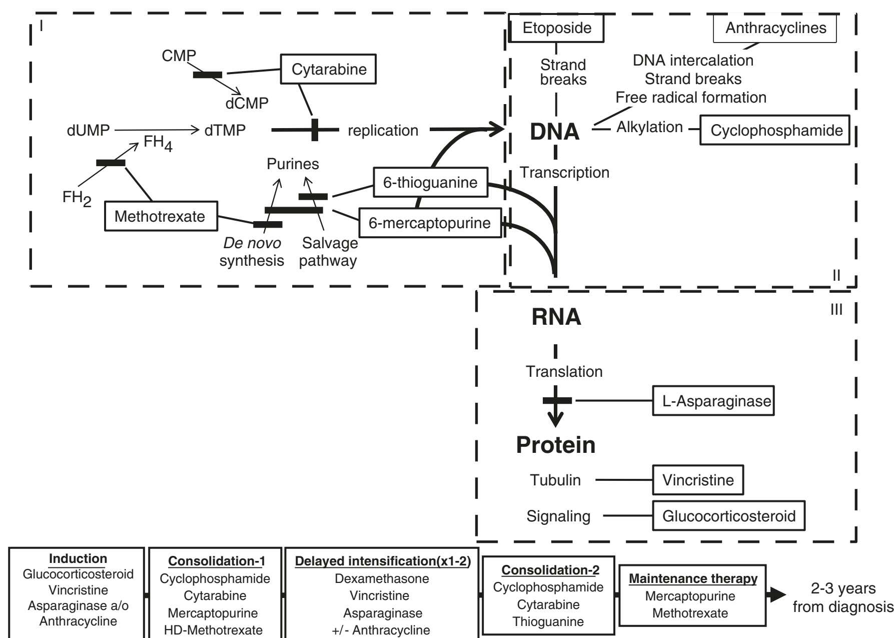

Fig. 5.1 Primary sites of action of *traditional* antileukemic drugs. Their use during antileukemic treatment program is outlined at the bottom. The antileukemic agents can roughly be divided into 3 groups. Goups II drugs interact with DNA. They generally cause profound acute nausea, hair loss, mucositis and myelo-/immunosuppression. They may also cause serious late effects, e.g. cardiotoxicity, urothelium damage, and second cancer. Group I, the antimetabolites, are analogues of normal folate or nucleotide precursors that interferes with DNA synthesis. Group III are the post-translational drugs. The acute toxicities of groups I and III are relatively limited compared to group II, including less nausea, hair loss, mucositis, myelo- and immunosuppression (can be significant for high dose methotrexate/cytarabine). In addition, each drug may be associated with specific toxicities (see text). Groups I and III drugs rarely lead to serious late effects, unless severe acute toxicities have occurred. CMP = cytidine monophosphate; dCMP = deoxycytidine monophosphate; FH_2 = dihydrofolate; FH_4 = tetrahydrofolate; dTMP = deoxythymidine monophosphate; dUMP = deoxyuridine monophosphate

L-asparaginase (Asp), thioguanine (6TG), cyclophosphamide, and cytosine arabinoside (AraC) in the 1950s, and ifosfamide and epipodophylloxins in the 1960s. Improved understanding of their pharmacokinetics (PK) and pharmacodynamics (PD) has made childhood ALL therapy one of the most impressive successes of modern medicine. This was not least due to (i) introduction of central nervous system (CNS) directed therapy in the 1960s, i.e. intrathecal (i.t.) chemotherapy, high-dose MTX and AraC (HD-MTX, HD-AraC) and irradiation [5, 6], (ii) introduction of intensive post-induction consolidation therapy and delayed intensification in the 1970s [7], (iii) observational studies linking biologically defined subsets of ALL patients to specific treatment requirements (e.g. lower propensity for MTX polyglutamation by T-lineage leukemia necessitating HD-MTX [8]), (iv) implementation of precise quantification of minimal residual disease (MRD) for risk/treatment

Table 5.1 Pharmacokinetics of traditional antileukemic drugs

Class of drug	Drug	Cell cycle specific	CNS penetration	Plasma half-life	Metabolism (primarily)	Route of elimination	Protein binding in plasma	Therapeutic drug monitoring	Toxicities
Glucocorti-costeroids	Prednisolone	No	Yes	2–4 h	Liver (CYP3A4, conjugation)	Urine	Yes	No	Hypertension, hyperglycemia, hepatotoxicity, osteonecrosis, neurobehavioural side effects
Glucocorti-costeroids	Dexamethasone	No	Yes, 4–5 times higher than prednisolone	3–6 h	Liver (CYP3A4, conjugation)	Urine	Yes	No	See prednisolone
Vinca alkaloid	Vincristine	Yes	No	Rapid steep decrease; subsequently terminal T½ of 15–155 h	Liver (CYP3A4)	Urine (10%) Bile (80%)	Yes	No	Neurotoxicity (peripheral/autonomic) Constipation SIADH (rare)
Anthracycline	Doxorubicin Daunorubicin	No	No (CSF/ plasma ratio very low)	T1/2 10–30 min T1/2 30–50 h	Liver (GSTs and conjugation)	Bile Urine (10%) Large proportion of the active drug is immediately bound to tissue DNA	Yes 75%	No	Nausea/vomiting, hair loss, immuno−/ myelosuppression, mucositis, cardiotoxicity

(continued)

Table 5.1 (continued)

Class of drug	Drug	Cell cycle specific	CNS penetration	Plasma half-life	Metabolism (primarily)	Route of elimination	Protein binding in plasma	Therapeutic drug monitoring	Toxicities
Asparaginase	E.coli asparaginase	No	No (<1%)[a]	1.24 ± 0.17 days	Reticuloendothelial system	–	No?	Asp activity	Hypersensitivity Allergic reaction Silent inactivation Thrombosis Pancreatitis Increased liver enzymes Hyperammonaemia Hypoalbuminaemia Hyperglycemia
Asparaginase	Pegylated E.coli asparaginase	No	No[a]	5.73 ± 3.24 days (Non-linear, time-dose dependent clearance $T_{1/2}$ varies from 11.8 days at day 1 to 2.4 days at day 20)	Reticuloendothelial system	–	No?	Asp activity	See E.coli asp , less immunogenic through pegylation
Asparaginase	Erwinia asparaginase	No	No[a]	0.65 ± 0.13 days	Reticuloendothelial system	–	No?	Asp activity	See E.coli asp, but immunologically different
Thiopurine	6-mercap-topurine	Yes	Yes (limited)	50 min	Inactive parent compound Xanthine oxidase in gut and liver, TPMT, NUDT15	Hepatic metabolism, urine	Yes (<20%)	Ery-TGN/MeMP DNA-TGN	Immuno−/myelosuppression, hepatotoxicity, second cancer

Thiopurine	6-thioguanine	Yes	Yes (limited)	90 min Terminal $T_{½,l}$ 5–9 h	Inactive parent compound. Xanthine oxidase in gut and liver, TPMT	Hepatic metabolism, urine	Yes	Ery-TGN/ MeMP DNA-TGN	Myelosuppression Hepatotoxicity Nausea/vomiting
Folate-analogue	Methotrexate oral dose <40 mg/m^2	Yes	No	3–10 h	Liver (storage)	Renal (90%) Bile (10%)	Yes (50%)	Ery-MTXpg	Myelosuppression Hepatotoxicity
Folate-analogue	Methotrexate high dose 1–5 g/m^2	Yes	No	8–15 h	Liver (storage)	Renal (90%) Bile (10%)	Yes (50%)	p-MTX	Myelosuppression Hepatotoxicity Renal toxicity Neurotoxicity
Arabinoside-cytidine	Arabinoside-cytidine	Yes	Yes	7–20 min	Inactivation by deamination	Metabolism (deamination in liver, plasma and tissue) renal	Limited (13%)	No	Myelosuppression, mucositis neurotoxicity, conjunctivitis
Oxazaphos-phorines	Cyclophos-phamide	No	Yes	4–8 h	Inactive parent compound. Activation by CYP450 enzymes (most important enzymes CYP2B6, CYP2C19 and CYP3A4) Detoxification mainly GSTs and alcohol dehydrogenase (ALDH1, ALDH3), but also by CYP3A4	Renal (<15% of the parent drug, 80% inactive drug)	Yes 20%, metabolites up to 60%	No	Nausea/vomiting, hair loss, immuno-/myelosuppression, mucositis, hemorarrhagic cystitis, second cancer

(continued)

Table 5.1 (continued)

Class of drug	Drug	Cell cycle specific	CNS penetration	Plasma half-life	Metabolism (primarily)	Route of elimination	Protein binding in plasma	Therapeutic drug monitoring	Toxicities
Oxazaphos-phorines	Ifosfamide	No	Yes	7–15 h	Inactive parent compound Activation by CYP450 enzymes (slower rate than cyclophosphamide)	Renal	Limited	No	Nausea/vomiting, hair loss, immuno−/myelosuppression, mucositis, hemorarrhagic cystitis, second cancer
Epipodophy-llotoxins	Etoposide	No	Yes (poorly)	Distribution $T_{1/2}$ 1.5 h Terminal $T_{1/2}$ 4–11 h	Hepatic (CYP3A4/5) Hepatic metabolism (glutathione/glucuronide conjugation)	Renal (10–70% unchanged drug) Biliary excretion (minor)	Yes (95%)	No	Myelosuppression Hepatotoxicity Nausea/vomiting Hypersensitivity Second cancer

[a]But asparagine markedly reduced

group stratification [9–13], and (v) exploration for acquired mutations allowing precision medicine approaches [14] (see Chaps. 7 and 9).

Contemporary treatment programs can roughly be divided into (i) a three to four drugs remission induction phase with VCR, Steroid, and Asp and/or anthracyclines [15], (ii) a consolidation phase with alternating of additional drugs combinations including HD-MTX, (iii) delayed intensification phases using drug classes similar to those used for remission induction followed by a short consolidation phase, (iv) CNS targeted treatment with or without cranial irradiation [6], and (v) maintenance therapy with oral daily 6MP and weekly MTX until 2.0–3.0 years from diagnosis, which in some protocols include VCR/Steroid pulses [16, 17] (see Chap. 8).

5.2 Pharmacokinetics and Pharmacodynamics

PK deals with drug (and metabolite) concentration-time courses in body fluids after administration of a specific dose, whereas PD covers the effects (efficacy and toxicity) resulting from a certain drug concentration (Fig. 5.2) [18]. Thus, PK is what the body does to the drug, and PD is what the drug does to the body. For all antileukemic agents there is a several fold interindividual variation in the so-called LADME parameters, i.e. Liberation (e.g. from a liposomal formulation), Absorption, Distribution, Metabolism and Excretion, where that latter four primarily reflect variations in liver and kidney function as well as patients' age, size and body composition. With few exceptions, PK parameters are not predictable, but need to be

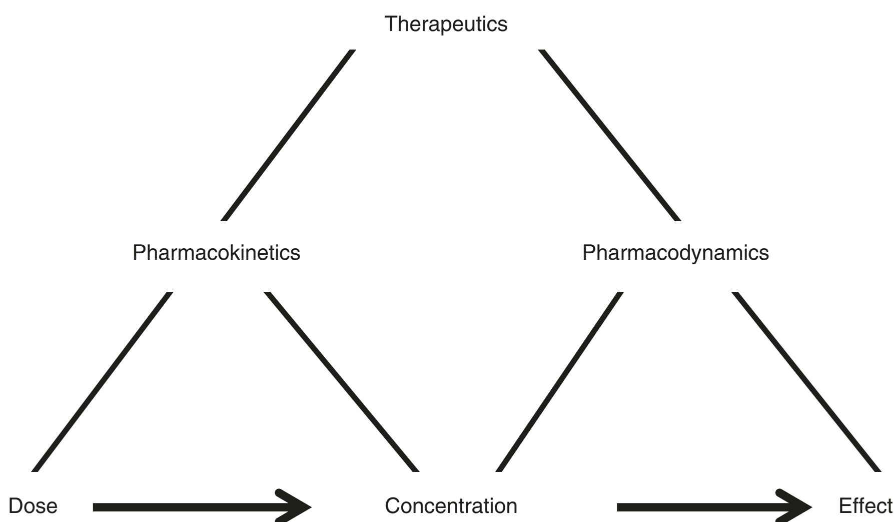

Fig. 5.2 Pharmacokinetics and pharmacodynamics

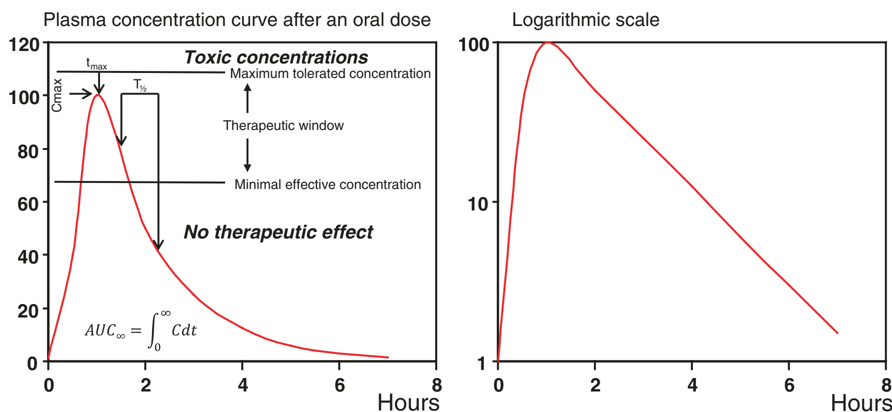

Fig. 5.3 Basic pharmacokinetics. With continuous intravenous administration (or repetitive oral dosing) the steady state concentrations is obtained after 3-5 times the elimination half life (t½). The steady state concentration is inversely correlated to drug clearance and volume of distribution (Vd). The Vd is defined as an apparent volume of distribution that would be needed to contain the total amount of an administered drug at the same concentration as that which is observed in the plasma. The clearance equals ke x Vd, where ke is the elimination rate constant, i.e. the rate at which the drug is eliminated from the body.

$$ke = \frac{\text{In}(2)}{\text{t}\frac{1}{2}} = \frac{\text{clearance}}{\text{Vd}};\ Cleareance = \frac{\text{dose (or absorbed dose fraction)}}{\text{AUC}}$$

AUC = area under the plasma concentration curve; C_{max} = peak plasma concentration after dose administration; t_{max} = time to reach C_{max}; T½ = elimination half life

measured and calculated directly. Such calculations can be performed using non-compartmental or compartmental methods in various PK models. For many drug dosing regimens (e.g. HD-MTX) the PK model can be approximated to a 2-compartment model. Although these are theoretical model compartments, the central compartment from where the maternal drug and its metabolites are eliminated generally represent the circulation, including the liver and kidneys. First order kinetics of elimination of antileukemic agents describe that the volume of blood cleared per time unit is constant (but not the absolute amount cleared). Accordingly, the plasma concentration will be linear on a logarithmic curve (Fig. 5.3). In zero order kinetics the amount eliminated per time unit is constant (e.g. ethanol), since the elimination capacity is saturated. If the rate of clearance of an infused drug, is independent of its concentration (approximately the case for HD-MTX), the steady state concentration (and the areas under the concentration curve, AUC) will change proportional to the dose given. Thus, a reduction of the infused dose by 50% will provide a 50% reduction in the steady-state concentration.

The therapeutic window for most anticancer agents is very narrow, and the s*tandard* dose of an antileukemic agent is determined by the maximum tolerated dose, i.e. the dose that in phase 1 studies caused unacceptable toxicities in an acceptable

very low percentage of patients [19]. However, due to wide interindividual variations in PK, this *standard* dose will provide insufficient systemic exposure to many patients and a potentially increased risk of relapse (Fig. 5.3).

Except for folinic acid rescue after HD-MTX [20], Asp dosing based on enzyme activity measurements [21, 22], and toxicity-targeted 6MP/MTX-based maintenance therapy [17], individualised drug dosing according to drug concentrations or to the limit of toxicity is not used outside research trials [23–25]. Thus, in spite of huge differences in tissue distribution and drug metabolism virtually all *traditional* antileukemic agents are rigorously dosed by body surface area (BSA). The few exceptions include i.t. chemotherapy (dose based on age) and infants (dose based on body weight). The original Du Bois formula for calculating BSA has nowadays been replaced by the far simpler Mosteller formula [26]: $BSA = \sqrt{\text{weight}(kg) \times \text{height}(cm) / 3600}$. BSA-based drug dosing does not provide equal or even predictable PK for individual patients, but its general use at least allows comparison of treatment intensity across ALL protocols [27, 28].

5.3 Pharmacogenetics

Pharmacogenetics covers genetic variations affecting PK and/or PD, i.e. treatment response phenotypes (review see Davidsen et al. [29] and Dulucq et al. [30]). Recent technological opportunities for low-cost, genome-wide analysis of millions of common host genome variants (primarily single nucleotide polymorphisms (SNPs) and indels) and easy-to-use bioinformatics online tools for data handling have created an expectation that mapping of host genome variants, will allow more precise dosing of anticancer agents [31, 32]. So far pharmacogenomics data has mainly focused on the widely used Steroid, MTX, and thiopurines, or on metabolic pathways and transport mechanisms that are common to several drugs, such as the glutathione S-transferases (GST) and cytochrome P450 enzymes (CYP) [29, 30, 33–35]. Although pharmacogenomic drug dosing may reduce toxicity [36, 37], no prospective studies have so far demonstrated that host genome based dosing of chemotherapy provides better cure rates in childhood ALL than drug dosing by BSA or by toxicity [37], and attempts to replicate genotype-phenotype associations in childhood ALL have often failed [29, 30]. However, it is noteworthy that variants associated with treatment response are frequently associated with PK and PD of the antileukemic drugs [38, 39].

ALL treatment strategies often include more than 10 different antileukemic agents, hundreds of genes, and thousands of common genome variants that can influence PK and PD, which questions the likelihood that single SNPs will have clinically significant impact on response phenotypes [29]. Complex bioinformatics analysis integrating the clinical impact of multiple variants in a pathway is doable, but has so far not been clinically implemented in health care [39, 40].

5.4 Glucocorticosteroid

The Steroids prednisone, prednisolone, and dexamethasone are among the most effective antileukemic agents, and in vitro Steroid sensitivity of leukemic cells are significant predictors of early treatment response and risk of relapse [41, 42]. Accordingly, many continental European groups use the reduction of blast count in peripheral blood after a seven days prednisolone prephase for risk group stratification, since in vivo prednisone poor response correlates with the likelihood of later leukemic recurrence [43].

Steroids are used during induction (prednis(ol)one or dexamethasone) and delayed intensification (generally dexamethasone), and as 5–6 days pulses in combination with VCR during maintenance therapy, although the efficacy of the latter is uncertain [25, 44, 45]. There is plenty of room for adjusting Steroid treatment intensity through choice of drug and dose-intensity. Continuous and discontinuous (1 week on, 1 week off, 1 week on) Steroid during induction phases, reduces the risk of osteonecrosis, but do not seem to interfere with the antileukemic effects [46, 47]. The in vivo antileukemic superiority of dexamethasone compared to prednisolone depends on the treatment intensity, with prednisolone (or prednisone) being less efficacious than dexamethasone, if administered less than six- to sevenfold higher doses, e.g. 60 mg prednisolone per m.sq. vs 10 mg dexamethasone per m.sq. [48, 49].

In vitro the antileukemic potency of dexamethasone is much higher than that of prednisolone [50], but due to the risk of toxicities associated with dexamethasone [46, 51], some collaborative ALL study groups restrict the use of a 10 mg/m^2 dose of dexamethasone during induction therapy to T-ALL, since it provides better event-free survival (EFS) rates as well as overall survival, whereas the latter may not the case for other ALL subsets [52]. Other groups such as the United Kingdom ALL group have used a lower dexamethasone dose (6 mg/m^2) during induction therapy for all non-infant ALL patients with acceptable toxicity rates and excellent cure rates [53, 54].

The lipophilic Steroids passively diffuse intracellularly, where they bind to the Steroid receptor (GR or NR3C1), which becomes activated by dissociation from the protein complex it is otherwise bound to [29, 55]. The Steroid-receptor complex is then translocated to the nucleus, where it binds to glucocorticosteroid responsive elements (GRE), which then up- or downregulates specific gene transcriptions leading to apoptosis of Steroid-sensitive leukemic blasts. Prednisolone and dexamethasone seem to regulate the same genes [56]. The Steroid-GR–mediated response can be modified by interactions between the inactive GR and several other proteins such as heat shock proteins, polymorphic hormone receptors and cytokines, including tumor necrosis factor (TNF) and interleukins (ILs). The binding of Steroid to the GR receptors up-regulates the expression of CYP3A and IL-10, but decreases expression of TNF[30]. Conversely TNFs reduces and IL10 increases the number of GRs and thus modulate Steroid sensitivity. Currently, there is little knowledge on how to score the combined interactions of these cytokines in the individual patient to further optimise glucocorticosteroid therapy [57].

Oral Steroids have almost complete bioavailability [58, 59]. After absorption, prednisone is rapidly converted to prednisolone in the liver, and these two drugs have similar PK [60]. The highly polymorphic genes encoding GSTs and CYP3A are involved in Steroid elimination, and Steroid are in themselves inducers of CYP3A enzymes, which subsequently may enhance the metabolism of others drugs, including VCR, epipodophyllotoxins, and cyclophosphamide [29]. Dexamethasone has better CNS penetration than prednisolone, a longer half-life in cerebrospinal fluid (CSF), but is also associated with a higher risk of neurotoxicity [51, 61]. Adding a physiologic dose of hydrocortisone to dexamethasone treatment may compensate for dexamethasone induced deficiency of cerebral mineralocorticosteroid signalling, and thus reduce the occurrence of serious neuropsychological adverse effects and sleep-related difficulties [62].

Multiple host genome variants influence Steroid ligands, receptors, and downstream effectors, and candidate gene studies have, although inconsistently, been associated with risk of hyperbilirubinemia, gastrointestinal toxicity, osteonecrosis, and risk of CNS relapse in high-risk ALL [29, 63]. However, due to the complexity of the Steroid responses, the power of the pharmacogenetics studies, and the diversity of results, no SNPs involved in Steroid PK, pharmacodynamics (PD) or downstream pathways have so far found a clinical role in the care of childhood ALL patients [57]. Patients with poor prednisone response, who are heterozygous for TNF -308G>A have been shown to have a significantly increased risk of relapse compared with the wild-type patients [64]. In addition, deletions of *GSTM1* and *GSTT1* has been associated with increased risk of prednisone poor response [34, 65]. Finally, a SNP in the *IL10* promoter region (IL-10 -1082A>G), leads to elevated plasma levels of IL-10 in patients homozygous for the G allele, and they are less likely to be prednisone poor responders, although this may not lead to an overall reduction in risk of relapse [29, 64].

5.5 Vincristine

The Vinca alkaloid VCR binds to β-tubulin and disrupts the mitotic spindle necessary for chromosome separation, which ultimately leads to apoptosis [66]. VCR is used in induction, consolidation, delayed intensification phases, and as reinduction pulses together with Steroid during maintenance therapy [45]. Doses vary from 1.5 to 2.0 mg/m^2, and are generally capped at a maximum dose of 2.0 mg to limit the risk of serious neurotoxicity, although some groups have capped the dose at 2.5 mg[1]. It is most commonly given as an intravenous (iv) bolus injection or as a brief diluted infusion to prevent the risk of accidental and fatal i.t. administration [67]. Extravasation of VCR results in local tissue damage, although less severe than with anthracyclines.

VCR is metabolised in the liver by CYP3A4 and CYP3A5 [68]. Fifty percent of excreted products are metabolites, the biliary system being the primary route of elimination [69]. Only 10% are excreted in the urine. *CYP3A4* can be induced by

multiple agents, including Steroid, VCR itself, phenytoin, and carbamazepine, thus reducing VCR exposure and potentially increasing the risk of relapse [70, 71]. On the other hand, inhibitors of CYP3A activity, such as the antifungal azoles, as well as significantly reduced liver or biliary function can decrease clearance of VCR, and thus increase the side effects of vincristine, but the effects in the individual patient is unpredictable and useful dosing guidelines are lacking. If clinically feasible, the administration of concomitant strong *CYP3A* inhibitors, such as azoles, should be interrupted, not least when VCR is administered weekly.

The inter- and even intraindividual variations in PK are large and unpredictable [72], which may explain their lack of correlation with in vivo antileukemic effect in some [73], although not all, studies [74]. Prolonged infusion seems to result in less neurotoxicity, but unchanged antileukemic efficacy. Recent use of slow release liposomal VCR supports that the risk of neurotoxicity, but not efficacy, is associated with high peak concentrations [75].

Peripheral neuropathy caused by interference with axonal microtubules is the primary and dose-limiting side effect giving VCR a narrow therapeutic index [76, 77]. The symptoms are generally symmetric and include sensory-motor polyneuropathy such as neuropathic pain, loss of tendon reflexes, motor dysfunction, foot/wrist drop, and paralysis [78]. The very rare occurrence of paresis of the vocal cords starts with hoarseness, but may result in severe airway obstruction. Autonomic neurotoxicity may cause constipation, abdominal pain and ileus, and prophylactic administration of laxatives and/or gut motility promotors should be used. Although VCR passes very poorly across the blood-brain-barrier, it can in rare cases affect the hypothalamic/pituitary axis directly and cause *syndrome of inappropriate secretion of antidiuretic hormone* (SIADH) with profound hyponatremia and convulsions. In a few patients neuropathy is very severe and indicates exploration for Charcot-Marie-Tooth syndrome [79], but common germline SNPs may also markedly increase the risk of dose-limiting neuropathy [77, 80].

5.6 Anthracyclines

The anthracycline antibiotics doxorubicin and daunorubicin are among the most effective antileukemic drugs, but may cause serious toxicities, not least cardiotoxicity [81]. They are generally used for remission induction and/or during intensification phases, including in high risk blocks. Due to risk of cardiotoxicity and severe myelo-/immunosuppression, the use of anthracyclines has been reduced or even abrogated in very low-risk patients as defined by younger age and low white blood cell count (WBC) at diagnosis, good prognosis karyotypes, and low MRD at the end of induction therapy. In contrast, it remains to be determined which subsets of higher risk ALL patients that similarly can be cured without anthracyclines [82, 83].

Anthracyclines mediate their cytotoxicity through free radical formation, inhibition of topoisomerase II, disturbance of helicase function, DNA intercalation, modification of signal transduction, and ultimately induction of apoptosis. The primary

effector mechanism is still not clarified, but most likely reflects its induction of DNA breakage, whereas free-radical formation is probably of less importance.

Doxorubicin and daunorubicin are usually administered at iv doses of 30 mg/m^2 per week or as 60 mg/m^2 every 3 weeks as prolonged infusion (1 or more hours) with caution to avoid extravasation of anthracyclines, since this results in severe local tissue damage.

The PK of anthracyclines is very variable [84, 85], but doxorubicin and daunorubicin display very similar PK. Liposomal and conventional daunorubicin have comparable plasma PK, but the liposomal formulation provide lower levels of the metabolite daunorubicinol and seems associated with a lower risk of later cardiotoxicity [86, 87]. In the blood 75% of doxorubicin and daunorubicin are bound to plasma proteins. Due to rapid binding to tissue DNA, the plasma concentration drops rapidly, but the terminal half-life is long. Neither the plasma concentrations of daunorubicin nor of daunorubicinol seem associated with outcome in ALL, whereas a higher intracellular AUCs rather than peak levels are associated with efficacy [88].

Doxorubicin and daunorubicin are inactivated by GSTs and by the conjugating enzyme NAD(P)H quinone oxidoreductase (NQO1), which make them more water-soluble and suitable for excretion, and deletions of *GSTM1* and *GSTT1* have been associated with reduced risk of relapse [29, 34]. The 13-hydroxylated metabolites doxorubicinol and daunorubicinol have only 5–10% of the cytotoxic activity of doxorubicin and daunorubicin, but may be more cardiotoxic [89]. Fifty percent of a dose is eliminated by hepatic aldo/ketoreductases and excreted by the biliary system, and only 10% by renal excretion. Systemic clearance of the anthracyclines is reduced in patients with decreased liver function and hyperbilirubinaemia. Accordingly, dose reduction may be indicated in patients with severe hepatic or biliary impairment or with exposure to drugs that diminish hepatic reduced glutathione pools (e.g. acetaminophen), but clear guidelines are not available.

The most studied *NQO1* polymorphism is the *NQO1* 609C>T, which reduces the enzymatic activity to only 2% of the wild-type protein [29]. Although this should increase anthracycline exposure and efficacy, a reduced leukemic relapse rate has not been reported [90].

Common adverse reactions include nausea, vomiting, mucositis, myelo- and immunosuppression with risk of serious infections. The risk of cardiomyopathy is associated with female gender, young age, higher cumulative doses and shorter infusion time. It has been suspected that longer infusion time (≥4 h) would reduce cardiotoxicity without compromising the antileukemic effect, but only the latter seems true [91]. Recent studies have identified host genome variants that may be associated with anthracycline-induced cardiotoxicity, but this awaits further validation [92].

5.7 Asparaginase

Asp has been part of childhood ALL treatment protocols for decades, but its optimal administration has only been clarified within the last 10–15 years.

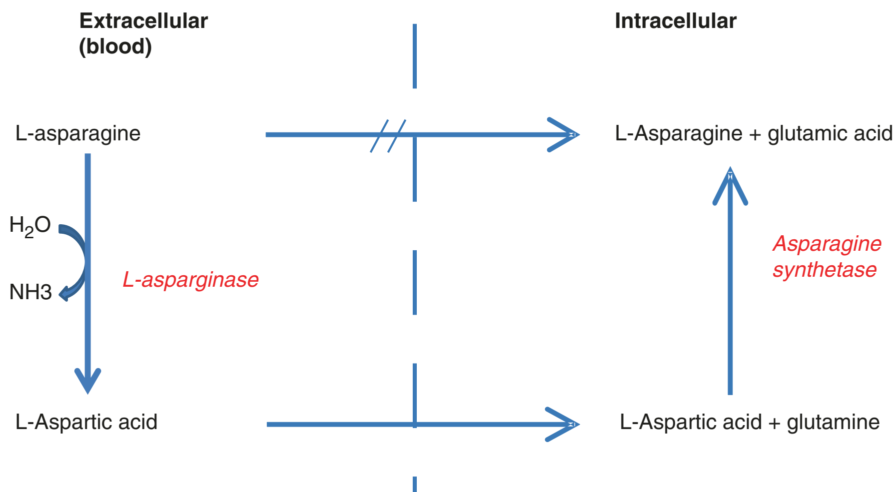

Fig. 5.4 L-asparaginase hydrolyses serum asparagine, but also has a low glutaminase activity. Most normal cells can synthetize L-asparagine from aspartic acid and glutamine and are therefore less susceptible to asparaginase than leukemic blasts. Leukemic blasts are restricted in their ability to up-regulate asparagine synthetase and have a higher need of asparagine due to enhanced proliferation rate

Asp can be extracted from two bacteriae, *Erwinia chrysanthemi* and *Escherichia coli*. To prolong half-life and decrease immunogenicity, *E-coli Asp* has been modified by covalent binding of polyethylene glycol (Peg-Asp). Peg-Asp has become the drug of choice in most first-line ALL treatment protocols. The various Asp preparations and recombinant analogs do not differ in their mode of action, but only in their biologic half-lives (shortest for the Erwinia preparation and longest for PEG-Asp due to reduced uptake in the reticuloendothelial system) [93, 94] and in their immunogenicity (lowest for PEG-Asp).

Asp reduces the extracellular pool of the non-essential amino acid asparagine by hydrolysing it into L-aspartic acid and ammonia, and to a much lesser extent Asp also catalyses glutamine (Fig. 5.4). The latter is important for de novo synthesis of purines and pyrimidines, but does not seem to be critical for the antileukemic effect. Asp does not enter cells or the CNS, but through depletion of extracellular asparagine Asp deprives these tissues of asparagine. E-coli Asp may give a more complete asparagine depletion in the CNS compared to Peg-Asp, suggesting that small amounts of E-coli Asp might enter the CNS [95, 96]. Asparagine depletion results in decreased protein and nucleic acid synthesis leading to inhibition of leukemic cell proliferation and induction of apoptosis. The specific L-Asp sensitivity of lymphoblasts reflects their restricted ability to up-regulate asparagine synthetase (ASNS) activity, and their higher need of asparagine due to their enhanced proliferation. Accordingly, high expression of ASNS in some ALL subsets or in normal bone marrow stroma may lead to resistance to Asp, and

down-regulation of ASNS can revert this resistance in human leukemia and lymphoma cell lines [97, 98].

Asp is metabolised by the reticuloendothelial system, independent of the hepatic CYP450 enzymes and renal function. Asp can be administered intramuscularly or iv. The intramuscular route results in lower peak levels and may be less immunogenic [97]. The differences in half-lives of the various Asp preparations determine dosing schedules. Due to its short half-life, Erwinia Asp is given three times a week or every other day in a dose of 20,000–25,000 IU/m^2 The dosing schedule of native E-coli Asp is 5000–10,000 IU/m^2 every 3–4 days. Peg-Asp is generally given every other week, at doses that vary from 1000 to 3500 IU/m^2. Real-time measurement of Asp activity level is currently used by several groups and allows dose adjustments to keep Asp activity levels above 100 IU/L to obtain complete and sustained depletion of serum asparagine [21, 22].

The toxicity of Asp can be divided in two major groups; the hypersensitivity reactions and toxicities caused by asparagine depletion. The most serious toxic reactions include hypersensitivity ranging from mild reactions to anaphylactic shock, hyperglycemia, pancreatitis liver toxicity such as hyperbilirubinemia, hypoalbumenia, coagulopathy, hyperammonemia and hypertriglyceridemia [99–103]. Since Asp is a foreign protein, it can cause antibody formation [97, 104]. These antibodies neutralise Asp with or without clinical signs of hypersensitivity. The latter is called silent inactivation, and can only be detected by the measurement of plasma Asp activity levels. In case of clinical allergy, Asp levels will generally be zero irrespective of the severity of the allergy, and these patients may, in addition of their lack of asparagine depletion have enhanced clearance of Steroid [105]. Premedication with glucocorticosteroid and antihistamines and increased infusion time can reduce allergic symptoms, but does not prevent Asp inactivation. Thus, symptoms of hypersensitivity indicate switch from E-coli derived preparations to Erwinia Asp (and vice versa) [106]. HLA-DRB1*07:01 and genetic variations in *GRIA1* on chromosome 5q33 are associated with a higher incidence of hypersensitivity and anti-Asp antibodies [107, 108].

Asp-induced hypoalbuminaemia can decrease the clearance of dexamethasone and other drugs [105], and Asp decreases MTX polyglutamation in a preclinical model, although the clinical significance of this is uncertain [109].

5.8 Thiopurines

The thiopurines 6MP and 6TG are essential drugs in the treatment of childhood ALL. They are included in consolidation therapy (6MP or 6TG in combination with low dose AraC), in combination with HD-MTX (6MP), and during maintenance therapy (6MP in combination with MTX). Although most groups only prescribe 25 mg/m^2 of 6MP, when given in combination with HD-MTX, most patient will tolerate 50 mg/m^2 or some even 75 mg/m^2 [110]. For 6MP/MTX maintenance therapy the starting dose of 6MP is 50–75 mg/m^2 dose of 6MP, which is then adjusted

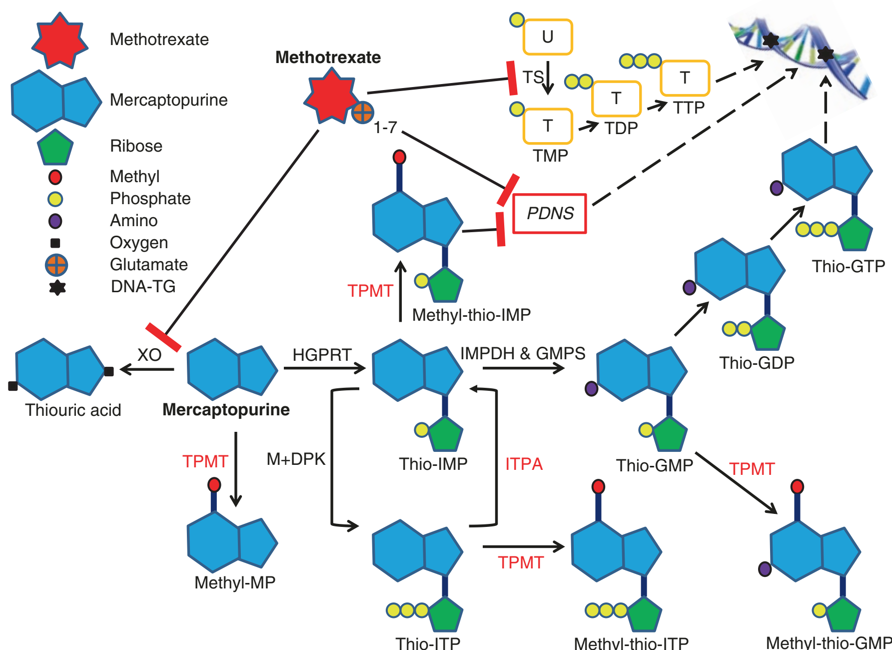

Fig. 5.5 Simplified outline of 6-mercaptopurine (6MP) metabolism, methotrexate (MTX) metabolism and their interactions. DNA-TG = Thioguanine nucleotides incorporated into DNA; GDP = Guanosine diphosphate; GMP = Guanosine monophosphate; GMPS = Guanosine monophosphate synthetase; GTP = Guanosine triphosphate: HGPRT = Hypoxanthine guanine phosphoribosyl transferase; IMP = Inosine monophosphate; IMPDH = Inosine monophosphate dehydrogenase; ITP = Inosine triphosphate; ITPA = Inosine triphosphate pyrophosphatases; M+DPK = mono- and di-phosphate kinases; MP = Mercaptopurine; PDNS = Purine de novo synthesis; TMP = Thymidine monophosphate; TDP = Thymidine diphosphate; TTP = Thymidine triphosphate; TPMT = Thiopurine methyltransferase; U = Uridine monophosphate; XO = Xanthine oxidase

to a set target WBC (usually 1.5–3.0 × 10^9/L) or ANC [16] as this is associated with reduced risk of relapse [17].

Both 6MP and 6TG are prodrugs that exert their cytotoxicity through hypoxanthine guanine phosphoribosyl transferase-mediated conversion into thioinosine monophosphate that are subsequently converted into mono-, di- and triphosphates of 6-thioguanosine (6TGN), which are incorporation into DNA (DNA-TGN) in competition with normal guanine (Fig. 5.5) [111]. During DNA replication and DNA repair, DNA-TGN will reliably match with cytosine. However, DNA-6TGN may become S-methylated which markedly enhance the likelihood of mismatching, specifically with thymidine. Although such mismatching can be recognised by the mismatch repair system, normal DNA sequence repair will be unsuccessful, since the methylated DNA-TGN will continue to mismatch, and the futile repetitive repair attempts will eventually either fail and induce point mutations or the multiple excisions and resynthesis attempts will lead to apoptosis [111].

6TG are more easily converted into 6TGN. 6TG also penetrates better into CSF than 6MP [112] and may be a superior drug for preventing leukemic relapse, but treatment with 6TG as the maintenance therapy thiopurine has in several studies been associated with a 10–20% risk of sinusoidal obstruction syndrome, which in a few patients has led to liver failure and need of liver transplantation [113, 114]. In some patients development of sinusoidal obstruction syndrome during 6TG-based maintenance therapy has been associated with reduced thiopurine methyl transferase (TPMT) activity [115].

Plasma levels of 6MP in children with ALL exert extensive inter- as well as intra-individual variability [116](for review see Schmiegelow et al. [16]). Due to a high first pass effect of xanthine oxidase in gut and liver, the median bioavailability of 6MP is less than 20% [117]. Although variants in the xanthine oxidase and hypoxanthine guanine phosphoribosyl transferase are known, neither have been shown to influence the risk of relapse in childhood ALL.

The conversion of thiopurines to active 6TGN competes with S-methylation of 6MP and several its metabolites mediated by TPMT, and dose increments of 6MP primarily leads to higher concentrations of methylated 6MP metabolites [118]. Methylmercaptopurine cannot be converted into active nucleotides. Methylthioinosine monophosphate is a strong inhibitor of purine *de novo* synthesis [119], and high cytosol 6TGN, methylated 6MP metabolites and MTX-polyglutamates, enhance DNA incorporation of 6TGN [120–122]. TPMT status can be determined by genotyping or phenotyping of erythrocyte TPMT activity. However, TPMT activity will be low at diagnosis, since the red blood cell pool is old, increased during maintenance therapy due to a reduced erythrocyte life span, and confounded in patients who have received allogeneic erythrocyte transfusion [123].

Erythrocyte levels of 6TGN (E-TGN) and methylated metabolites (E-MeMP) have been used to monitor the treatment intensity of 6MP, and although E-TGN initially seemed promising in this respect [124], this parameter tended to lose its significance as intensified 6MP/MTX maintenance therapy gained attention [16, 24]. Still, low levels of both E-6TGN and E-MeMP (or high WBC and lack of elevated alanine aminotransferase levels) in spite of 6MP dose increments can be an indicator of poor treatment adherence [16, 125].

Patients with inherited low TPMT activity will have higher erythrocyte 6TGN levels and be at increased risk of hematopoietic toxicity, and thus tolerate lower doses of 6MP during maintenance therapy [36, 126–128].

Numerous SNPs have been described in the TPMT gene, of which TPMT*2 238G>C, TPMT*3B 460G>A and TPMT*3C 719A>G are the most common variants [129], leading to reduced enzyme activity and tolerance to 6MP and to a lesser extent 6TG [130]. Five to 10% of white are heterozygous for low activity TPMT alleles, and 1 in 300 individuals is TPMT deficient with two low activity alleles. Patients with low activity *TPMT* alleles have more rapid reduction in their MRD [131] and a reduced risk of relapse when treated with 75 mg/m^2 of 6MP [127, 132]. However, the relapse rate for TPMT low activity and wild type patients may be similar, if the maintenance therapy starting dose of 6MP is reduced for the patients with TPMT low activity alleles [37].

The natural substrate for TPMT is unknown, and TPMT deficient patients are otherwise phenotypically normal. So far, TPMT genotype is the only example of routine implementation of pharmacogenetics drug dosing in ALL treatment [36, 37, 130], although most collaborative ALL groups will only test for TPMT variants in patients that demonstrate excessive myelotoxicity [123].

During 6MP-based maintenance therapy a median of ~1:8000 DNA nucleotides are replaced by 6TGN in nucleated cells [121]. When children on maintenance therapy are dose adjusted by WBC, *TPMT* wild type and heterozygous patients will differ in their E-6TGN and E-MeMP, but obtain very similar DNA-TGN levels [121]. Low DNA-TGN has recently been associated with an increased risk of relapse [133, but prospective clinical trials are needed to determine, if DNA-TGN can supplement or even replace WBC/ANC as guide for 6MP dose adjustments.

In Asian and South American populations low activity TPMT variants are rare, whereas low activity *NUDT15* variants are common, with allele frequencies up to 20% [134, 135]. NUDT15 mediates dephosphorylation of thioguanine nucleotides, and patients with reduced activity have a phenotype similar to that of TPMT low activity patients, with reduced tolerance to 6MP [135].

Bone marrow suppression is the primary dose-limiting side effect of thiopurines and primarily reflects intracellular TGN levels, whereas methylated metabolites are correlated with hepatotoxicity with a rise in alanine aminotransferase [136, 137]. Patients with low activity alleles of *TPMT* or *NUDT15* experience more myelotoxicity at standard 6MP doses. Thus, the cumulative incidence of 6MP dose reductions during maintenance therapy is highest for TPMT and/or NUDT15 deficient patients (100% of patients), lower for heterozygous, and lowest in wild-type TPMT and NUDT15 patients [135]. Accordingly, some groups reduce the starting doses of 6MP for patients with low activity *TPMT* alleles to reduced toxicity, but similar guidelines for NUDT15 are lacking [123].

Evening dosing of 6MP and MTX was in the 1980'ies associated with a reduced risk of relapse [138, 139], and an evening schedule may provide more favorable PK [140, 141] but with contemporary more effective antileukemic therapy that is no longer the case [142].

Treatment-related second malignant neoplasm (SMN) is a rare toxicity of thiopurine therapy that is associated with longer maintenance therapy and higher 6MP doses and associated with higher 6MP doses and longer maintenance therapy [143, 144]. The risk of treatment-related myeloid neoplasia (t-MN) has been associated with low *TPMT* activity in some [145], but not all studies [132, 146].

5.9 Methotrexate

The folate analogue MTX (4-amino-10-methyl-pteroylglutamic acid) plays a key role in antileukemic therapy. Its complex pharmacology mirrors that of natural folates with a marked interpatient variability in pharmacokinetics, efficacy, and toxicity [20]. In childhood ALL, MTX is administered widely during ALL therapy as (i)

intrathecal (i.t.) therapy in age-adjusted doses, (ii) intravenous escalating doses without folinic acid rescue, (iii) higher intravenous doses (1.0–5.0 g/m^2) necessitating high dose folinic acid rescue, and finally as (iv) oral or parenteral low doses of 20–40 mg/m^2 at 1–2 week intervals as part of the backbone of maintenance therapy.

MTX enters the cell by active transport via the reduced folate carrier 1 (RFC1), coded on chromosome 21, although other influx and efflux mechanisms mediated by ABC transporters (specifically the ABCC1-4) and breast cancer resistance protein also play a role for cellular MTX concentrations [147]. At high MTX doses passive diffusion across the cell membrane also plays a significant role. MTX interferes with the natural folate-homocysteine cycle and inhibits multiple folate-dependent enzymes and pathways, including dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), 5,10-methylene-tetrahydrofolate reductase (MTHFR), and purine de novo synthesis, which leads to lack of reduced folate, inhibition of DNA synthesis, apoptosis, increased adenosine levels and potentially life-threatening toxicities [29, 148]. When at least 95% of DHFR the synthesis of tetrahydrofolate is compromised [149], but folinic acid (reduced folate) can then counteract the effect. Intracellularly, the enzyme folylpolyglutamate synthetase (FPGS) polyglutamates MTX (as well as normal folate) to polyglutamates forms with 2–6 polyglutamate residues (MTXpg), which increase intracellular retention as well as affinity (and thus efficacy) for its target enzymes proportional to glutamyl chain length [150–154]. In contrast, MTXpg hydrolysis by gamma-glutamyl hydrolase will reduce the pool of MTXpg due to efflux of the maternal drug and the short-chained polyglutamates [154].

The ability for MTX polyglutamation is reduced in T-cell leukemia, probably since T-ALL blasts have lower expression of FPGS and higher breast cancer resistance protein and gammaglutamyl hydrolase (GGH) activity, which reverses polyglutamylation process and favorises MTX efflux [147]. Accordingly, most T-ALL require higher HD-MTX doses (5 g/m^2), whereas B-ALL, especially high-hyperdiploids, can do with lower doses [23, 155].

Measurement of plasma MTX concentrations during HD-MTX to adjust dosing of folinic acid rescue was the first example of routine therapeutic drug monitoring in pediatric oncology. Folinic acid rescue is usually postponed until hour 42 after the start of the MTX infusion in order to avoid rescue of leukemic blasts [156, 157], although this is not well documented. Depletion for more than 42–48 h will cause irreversible cytotoxicity to normal tissue [158].

HD-MTX is widely used in childhood ALL therapy, and therapeutic drug monitoring of HD-MTX has been shown to reduce relapse rates of B-cell precursor ALL [23], although its role is yet not completely clarified. Although it enhances cellular uptake and is important for MTX-polyglutamation, a 300-fold increase in MTX dose only leads to a 2.0–2.5 fold difference in intracellular MTX content [20]. In addition, exposure time, including timing and intensity of folinic acid rescue, rather than peak concentrations, are of importance. Thus, 1 g/m^2/24 h with two doses of folinic acid rescue gives 100-fold lower peak concentrations than 12 g/m^2 over 4 h (7.2 μM versus 700 μM), but more toxicity, longer lasting serum MTX concentrations higher than 1.0 μM, and similar event-free and overall survival [159, 160].

When HD-MTX is given, MTX clearance is dependent on a normal renal and liver function. A rapid clearance has been linked to reduced cure rates [23, 161], although not all studies have confirmed this association [157] potentially reflected dosing strategies for both HD-MTX and folinic acid rescue [156]. The liver is responsible for degrading approximately one-third of a dose of 5 g/m^2 to 7-hydroxy-MTX (7-OH-MTX) by hepatic aldehyde oxidase. The plasma concentration of (7-OH-MTX) can exceed that of MTX, and even enhance MTX-induced toxicity including nephrotoxicity [20, 162]. A smaller proportion is metabolised in the liver to the inactive metabolite (4-[[2,4-diamino-6-(pteridinyl)methyl]-methylamino]-benzoic acid) (*DAMPA*), while the remainder is being excreted unmetabolised by the kidneys.

MTX and its metabolites are weak acids that can crystallise in the acidic renal environment and cause acute, although reversible, severe reduction in renal function [163–165]. The acute renal failure cannot be predicted prior to HD-MTX therapy, but can be recognised early by a rise in serum creatinine. Thus, a rise of >50% within 24 h from the baseline value has a sensitivity of 0.32 and a specificity of 0.99 to predict delayed MTX elimination, and 99% of courses with normal clearance have a rise in serum creatinine of less than 50% [166]. The severe acute renal toxicities with significantly delayed MTX clearance most frequently occur after the first or second HD-MTX courses, and rarely recur [167]. When a significant delay in MTX clearance occurs, hydration should be increased from the usual 3000 to 4500 ml/m^2/24 h accompanied by proportional intensification of alkalinisation to increase solubility of MTX in the urine. With very severely delay in MTX clearance and kidney dysfunction the enzyme carboxypeptidase can be administered, since it rapidly degrades MTX to DAMPA and glutamate, although it will not change the time to normalisation of the renal function [168, 169].

The efficacy and toxicity of low-dose MTX may be mediated by different mechanisms. Oral MTX is rapidly absorbed by an active, but saturable, transport mechanism with a bioavailability of 50–95%, a peak concentration of 0.3–2.2 mM within 1.5–2.5 h from intake, and an elimination half-life of 4–6 h, which mainly reflects renal excretion of unmetabolised MTX within 24 h [16, 117]. Thus, parenteral administration at these doses will only increase systemic exposure slightly and has not been shown to reduce the relapse rate [170]. Rheumatologist have routinely supplemented patients with folic acid (5 mg per week) to avoid gastrointestinal toxicity, hepatotoxicity and hyperhomocysteinaemia while preserving efficacy [171]. Although hardly studied in childhood ALL, children who receive folic acid supplementation have higher folate levels and significantly less myelosuppression [172].

Measurements of MTX polyglutamates in erythrocytes (Ery-MTX) has been explored for monitoring of maintenance therapy intensity, since Ery-MTX is related to the dose of MTX in the preceding weeks, the cellular MTX incorporation in the bone-marrow, and the degree of myelosuppression [16, 124]. E-MTX is also strongly associated with DNA-TGN levels during 6MP/MTX maintenance therapy [122, 133]. However, a recent large randomised study could not demonstrate the a benefit of MTX dose adjustments according to Ery-MTX [24].

MTX passes poorly into the CNS, with a concentration ratio in the order of 1%. Accordingly HD-MTX was initially introduced to improve penetration into CNS and testicular tissue and to overcome cellular MTX resistance. However, HD-MTX is costly, requires several days of hospital admissions until the p-MTX is below a set threshold (200 or 400 nM), carries a risk of severe bone-marrow suppression and thus treatment interruptions, and recent meta-analyses have questioned its role for prevention of CNS relapse [173].

Expression patterns in leukemic cells of the multiple genes involved in folate (and MTX) disposition have been strongly associated with leukemia subtypes, and some correlate with MTX response in vivo [174]. However, the gene expression patterns are poorly correlated with host genome polymorphisms of the same genes.

MTX disposition in the individual patient mirrors the many genes involved in the folate-homocysteine pathway, and numerous pharmacogenetic studies have been conducted to explore the impact of host genome variants on MTX PK and PD (Table) (see Davidsen et al. and Schmiegelow et al. for reviews [20, 29]). However, most of the studies are low powered and include only candidate genes, and the few well-powered GWAS studies have, with few exceptions, not been replicated in independent cohorts [32]. Interindividual variations in HD-MTX PK have a genetic component of more than 50%. *SNPs* in *SLC01B1* is one of the strongest and best validated determinators of renal clearance of HD-MTX (2.0–5.0 g/m^2), but still accounts for less than 10% of the interindividual differences in MTX clearance [175, 176]. These and other variants, e.g. thymidylate synthase tandem repeat polymorphism, have been associated with risk of MTX-related toxicities [176].

The RFC1 80G>A is one of the most extensively investigated polymorphism in the *RFC1* gene (also named *SLC19A1*), and several clinical implications of these alleles have been reported. The A allele has a frequency of ~50% and has been associated with better cellular uptake [177], higher end-of-infusion plasma levels of MTX during HD-MTX therapy methotrexate, as well as a reduced relapse rate compared to patients with one or two G alleles among ALL patients repetitively exposed to HD-MTX (5 g/m^2/24 h) [178], but only among patients disomic for chromosome 21 (where RFC1 is coded). The RFC1 80G allele has been associated with hepatotoxicity, including hyperbiliruinemia, and vomiting.

Methyltetrahydrofolate reductase (MTHFR) is also an important enzyme in the folate-homocysteine cycle, and two SNPs in the gene encoding MTHFR have been extensively studied: MTHFR 677C>T and MTHFR 1298A>C, both of which reduce the enzyme activity [179]. In a study of 520 patients with childhood ALL, the T allele of MTHFR 677C>T was shown to be associated with an increased risk of relapse [180], but only some studies have been able to confirm this [29]. In contrast, the data linking *MTHFR* polymorphisms to hepatotoxicity, myelosuppression, oral mucositis, gastrointestinal and skin toxicity are more solid [179].

Trimetroprim-sulfamethoxazole (TMP/SMX) is generally used as Pneumocystis jiroveci prophylaxis during ALL therapy [181, 182] and there has been a worry that

it could interfere with MTX pharmacokinetics and/or efficacy. However, it seem to interfere with neither low dose [183] or HD-MTX PK [184], and although is does reduce tolerance to oral MTX-based maintenance therapy, this does not influence relapse rate [185].

When oral 6MP is given concurrently with HD-MTX, it seems to be the primary mediator of bone-marrow suppression [186, 187]. This interaction is biochemically and clinically well supported, since MTX increases the bioavailability of 6MP [189, 190], inhibits de novo purine synthesis with increased intracellular levels of phosphoribosyl pyrophosphate, and thus increased formation of 6-thioguanine nucleotides (the primary mediator of 6MP cytotoxicity)(Fig. 5.5) [119]. When HD-MTX (5.0 g/m^2/24 h with folinic acid rescue) is given together with oral 6MP (75 mg/m^2), approximately 40% of the patients will experience treatment interruption of a median of 10 days due to severe myelotoxicity [186]. This myelosuppression can be avoided by reductions of the dose of 6MP 1–2 weeks before and after HD-MTX [190].

5.10 Cytosine Arabinoside

AraC is used either as a low dose 4-days schedule together with a thiopurine, or as HD-AraC for subsets of ALL patients with a significantly increased risk of relapse. The PK of AraC varies widely and the half-life is short with a median T½ of minutes.

Nucleoside transporters, primarily the human equilibrative nucleoside transporter 1 (hENT1 or SLC29A1), play a major role in uptake of AraC by leukemic cells, and a decrease in hENT1 expression is associated with AraC resistance [191]. Intracellularly, AraC undergoes phosphorylation, mediated by deoxycytidine kinase (dCK), to arabinoside-cytidine monophosphate (ara-CMP), which by other kinases is converted into the cytotoxic form ara-CTP, which then competes with natural deoxycytidine triphosphate (dCTP) for incorporation into DNA leading to inhibition of DNA polymerase, blocking of DNA synthesis and repair, and eventually apoptosis. Alternatively, AraC may undergo deamination to the nontoxic uridine arabinoside (ara-U) by cytidine deaminase (CDA), and high levels of CDA correlate with in vitro and in vivo AraC resistance.

Several SNPs in the hENT1 gene and the promoter region of dCK, and some of these have been associated with increased promoter activity and a better outcome for myeloid leukemia patients [29]. However, for both hENT1 and dCK clinical pharmacogenetic studies in childhood ALL are lacking. High levels of 5NT enzyme activity have been associated with a higher relapse rate in childhood ALL [192]. Several SNPs in the CDA gene may also affect expression levels, activity, and risk of toxicity.

The primary dose-limiting toxic effects of AraC are myelosuppression, mucositis, and in addition a risk of encephalopathy when HD-AraC is given.

5.11 Cyclophosphamide and Ifosfamide

The nitrogen mustards, especially cyclophosphamide, are the most commonly used alkylating agents in ALL treatment [193], and applied during consolidation (cyclophosphamide) and as part of intensive blocks for high risk patients by some collaborative groups (both cyclophosphamide and ifosfamide). Although oral dosing is feasible, cyclophosphamide is generally given iv at doses of 500–1000 mg/m^2.

The active metabolites of cyclophosphamide attach an alkyl group to the guanine base of DNA, which interferes with DNA replication by forming irreversible intra- and inter-strand DNA crosslinks, thus inhibiting DNA replication, which eventually leads to apoptosis.

There are large interindividual variations in the PK and metabolism of cyclophosphamide, ifosfamide and their cytotoxic metabolites. Cyclophosphamide is a prodrug that becomes active after metabolic transformation by 4-hydroxylation activation to 4-hydroxycyclophosphamide, which exists in equilibrium with its tautomer aldophosphamide that is spontaneously hydrolysed to phosphoramide mustard and acrolein. The former is an active alkylating agent, while the latter causes hemorrhagis cystis. The conversion of cyclophosphamide to its active metabolites is mediated by several CYP enzymes with CYP2B6 playing the major role, since it has higher affinity for cyclophosphamide and higher metabolic capacity than the other CYP activators CYP2A6, CYP2C9, CYP2C19, CYP3A4 and CYP3A5 [29]. The metabolites are highly protein bound and distributed to all tissues. Detoxification of 4-hydroxycyclophosphamide is mainly by GSTA1 and GSTP1, whereas aldophosphamide, in addition to spontaneous elimination, can be oxidised to inactive carboxyphosphamide by aldehyde dehydrogenase variants ALDH1 and ALDH3. ALDH1 is the most efficient gene variant, and overexpression of ALDH1 has been shown to induce cyclophosphamide resistance in vitro.

No childhood ALL studies have explored in detail the clinical impact of host genome variants in the CYP and ALDH genes on the effect of cyclophosphamide therapy. However, several SNPs have been associated with increased transcriptional activity in vitro and PK of cyclophosphamide in other cancers, including CYP2B6 -82T>C, CYP2B6 516G>T, CYP2B6 785A>G, and CYP2C19*2 681G>A. It remains unclear to what extent GST polymorphisms can be correlated to effects of cyclophosphamide treatment and prognosis in childhood ALL [33, 34].

Drugs inducing hepatic P450 enzyme activity may result in accelerated metabolism of cyclophosphamide to its active metabolites, increasing both efficacy and toxicity of the drug. In contrast, drugs that inhibit hepatic enzymes (table, e.g. corticosteroid and azoles) and severe hepatic impairment result in reduced effect of cyclophosphamide. Cyclophosphamide and its metabolites are primarily excreted in the urine, and the dose should be reduced in patients with impaired renal function.

Cyclophosphamide causes nausea and vomiting, bone-marrow suppression, hemorarrhagic cystitis, and the two latter toxicities are the primary dose-limiting factors [193]. During and for at least 8 h after the administration, adequate iv

amounts of fluid (3000 mL/m^2) and mesna should be administered to reduce the risk of urinary tract toxicity. The most serious long-term toxicity is an increased risk of developing secondary cancer [143].

5.12 Epipodophyllotoxins

Among the epipodophyllotoxins, etoposide (VP16) is the primarily used antileukemic agent, but is currently only used for high risk patients during consolidation blocks.

DNA topoisomerase I and II are essential for DNA replication, transcription, chromosomal segregation, and recombination, and epipodophyllotoxins stabilises cleavable topoisomerase II/DNA complexes, thus preventing re-ligation of DNA strands and causing DNA strand breaks and apoptosis.

The interindividual variability in PK of epipodophyllotoxins in children is significant, and this may play a role for efficacy and toxicity [194–197]. The median bioavailability of oral VP16 is only 50% and oral VP16 is not used in ALL therapy. In plasma, VP16 is more than 95% bound to proteins, and albumin infusion prior to VP16 should be considered in patients with profound hypoalbuminemia to avoid excessive bone-marrow suppression. Ten to seventy percent are excreted unmetabolised in the urine [198].

Penetration into the CSF is quite limited (0.5%), but it will be far less bound to proteins in CSF and may have antileukemic effects [199].

The main non-renal elimination route is hepatic metabolism, and VP16 is a substrate for CYP3A4 and CYP3A5. Mediated by GSTT1/GSTP1 and UGT1A1, respectively, glutathione and glucuronide conjugation can inactivate VP16 and several of its metabolites. VP16 and its metabolites are mainly excreted by the kidney, while biliary excretion plays a minor role. In case of kidney and liver dysfunction the dose of VP16 should be reduced proportionate to the creatinine clearance and hyperbilirubinaemia.

The efficacy of epipodophyllotoxins and other topoisomerase II targeting antineoplastic agents (e.g. anthracyclines) may vary according to polymorphisms in the topoisomerase II genes, but few studies have explored this, even though many candidate genes and SNPs have been identified [29](Table). Epipodophyllotoxins are substrates for both GSTs and CYP enzymes (primarily CYP3A4, but also CYP3A5) and low-activity G alleles of GSTP1 313A>G and of CYP3A5*3 has been association with a higher clearance of etoposide, whereas no significant effect of CYP3A4*1B polymorphism have been demonstrated. UDP-glucuronosyltransferase 1 (UGT1A1) glucuronidates VP16, making it more water-soluble and more suitable for excretion. A polymorphism with 7 (TA) repeats in the promoter region of UGT1A1 (UGT1A1*28) reduces expression of UGT1A1 compared with the wild type with six repeats (6TA), and is associated with lower VP16 clearance in children with ALL. Furthermore, UGT1A1*28 has been reported to be a strong predictor of

hyperbilirubinemia in children with ALL. Finally, VP16 is a substrate for the multiple drug resistance gene P-glycoprotein, and high activity CC genotype of MDR1 3435C>T has been associated with higher VP16 clearance. However, the impact of these polymorphisms on cure rates remains to be demonstrated, and none are currently integrated into clinical care of children with ALL.

Concomitant administration of CYP3A4/5 inducers (e.g. Steroid) can increase clearance of VP16 and potentially reduced efficacy. However, preemptive dose adjustments are not routinely recommended. Drugs that inhibit CYP3A4/CYPA5, such as azoles might also interfere with VP16 metabolism.

The most frequent adverse reactions are nausea and vomiting, mucositis with stomatitis and diarrhea, myelosuppression, hepatotoxicity and allergy-like reactions. The latter can be avoided by giving VP16 as a slow infusion of 30–60 min to prevent unspecific mast cell activation, hypotension and/or bronchospasm. Epipodophyllotoxin associated second myeloid malignancy is a rare toxicity in childhood ALL that frequently involves the *MLL* gene [143].

5.13 Intrathecal Chemotherapy and Central Nervous System Leukemia

When CNS-targeted therapy was not provided in the 1950s and 1960s, 80% of all patients relapsed in the CNS, and although the overall risk of CNS relapse is low with contemporary antileukemic therapy, 30–40% of all relapses still involve the CNS. At diagnosis of ALL leukemic blasts in CSF with leukocyte levels $\geq 5 \times 10^6$/l can be demonstrated by cytospin preparations (so called CNS3) in a few percent of all children with ALL at diagnosis, and these patients have an increased risk of relapse. But if CSF is explored by sensitive methods (e.g. flow cytometry) or morphologically explored before cells decay in CSF, at least 30% have CNS involvement, although at levels far below 5×10^6/L (so called CNS2) [200]. The clinical significance of such limited CNS involvement for risk of later relapse is yet to be determined. However, these findings all underscore the necessity of CNS-targeted therapy. Until recently this included cranial irradiation, but with the improvements of both systemic and i.t. chemotherapy, many groups currently have substituted irradiation with i.t. chemotherapy to reduce the risk of neurotoxicity (see Chap. 12) [6, 201].

Although many anticancer agents can be administered i.t., only three antileukemic drugs are used in front-line antileukemic therapy, i.e. MTX, Steroid, and AraC. Most collaborative ALL treatment groups have chosen i.t. MTX as the standard drug, whereas the combination of MTX, Steroid, and AraC (triple intrathecal therapy, TIT) has been reserved for higher risk patients and for patients with CNS3 at diagnosis. For the latter patients, additional doses of TIT are given during induction therapy until the CSF is free of leukemic blasts.

Since the brain and CSF volume grows rapidly during the first years of life, and the CSF approaches adult volume by the age of 3 years, the dosing of i.t.

chemotherapy is by age groups; i.e. <1.0 years, 1.0–1.9 years, 2.0–2.9 years, and ≥3.0 years.

After lumbar administration of an anticancer agent it must diffuse against the normal CSF flow, which goes from the lateral ventricles to the third, then the forth ventricle, and finally to the subarachnoid space. Thus, only 10% of an i.t. dose will reach the lateral ventricles [202]. Furthermore, ATP-binding cassette (ABC) transporters will actively pump MTX out of CSF. I.t. MTX causes more bone-marrow suppression than an oral MTX at a similar dose, which reflects longer systemic exposure above the cytotoxic threshold [203].

Liposomal AraC (Depocyte) has been used in many protocols for second line therapy of ALL, but there is a lack of studies with de novo childhood ALL patients [204]. A small controlled trial indicated that it may provide superior outcome compared with intrathecal TIT, but with higher risk of short term CNS toxicities, not least arachnoiditis [205].

5.14 Patient Adherence and Physician Compliance

Since childhood ALL is highly chemosensitive, interindividual differences in drug disposition as well as physician compliance to dose adjustment guidelines and/or patient adherence to orally prescribed chemotherapy may influence risk of relapse [125, 206]. During maintenance therapy blood counts and aminotransferase levels have been used to target treatment intensity and monitor patient adherence, but this strategy is challenged by wide inter-ethnicity, -age and -gender associated difference in normal blood counts. E-6TGN/MeMP/MTXpg and DNA-TGN can be applied to identify lack of patient adherence, but these are not generally available and guidelines for individual dose adjustments based on such pharmacological measurements are lacking [16].

5.15 Treatment of Infants

In the first year of life, significant changes in PK and PD occur as a consequence of normal development in body composition, organ maturation and their maturation of drug elimination pathways. Although infants differ as much as older children in drug disposition, antileukemic drug dosing in infants are generally adjusted by on age: three-fourths for patients 6–12 months old and two-thirds for patients <6 months, respectively, and furthermore based on body weight (equalising 1 m^2 with 30 kg). The clearance of MTX tends to increase in the first year of life which may affect risk of MTX-related toxicities [207–209], and VCR neurotoxicity seems to be enhanced although infants do not seem to differ in PK from older children [210]. PK studies of Steroid, Daunorubicin, and asparaginase have not indicated dose reductions for these drugs [211, 212].

5.16 Treatment of Adolescents

Adolescents with ALL have generally been reported to have an inferior outcome compared to younger children, but the gap in outcome is being closed [213, 214]. Although they are likely to differ from younger children in PK of some anticancer agents [215] it does not seem to be the case for VCR [216], Steroid [59], Asp [217], and i.t. Depocyte [218], whereas adolescents do seem to have slower clearance of HD-MTX [219] and accumulate higher levels of cytotoxic metabolites of 6MP and MTX [17]. They also more frequently have higher risk features, including T-cell leukemia and higher MRD at the end of induction therapy [214, 220], but not necessarily more toxicity, except for the risk of thrombosis, pancreatitis and osteonecrosis [46, 221, 222]. Furthermore, a poor adherence to oral chemotherapy may be risk factor for relapse [223].

5.17 Treatment of Obese Patients

Worldwide the prevalence of childhood obesity is increasing at an alarming rate, and during ALL treatment it may furthermore increase due to exposure to Steroids. Except for capping the VCR dose at 2.0–2.5 mg, capping the dose of antileukemic agents is not routinely recommend in obese patients, primarily since BSA is a poor measure of body composition and a poor predictor of drug disposition although this has only been studied in few patients [224]. Furthermore, obesity have been associated with decreased EFS, increased relapse rate and unchanged toxicity rates in childhood ALL, potentially either due to cytokines released from adipocytes or due to treatment adherence factors associated with cultural patterns of excessive eating and limited physical activity [225]. PK data in obese children are limited, but liver and kidney function and clearance (per m.sq.) of antileukemic agents would not be expected to change markedly in obese patients, although the role of hepatic steatosis is unexplored [226]. The guidelines of the American Society of Clinical Oncology suggest the use of actual body weight for appropriate dosing of chemotherapy of adult obese cancer patients, but similar guidelines have not been validated for children [227].

5.18 ALL Predisposition Syndromes and Chemotherapy

Approximately 5% of children with ALL harbour germline mutations that strongly predispose them to development of ALL (see Chap. 1). Treating a malignancy in a child with an ALL predisposition syndrome is a challenging balance between efficacy and toxicity, since many of these patients are already burdened by their medical condition and may in addition be at increased risk for chemo- and radiotherapy-induced toxicities [228]. It adds to the problem that such patients are generally excluded from collaborative clinical trials, and with few exceptions little is known with respect to their optimal treatment.

For children with Down syndrome and ALL, both smaller studies and wide international collaborations have shown that 6TG and MTX may have different PK in children with Down syndrome and provided some guidelines for the treatment of these patients [229, 230]. In addition to ALL associated risk factors and PK of antileukemic agents, poor physician compliance to protocol recommendations of dose adjustments may contribute to their increased risk of relapse [206]. Among children with Down syndrome and ALL, HD-MTX PK does not predict the increased risk of MTX-related gastrointestinal toxicity in these patients [231].

For children with ataxia telangiectasia and Nijmegen Breakage Syndrome, a recent study indicated that many of these patients stand a good chance of cure with conventional chemotherapy with acceptable toxicity profiles, and that they should be offered chemotherapy with the intention to cure [232]. Almost half of all patients with low-hypodiploid ALL harbor germline *TP53* mutations, and there may be indication to explore for *TP53* mutations in such patients, not least in case of excessive toxicity [233].

5.19 Conclusions and Future Perspectives

Many childhood ALL patients are at risk of relapse or excessive toxicity due to adverse PK and/or tissue tolerance to chemotherapy, and host genome variants are likely to explain much of these diversity. The costs of performing genome-wide exploration of hundreds of thousands of common germline variants has become low and SNP profiling of large patient cohorts on contemporary ALL protocols are expected to clarify the critical genotype-phenotype interactions relevant for efficacy and toxicity, which eventually may lead to implementation of germline variants into future treatment stratification. In addition to genotyping, this will require deeper phenotyping than currently performed both with respect to PK and acute toxicities [105]. In addition, the benefits of individual dose adjustments based on drug monitoring should be explored further and more systematically, not least for Asp, HD-MTX, and oral 6MP/MTX maintenance therapy.

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Chapter 6
Minimal Residual Disease (MRD) Diagnostics: Methodology and Prognostic Significance

J.J.M. van Dongen, V.H.J. van der Velden, M. Brüggemann, and A. Orfao

6.1 Introduction

Minimal residual disease (MRD) diagnostics is currently applied to a vast majority of pediatric acute lymphoblastic leukemia (ALL) patients [1–9]. MRD monitoring assesses in-vivo treatment efficacy and assigns patients to MRD-based risk groups.

Over the past 30 years, many technologies have been evaluated for MRD detection [10–12]. For accurate and sensitive detection of low frequencies of ALL cells, such techniques should be able to reliably discriminate ALL cells from normal leukocytes in blood and BM below or equal to one ALL cell in 10,000 normal cells ($\leq$0.01% or $\leq 10^{-4}$). Leukemia-related characteristics are being used for this purpose, such as aberrant immunophenotypes, specific genetic aberrations, and/or specific immunoglobulin (IG) or T-cell receptor (TR) gene rearrangements, which are detectable by flow cytometry or polymerase chain reaction (PCR)-based molecular techniques. Over a period of 25 years, several PCR-based and flow cytometric

J.J.M. van Dongen, MD, PhD (✉)
Department of Immunohematology and Blood Transfusion, Leiden University Medical Center LUMC, Albinusdreef 2, 2333, ZA, Leiden, The Netherlands

Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands
e-mail: J.J.M.van_Dongen@LUMC.nl

V.H.J. van der Velden
Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

M. Brüggemann
Department of Hematology, University Hospital Schleswig Holstein, Campus Kiel (UNIKIEL), Kiel, DE, Germany

A. Orfao
Department of Medicine, Cancer Research Center (IBMCC-CSIC-USAL) and Cytometry Service (NUCLEUS), University of Salamanca (USAL) and IBSAL, Salamanca, ES, Spain

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Table 6.1 Characteristics of the three standard MRD methods

MRD technique	Conventional flow cytometry	RQ-PCR of IG/TR genes or breakpoint regions of	RQ PCR of fusion transcripts and other aberrances
Estimated sensitivity	3–4 colors: 10^{-3}–10^{-4} 6–8 colors: 10^{-4}	10^{-4}–10^{-5}	10^{-4}–10^{-6}
Applicability	BCP-ALL: >90%	BCP-ALL: 95%	BCP-ALL: 25–40% (age dependent)
	T-ALL: >90%	T-ALL: 90–95%	T-ALL: 10–15%
Advantages	Fast Analysis at cell population level or single cell level Easy storage of data Information about the whole sample cellularity	Applicable in virtually all BCP-ALL and T-ALL Sensitive Fairly standardized + regular international QA rounds	Relatively easy Sensitive Applicable for specific leukemia subgroups, such as BCR-ABL or MLL-AF4
Disadvantages	Variable sensitivity, because of similarities between normal (regenerating) cells and malignant cells Limited standardization, no QA results	Time consuming Expensive Requires extensive experience and knowledge	Limited standardization (only "harmonization") Limited QA rounds (with conversion factors) Limited applicability in ALL (absence of targets in more than 50% of cases) Risk of contamination

Adapted from Van Dongen et al. [12]

(flow-MRD) technologies have step-wise developed into routinely applicable MRD tools, particularly thanks to long-term international collaboration with open exchange of knowledge and experience and collaborative experiments [1, 9, 13–23]. The principles and characteristics and the pros and cons of these MRD techniques are summarized in Table 6.1 and briefly discussed below [12].

6.2 Standard MRD Methods

6.2.1 Quantitative PCR of Immunoglobulin and T Cell Receptor Gene Re-arrangement (IG-TR) Targets (DNA Level)

From 1989 to 1991 onwards, many laboratories started to use PCR analysis of IG-TR gene rearrangements for MRD detection [24–27], taking advantage of the highly diverse size and composition of the junctional regions (Fig. 6.1a), which

resulted in high sensitivities of 10^{-4} to 10^{-5} [28]. This so-called allele-specific oligonucleotide (ASO) PCR further improved by the introduction of real-time quantitative PCR (RQ-PCR) technologies in 1997–1998, which use fluorescent-labeled probes as reading system for improved quantitation (Fig. 6.1b–d) [28–32].

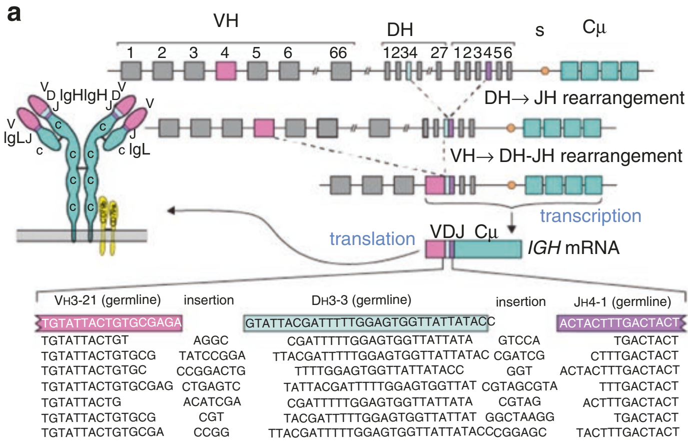
The first large scale PCR-based MRD studies were performed in childhood ALL, using IGH (VH-JH), TRG and TRD gene rearrangements as PCR targets, mainly because of the limited number of primers needed to detect these rearrangements [1, 2]. Soon it appeared that multiple IGH and TRD gene rearrangements occur in a substantial fraction (25–40%) of BCP-ALL patients, implying that multiple subclones (with different IG-TR rearrangements) are present [33, 34]. Such subclones might differ in treatment response. Indeed, clonal evolution with changed IG-TR rearrangement patterns at relapse particularly occurs in patients with oligoclonal rearrangements at initial diagnosis [34, 35]. Therefore several European consortia (BIOMED-1, I-BFM-SG, and BIOMED-2 Concerted Actions) introduced additional PCR-targets to solve at least part of the oligoclonality issue, such as IGK, TRB, incomplete IGH (D-J) and unusual TRD (Vδ2-Jα) rearrangements [31, 36–40]. Thanks to these additional targets, the majority of ALL patients (>95%) can now be monitored with at least two sensitive MRD-PCR targets [14, 31]. Since 2001, the RQ-PCR MRD method has been harmonised between ~60 diagnostic laboratories worldwide and is subjected to biannual international quality assurance (QA) rounds (www.EuroMRD.org) [14].

However, ASO-RQ-PCR MRD methods require extensive knowledge, experience and a degree of operator dependency, and are laborious and time consuming. Detection and sequencing of the ALL-related IG-TR rearrangements at diagnosis and design and selection of the corresponding ASO primers takes 2–3 weeks, while analysis of follow-up samples takes a few days [14, 22].

6.2.2 *Classical Multicolor (4–6-Color) Flow-MRD*

In parallel to the ASO-RQ-PCR methods, flow cytometry was explored as less labor-intensive and faster MRD technique, when 4- and 6-color cytometers became available in 1998–2002 (Table 6.1) [3, 8, 13, 41–44]. These multi-color approaches followed classical concepts with emphasis on the detection of aberrant immunophenotypes in the "empty spaces" (not overlapping with normal leukocytes) in 2-dimensional dot plots, particularly based on the experience of the BIOMED-1 Concerted Action [13, 15, 42–44]. Good sensitivities were achieved, but many comparative flow-PCR studies consistently showed that flow-MRD did not allow for reliable MRD measurements at levels below 10^{-4} in all cases [45–48], particularly at post-induction time points when regenerating BCP cells ("hematogones") are present in abundance [49, 50].

Another disadvantage of flow-MRD is that the applied immunostaining protocols, antibody panels, and gating strategies differ significantly between centers and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insertion
DH3-3 (germline)
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GTATTACGATTTTTGGAGTGGTTATTATACC
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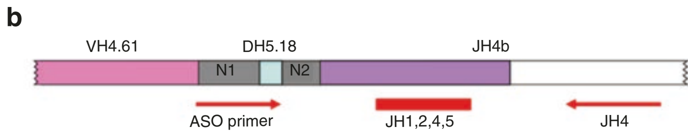
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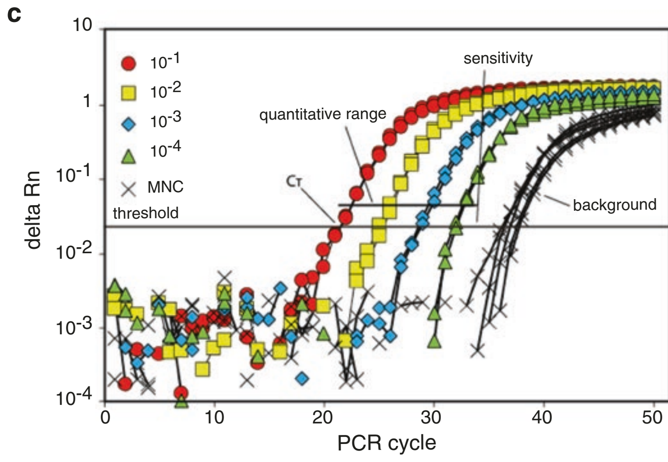
N1
N2
ASO primer
JH1,2,4,5
JH4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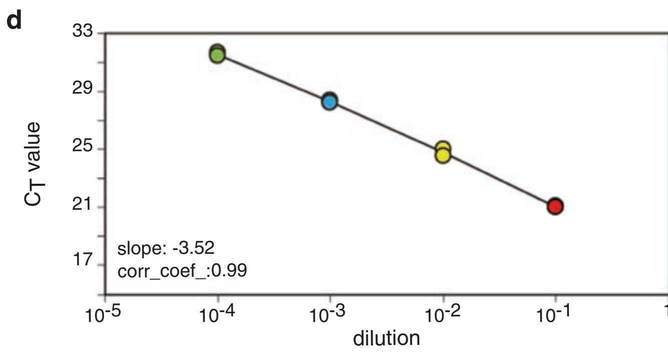
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between treatment protocols and are highly operator dependent procedures, causing substantial inter-laboratory variation. This is a major concern for all clinical studies that wish to exploit MRD measurements.

6.2.3 Real-Time Quantitative Reverse Transcriptase (RQ-RT)-PCR of Fusion Gene Transcripts

PCR methods for detection of fusion gene transcripts became an important MRD tool in myeloid leukemias (BCR-ABL+ chronic myeloid leukemia and PML-RARA+ acute promyelocytic leukemia) as well as in BCR-ABL+ ALL, because of its age-related high frequency [51–53]. In childhood ALL, RQ-RT-PCR is much less used, albeit that it can have added value in well-defined homogeneous subgroups such as BCR-ABL+ ALL [51, 52]. The RQ-RT-PCR methods are sensitive (10^{-4}–10^{-6}) and relatively easy to perform with standardized PCR protocols and primer-probe sets already available for more than a decade [51, 54]. Nevertheless, full standardization of all steps and international External quality assurance (EQA) systems are not yet available (Table 6.1). This is why the EuroMRD consortium is building such a program.

6.3 Sample Requirements

For reliable monitoring of MRD, not only sensitive methods are crucial, but the choice of sample and its quality are important as well. Therefore several sample requirements should be taken into account [12].

6.3.1 Monitoring of Bone Marrow Samples, Not Blood Samples

Several large-scale clinical studies evaluated MRD levels in paired blood/BM samples in both BCP-ALL and T-ALL [55–57], revealing that blood MRD levels in T-ALL patients were comparable or up to one log lower than in BM (Fig. 6.2a, b).

Fig. 6.1 Basic principles of RQ-PCR-based MRD analysis using rearranged IG and TR genes as targets. (**a**) Schematic diagram of an IGH gene rearrangement, resulting in an V-D-J exon with highly diverse junctional regions, which differ in each individual B-cell, even if by coincidence the same gene V, D, and J genes are used. (**b**) Design of a TaqMan probe-primers set for VH4.61-DH5.18-JH4B rearrangement with the upstream primer fully matching the junctional region sequence. (**c**) RQ-PCR analysis of a dilution experiment. The amplification plot shows the position of the threshold and obtained Ct values, a quantitative range of 10^{-4}, sensitivity, and the background signal (*black* x). (**d**) Standard curve, based on the dilution experiment of the VH4.61-DH5.18-JH4B rearrangement

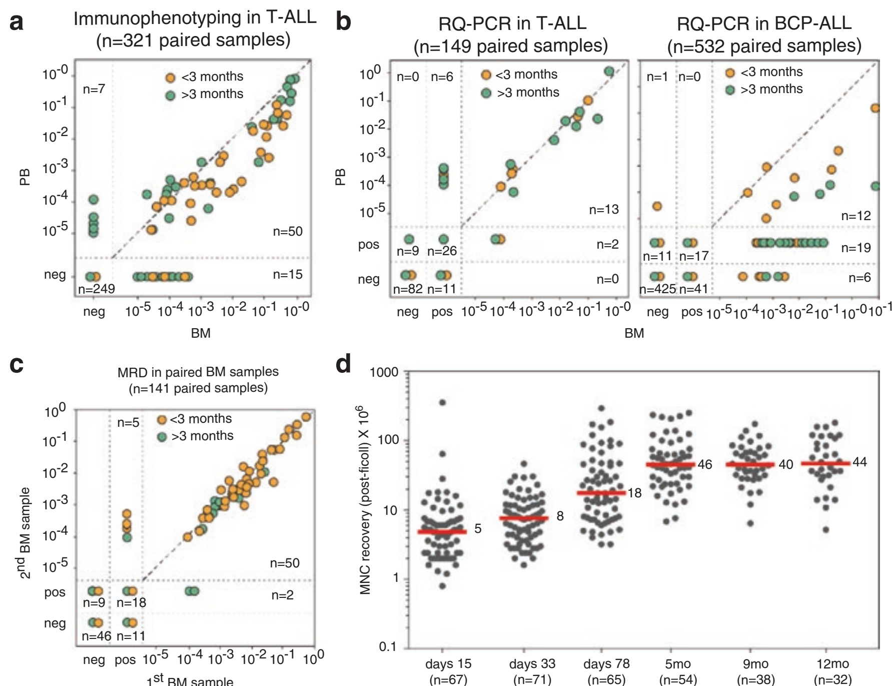


Fig. 6.2 ALL cell frequencies in blood and BM samples during follow-up. (**a**) Frequencies of T-ALL cells, as detected by immunofluorescence microscopy with staining for a T-cell marker and terminal deoxynucleotidyl transferase (TdT) in 321 paired blood and BM samples, obtained from 26 patients [56, 111]. The T-ALL cell frequencies are comparable in many pairs, but differences can occur up to one log. Orange: sample <3 months of follow-up; green: >3 months of follow-up. (**b**) *Left***:** frequencies of ALL cells in 149 paired blood and BM samples from 22 T-ALL patients, analyzed by RQ-PCR of TR gene rearrangements and TAL1 deletions [56]. A strong correlation was observed between the blood and BM frequencies in T-ALL. *Right***:** frequencies of ALL cells in 532 paired blood and BM samples from 62 BCP-ALL patients, analyzed by RQ-PCR of IG and TR gene rearrangements [56]. The MRD levels were significantly higher in BM as compared to blood. Moreover the ratio between the MRD levels in BM and blood was highly variable, ranging from one log up to three logs. Orange: sample <3 months of follow-up; green: >3 months of follow-up. (**c**) Frequencies of ALL cells in 141 paired BM samples (*left-right*) from 26 patients, showing a very high concordance [58]. Only in case of very low MRD levels, variation was seen, mainly because of levels outside the quantitative range of the RQ-PCR assay. Orange: sample <3 months of follow-up; *green*: >3 months of follow-up. (**d**) Recovery of BM mononuclear cells (MNC) after ficoll density centrifugation at different time points during follow-up in the DCOG-ALL11 protocol. Recovery of MNC is relatively low at day 33 and day 78 (median values of 5 to 8×10^6). Recovery at day 78 and at later time points is much higher (median of 18 to 40×10^6)

However, in BCP-ALL patients, peripheral blood MRD levels were between one and three logs lower than in BM (Fig. 6.2b), making quantitative MRD studies via blood sampling impossible in BCP-ALL patients [55–57]. Consequently, for both BCP-ALL and T-ALL patients BM sampling is currently recommended.

6.3.2 Homogeneous Distribution of ALL Cells over BM During Treatment

For a long time it has been assumed that ALL is relatively homogenously distributed throughout the BM at diagnosis, but that treatment might cause differential degrees of tumor load decrease in different parts of the BM compartment, which might result in different MRD levels in different BM aspirates during follow-up. Therefore, we performed 141 paired (left-right) BM studies in 26 patients during the first year of treatment, showing highly concordant results between the paired BM samples (Fig. 6.2c) [58]. Consequently, during the first phases of ALL treatment no signs for unequal distribution of ALL cells were found.

6.3.3 Always Use the First Pull Aspirate for Obtaining Reliable MRD Measurements

Sensitivities of $\leq 10^{-4}$ require sufficient numbers of BM cells to be evaluated. Early studies indicated that only the first pull sample should be used, because of significant hemo-dilution in subsequent aspirates at the same spot. For the same reason, also aspiration of large volumes is discouraged and optimal sample volume is 2–5 mls e.

RQ-PCR based MRD studies require at least 2×10^6 cells for each follow-up time point, which is sufficient to extract ≥6 μg of DNA, needed for analysis of at least two MRD-PCR targets in triplicate and the control gene in duplicate [14]. Note that generally only 50% of DNA is recovered from the theoretical 13 μg of DNA, present in 2×10^6 cells. Current flow cytometric MRD studies require even more cells, preferably $\geq 5 \times 10^6$ cells (see later).

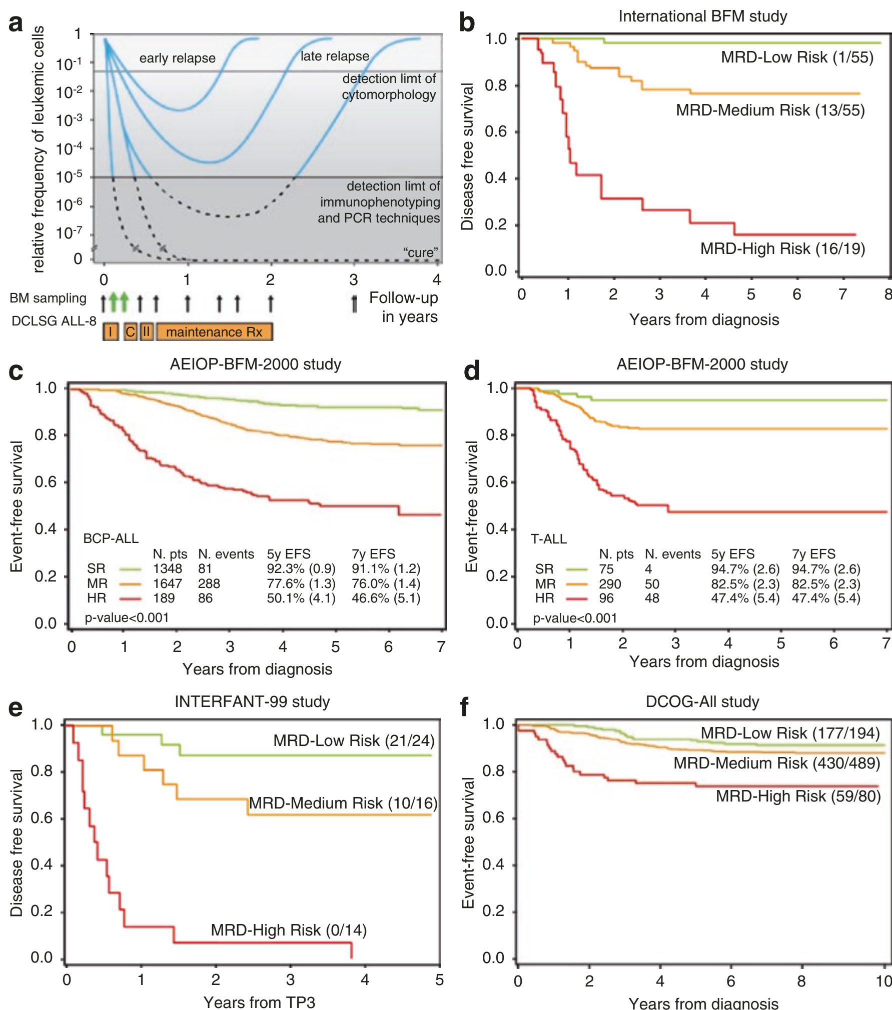
Of note, the overall cell recovery directly relates to the treatment time point, with low cell yields at day 15 and day 33 after starting therapy, but higher cell yields at day 79 and later time points (Fig. 6.2d). The lower cell yields at day 15 are generally not a problem, because at that time most patients still have clearly detectable MRD levels. Lack of sufficient cells at day 33 is a potential problem, because at that time it is important to identify patients with undetectable MRD levels, using MRD-PCR targets with a quantitative range of $\leq 10^{-4}$. Consequently, appropriate BM sampling is a critical part of MRD-based clinical studies.

6.4 Prognostic Value of MRD Diagnostics

6.4.1 Frontline Treatment

MRD diagnostics has proven to be the strongest independent prognostic factor in ALL patients, allowing for risk group assignment into different treatment arms, ranging from low-risk/standard-risk with treatment reduction to medium-risk or high-risk

with mild or strong treatment intensification, respectively. The first large-scale multicenter clinical MRD studies in childhood ALL evaluated the prognostic value of different MRD levels at multiple follow-up time points (Fig. 6.3a) [1–3]. MRD measurements at 1 month ("day 33") and at 3 months ("day 78") after starting therapy, appeared to provide the most important prognostic information (Fig. 6.3b) [1]. MRD-based low-risk patients were MRD negative at both time points (defined as no detectable MRD, using methods that reach a sensitivity of $\leq 10^{-4}$); MRD-based high-risk patients had high MRD levels ($\geq 5\times 10^{-4}$) at the 3 month time point; MRD-based medium-risk patients had moderate to low MRD levels ($<5\times 10^{-4}$) at month 3 after starting therapy (Fig. 6.3b) [1]. Note that the $\geq 5\times 10^{-4}$ cut-off level in RQ-PCR MRD analysis is the same as the original 10^{-3} cut-off level in the classical dot-blot hybridization technique. [1, 59] Subsequent studies confirmed the prognostic significance of



MRD-negativity at early time-points (during induction therapy) for recognition of low-risk patients and the prognostic value of MRD-positivity at later time-points (after induction therapy) for the identification of high-risk ALL. Early MRD measurements at day 15 in childhood ALL can provide additional information for identification of very early good responders ($<10^{-3}$) and a small subgroup of poor responders ($\geq 10^{-2}$) [19, 60, 61]. However, MRD-based risk-group definition at 2 weeks will have a different level of accuracy as compared to the day 78 MRD information, when the response to the complete treatment induction block is evaluated.

Based on the promising data of retrospective studies, subsequent studies used MRD diagnostics to stratify patients in different treatment arms, aiming at improved relapse-free survival in high-risk patients and therapy reduction (with reduced toxicity while maintaining excellent outcome) in low-risk patients . The large-scale AEIOP-BFM 2000 studies have shown that MRD-based treatment strategies indeed further improve outcome in both BCP-ALL and T-ALL patients (Fig. 6.3c, d) [1, 62, 63]. The UKALL-2003 randomized controlled trial demonstrated that treatment can be reduced in MRD-based low-risk patients [64], and that it can be augmented in MRD-high risk patients, albeit at the cost of more adverse events [65].

Even within relatively homogeneous high-risk patient groups, such as infant ALL patients with MLL gene aberrations (Fig. 6.3e), children with BCR-ABL+ ALL and BCR-ABL1-like ALL treated with tyrosine kinase inhibitors plus chemotherapy, MRD levels predict outcome in a comparable way as in childhood ALL [66–69]. Only IKZF1 alterations (deletion or mutations) had added independent value in the MRD-based medium-risk group by identifying a subgroup of poor-prognosis patients [70].

Fig. 6.3 Longterm follow-up in childhood ALL patients, classified according to MRD measurements. (**a**) Schematic diagram of relative frequencies of ALL cells in BM during and after treatment. *I* Induction treatment, *C* consolidation treatment, *II* Reinduction treatment. The detection limit of cytomorphology and the detection limit of immunophenotyping and PCR techniques is indicated. (**b**) Disease-free survival of 129 ALL patients, classified according to three MRD-based risk groups in the International BFM study [1]. Patients were classified as MRD-low-risk, if no MRD was detected at day 33 (TP1) and at day 78 (TP2); patients with MRD $\geq 10^{-3}$ at TP2 were classified as MRD-high-risk; all other patients had MRD $<10^{-3}$ at TP2 and were classified as MRD-intermediate-risk. (**c**) Event-free survival of 3184 BCP-ALL patients of the AEIOP-BFM 2000 study (with kind permission by dr. V. Conter, Monza, IT) [62]. Patients were classified as MRD standard risk (SR) if no MRD was detected at day 33 (TP1) and at day 78 (TP2), as MRD intermediate risk (IR) when MRD was positive at one or both TPs, but $<10^{-3}$ at TP2. Patients with MRD $\geq 10^{-3}$ at TP2 were classified as MRD high risk (HR). (**d**) Event-free survival of 464 T-ALL patients of the AEIOP-BFM-ALL 2000 study (with kind permission by M. Schrappe, Kiel, DE) [63]. The MRD-based classification is the same as for panel C. (**e**) Disease-free survival of 54 infant ALL cases, treated according to the INTERFANT-99 treatment protocol [66]. Patients were considered MRD high-risk if the MRD level at TP3 was $\geq 10^{-4}$; patients were considered MRD-low-risk if MRD levels were $<10^{-4}$ at both time points; all remaining patients were considered MRD-medium-risk. Only 3 out of 24 MRD-low-risk patients relapsed, while all 14 MRD-high-risk patients relapsed. (**f**) Event-free survival ALL patients, stratified according the DCOG-ALL10 treatment protocol (with kind permission by dr. R. Pieters, Utrecht, NL) [72]. MRD-based low-risk patients: 5-year event-free survival of 93% (SE 2%), 5-year overall survival of 99% (SE 1%) and 5-year cumulative incidence of relapse of 6% (SE 2%); the medium-risk patients had a 5-year EFS rate of 88% (SE 2%); the high-risk patients had a 5-year event-free survival of 78% (SE 8%)

6.4.2 Treatment Reduction in MRD-Based Low-Risk Patients?

Already in the 1980s, it was clear that a substantial group of childhood ALL patients (35–45%) survived on less toxic treatment protocols, implying that the more intensive (and more toxic) treatment protocols of the last 15–20 years are not needed in a significant fraction of the patients. However, in an era of progressive treatment intensification with progressively better outcomes, therapy reduction has been an issue of debate at many childhood oncology meetings. Nevertheless it is fair to assume that the MRD-based low-risk patients (MRD-negative at 1 and 3 months) might benefit from treatment reduction.

Identification of *truly low-risk patients* (with a relapse risk <5%) requires an MRD technique that measures low MRD levels (quantitative range: $\leq 10^{-4}$), otherwise it is not possible to consider therapy reduction. Whereas many flow cytometry and PCR-based MRD studies claim a sensitivity of $\leq 10^{-4}$, most standard flow-MRD studies reach such sensitivity only in a subset of patients, depending on the specific aberrant phenotypes and the level of background BM regeneration at different time points [45–48]. This is clearly illustrated by the high numbers of relapses in the "MRD-negative" low-risk patients in flow-MRD vs PCR-based studies [4, 7].

In the DCOG-ALL10 treatment protocol, the strict criteria of the MRD-PCR-based low-risk group of the original I-BFM-SG study have been retained to define MRD negativity, using *at least two different types of sensitive IG-TR PCR targets*, thereby avoiding or reducing oligoclonality problems and related false-negative results [1, 14, 71]. This made the MRD-based low-risk group one-third smaller than previously (~28% instead of ~43%), but resulted in a 5-year cumulative incidence of relapse (CIR) of only 6% with an excellent 5-year overall survival (OS) of 99% despite significant therapy-reduction with virtually no toxicity (Fig. 6.3f) [72]. MRD-based medium-risk patients had a significant higher 5-year event-free survival (EFS) of 88% with therapy intensification compared to historical controls (76%). The highly-intensive chemotherapy and stem cell transplantation in MRD-based HR patients resulted in a significantly better 5-year EFS of 78%, but at the cost of greater toxicity. The overall outcome improved significantly (5-year EFS 87%, 5-year OS 92%, 5-year CIR 8%) compared to preceding DCOG protocols (Fig. 6.3f) [72].

6.4.3 Stem Cell Transplantation, Relapse Treatment, and Innovative Drugs

MRD measurements also identify good and poor responders and correlate with outcome in relapsed ALL patients and post stem cell transplantation (SCT) [73–76]. MRD diagnostics before allogeneic SCT in childhood ALL was the most important predictor post-SCT relapse [74, 75, 77], while rising MRD post-SCT is also a strong predictior of relapse [78, 79]. Consequently, MRD measurements are now guiding treatment decisions in childhood ALL patients undergoing SCT [80, 81]. Because

of its high prognostic value, MRD diagnostics is currently also used for evaluation of treatment effectiveness in clinical trials with innovative drugs, such as antibodies and small molecules [82–87]. In these clinical trials MRD measurements might be used as a surrogate endpoint, thereby shortening the study end-point assessment [88] and helping bring those drugs to market more quickly.

6.4.4 Continuous Monitoring After Induction Treatment?

Continuous MRD monitoring of pediatric ALL patients is not practicable in routine practice in MRD-based low-risk and medium-risk patients, since remission duration is highly variable and the kinetics of leukemic cell regrowth differs significantly among patients (from gradual regrowth over multiple months to rapid progression in only a few weeks) [58, 89]. Additional monitoring might have added value in MRD-based high-risk patients for early treatment intervention, since most relapses in this group occur while on treatment.

Innovative Drugs, Deeper Remission, More Sensitive MRD Techniques

The outcome of ALL treatment has improved at the cost of higher toxicity, particularly for the high-risk patients. Therefore new targeted treatment strategies with innovative drugs, such as antibodies, CAR T-cells and checkpoint inhibitors, are currently being tested [86, 87]. These intervention may induce a "deeper remission" and will require MRD monitoring with a more sensitive assay. Consequently the limit-of-detection will need to be 10^{-5} or to 10^{-6} for which new high-throughput MRD technologies and analysis of more BM cells or greater amounts of DNA will be necessary.

6.5 New High Throughput MRD Technologies

So far, most European clinical trials use PCR-based MRD techniques, while in US and several Asian countries flow-MRD approaches are preferred. In the last few years, new high-throughput PCR-sequencing and flow-MRD techniques have been developed, which in part employ the basic knowledge and experience of standard MRD techniques [12]. These new approaches aim at higher sensitivities and at easy and broad applicability. The advantages and disadvantages of the two high-throughput MRD techniques are clearly different and need further evaluation (Table 6.2).

6.5.1 EuroFlow-Based (≥8-Color) Next Generation Flow-MRD (NGF-MRD)

The EuroFlow consortium has developed high-throughput techniques in flow-MRD, based on multivariate analysis, e.g. principal component and canonical analysis [90, 91]. Another important feature is the development of MRD antibody combinations that map

Table 6.2 Characteristics of high throughput MRD techniques [12]

MRD technique	EuroFlow-based flow cytometry (≥8 colors)	PCR-based HTS of IG-TR genes
Targets	N-dimension (e.g. PCA) based deviations from normal leukocytes (normal differentiation/ maturation pathways) using novel software (e.g. Infinicyt)	Rearranged IG/TR genes Specific onco-genetic aberrations
Estimated sensitivity	Limit-of-Quantification: 10^{-5}; Limit-of-Sensitivity: $<10^{-5}$ ($\geq 5.0 \times 10^{6}$ cells analyzed)	10^{-4}–10^{-6} (depending on amounts of DNA analyzed)
Applicability	BCP-ALL: >95% T-ALL: >90%	BCP-ALL: >95% T-ALL: >90%
Availability	Multiple labs in Europe, South America, Asia, South Africa, and Australia; still limited in US	Limited no. of labs; mainly centralized in companies
Standardization/ assay verification	Full technical EuroFlow standardization and assay verification	No standardization between labs No guidelines for data analysis
QA rounds	External technical QA (will be increased to 2–4 EQA rounds per year)	No external QA rounds yet
Clinical validation	Ongoing	Ongoing
Advantages	Rapid (within 3–4 h) Highly standardized with possibilities for automated gating (Infinicyt software) Efficient data storage and management with easy data comparison. Accurate quantitation Provides information on normal and malignant cells Broadly available around the world Ready for IVD development	High sensitivity Not dependent on primers for patient-specific junctions Potential for IVD development Provides information on background repertoire of B- and T-cells Potential to identify oligoclonality and clonal evolution phenomena
Disadvantages	Continuous education and training required Many cells needed to reach the required sensitivity, e.g. $\geq 5.0 \times 10^{6}$, if Limit-of-Quantification of 10^{-5} is needed with Limit-of-Sensitivity of $<10^{-5}$	Super-multiplex PCR, prone to disproportional target amplification Discrimination from normal clonal background Complex bioinformatics pipeline + need for error correction Turnaround time of ~1 weeks per sample Prone to contamination problems (if no barcoded primers are used) No clear definition for positivity Limited experience in the field

Adapted from Van Dongen et al. [12]

the entirety of the normal BCP pathway in BM, allowing definition of the degree of immunophenotypic deviation of BCP-ALL cells from normal BCP (also in regenerating BM), visualized in multivariate analysis plots (Fig. 6.4) [90, 91]. This development required five rounds of design-testing-evaluation-redesign (with 50–100 BCP-ALL cases per testing round) in order to define reliable combinations of fluorochrome-conjugated antibodies. Also flow-MRD in T-ALL requires discrimination from various types of normal T-cells and other cells with cross-lineage marker expression.

To reach high sensitivity, new cell sample processing was introduced, aiming at analysis of $\geq 5 \times 10^6$ cells to detect a population of ≥40 cells at quantifiable MRD levels of 10^{-5}. This requires fully standardized approaches, including instrument settings, sample processing with bulk lysis procedure, immunostaining, data acquisition, and data analysis with standardized (even automated) gating strategies for definition of normal vs aberrant cell populations [92, 93]; see www.EuroFlow.org for standard operating procedures (SOP) (Table 6.2). The EuroFlow quality assurance (QA) program helps to identify technical failures or inconsistencies and is available for all EuroFlow users since 2015 [94].

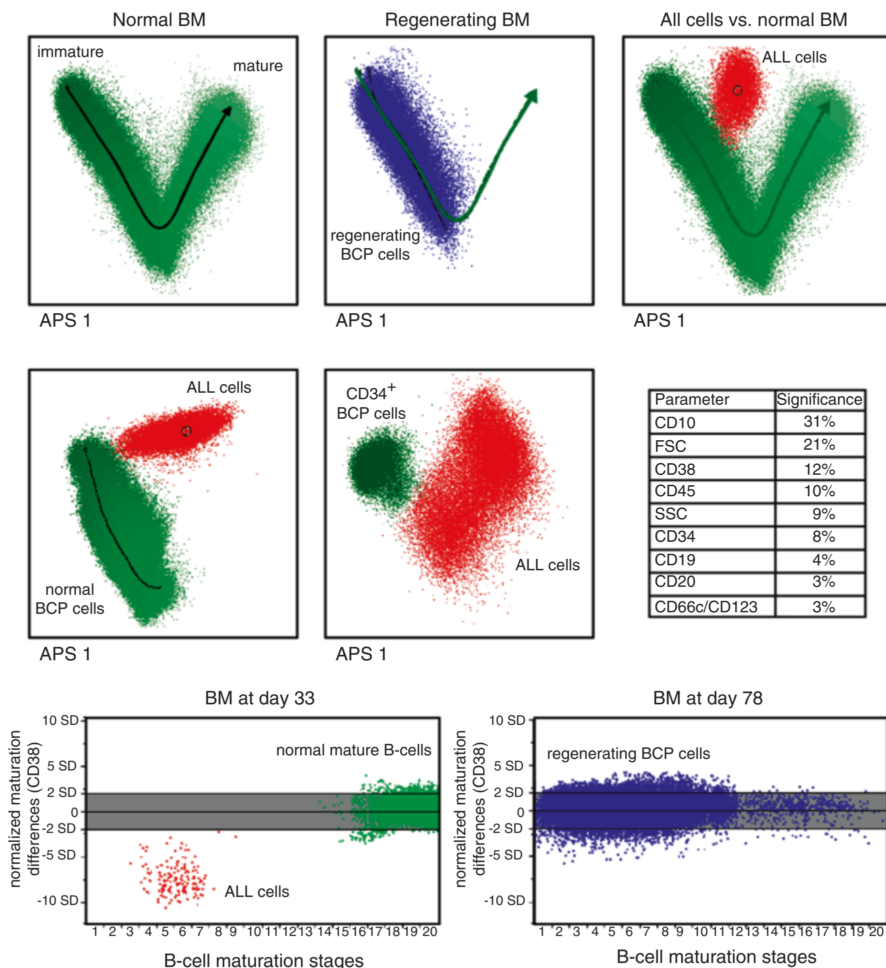
Importantly, EuroFlow-based NGF-MRD strategies provides a full visualisation of the composition of both normal cells and aberrant cells, such as:

- Treatment-induced immunophenotypic "maturation" shifts within the ALL cell population [95, 96], including lineage shifts in ~5% of pediatric cases, such as CD2+ BCP-ALL cases with an early switch to the monocytic lineage [97, 98].
- Heterogeneity in the blast cell population with "dedifferentiation" to immature even CD19-negative "stem-like cells" in BCP-ALL [99].
- Aberrancies in other lineages, pointing to the possibility that more lineages are affected by the disease process or by toxicity of the treatment [100].

Finally, within the last decade, most diagnostic laboratories have moved from 3- and 4-color flow cytometers to 8- and 10-color flow cytometers. This will contribute to the rapid implementation of sensitive flow-MRD measurements.

6.5.2 High-Throughput Sequencing (HTS) of IG-TCR Targets (DNA Level)

PCR-based HTS of IG-TR gene rearrangements to quantify MRD in lymphoid malignancies is currently the focus of intense research. For this purpose, multiplex PCR V-, D- and J-primer sets [37, 101–103] are used to amplify all potential rearrangements in a sample and to subsequently sequence them with high depth of more than 1×10^6 sequences. Comparable to RQ-PCR approaches, the first step is identification of clone specific IG-TR index sequences using the diagnostic sample (Table 6.2). However, in contrast to RQ-PCR the laborious design and testing of patient specific assays is avoided as the same multiplex approach is applied to follow-up samples, with re-identification of the index sequence(s) allowing for MRD quantification. Moreover, the readout is more specific than RQ-PCR where



Parameter	Significance
CD10	31%
FSC	21%
CD38	12%
CD45	10%
SSC	9%
CD34	8%
CD19	4%
CD20	3%
CD66c/CD123	3%

Fig. 6.4 EuroFlow-based multidimensional analysis of normal and malignant BCP cells. (**a**) *Left*, Automated population separation (APS) of normal B cell differentiation in BM (BCP cells and more mature B-cells). *Middle*, APS view of BCP cells in regenerating BM (*blue dots*), plotted against the normal B-cell differentiation (*green arrow*), showing that regenerating BCP cells ("hematogones") are fully comparable to BCP cells in normal BM. *Right*, Plotting of ALL cells (*red dots*) against normal B-cell differentiation (*green*), showing that the ALL cells differ from normal B-cells. (**b**) *Left***,** ALL cells (in *red*) plotted against normal BCP cells (*green*). *Middle*, ALL cells (*red*) plotted against immature CD34+ BCP cells only, showing that the ALL cells separate from their normal counterparts. *Right*, The separation is not based on a single marker, but on multiple markers (in this case: CD10, FSC, CD38, etc.). (**c**) Normalized B-cell maturation pathway (*grey* zone), allowing to assess differences in CD38 expression between ALL cells and normal cells to support MRD detection. *Left*, MRD analysis in BM at day 33, showing complete deletion of the normal BCP cells, but presence of normal more mature B-cells (*green*) within the normal B-cell pathway as well as a small population of ALL cells with aberrant (low) CD38 expression. *Right*, MRD analysis of BM at day 78 of the same patient as in the right panel, now showing regeneration of normal BCP cells (*blue dots*), which fit with the normalized B-cell differentiation pathway (*grey* zone). No aberrant cells were detected at day 78 in this patient sample

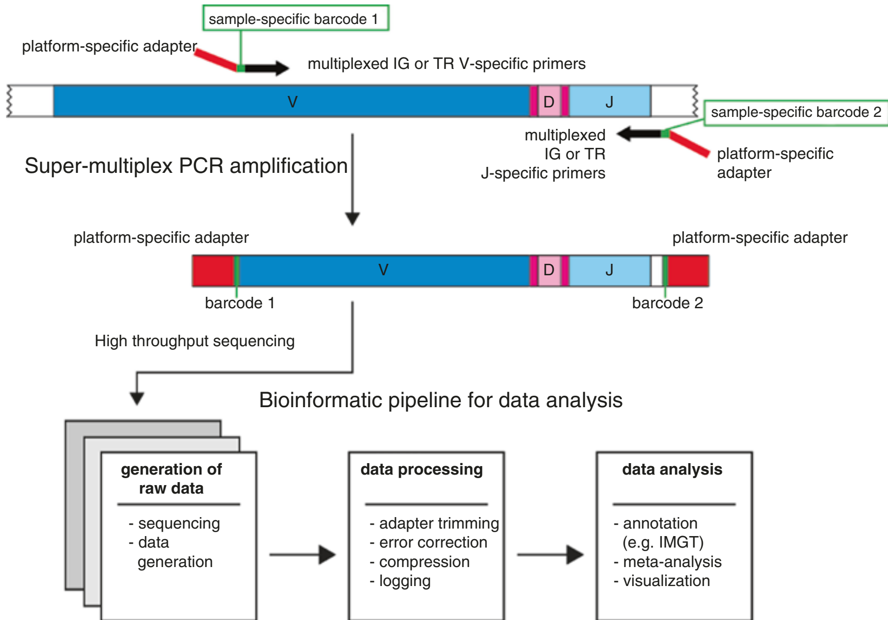


Fig. 6.5 Schematic diagram showing the various steps in HTS of IG and TR for MRD detection. *Top panel*: The IG or TR gene rearrangements are amplified in a single step using a super-multiplex PCR with many different primers, which match with one or more individual V and J genes of the IG and TR genes. The primers contain a platform specific adapter (*red*) as well as a unique identifier (*barcode*) for each sample (*green*). *Middle panel* After PCR amplification, HTS is being performed, using sequence primers directed against the platform-specific adapters. *Lower panel*: The obtained sequencing data are processed via a specially designed bioinformatics pipeline, which includes error correction, annotation of the gene segments, meta-analysis and visualization of the results (www.EuroClonality.org)

false positive results may be caused by non-specific binding of the ASO primer, particularly in situations with massive BCP regeneration [104, 105]. HTS IG-TR can also detect clonal evolution of IG-TR rearrangements [106] and provides insight into the background repertoire of normal (non-malignant) B- and T-cells [107]. Overall, HTS can speed-up the process of molecular MRD quantification and provide results at early time points of treatment, which has not been possible before due to time-consuming ASO-RQ-PCR preparations.

One of the main concerns in using HTS for MRD assessment is the correct identification of the index leukaemia specific IG-TR gene rearrangements (Table 6.2). Published studies use an arbitrary cut-off of 5% of all sequences [102, 108, 109]. This procedure is error-prone, because (depending on the clinical setting) IG-TR rearrangements of unrelated B- and T-cell clones can account for a considerable fraction of amplified sequences and might be misinterpreted as “leukemia-specific” rearrangements, particularly when the applied primer set does not detect the IG-TR rearrangements of the ALL cells; in such situation only IG-TR rearrangements of the remaining lymphoid cells will be detected by HTS. Also the assumption of

absolute specificity of the ALL sequence has to be revisited, because (depending on the rearrangement) background frequencies might occur, limiting the sensitivity of HTS [110]. Another issue, rarely discussed, is the fact that most PCR-HTS approaches use a two-step procedure with the necessity of post-PCR processing with non-barcoded PCR amplicons, which is prone to contamination and in this respect a step backwards, comparable to nested PCR methods of previous times. This is why several groups are now redesigning primers directly linked to sample-specific barcodes in a one-step procedure (Fig. 6.5).

Like other MRD methods, the sensitivity of HTS is dependent on the number of analyzed cells and the corresponding amount of DNA. Therefore a sensitivity of 10^{-6} cannot be reached, if only 2–4 μg of DNA is used. Furthermore, DNA is extracted from all cells in the sample, thus the target cell DNA is mixed with that of normal counterparts and other haemopoietic cells. As a consequence only a small fraction of the DNA of interest is amplified, e.g. only the IG rearrangements of 50,000 B-cells out of a total of 10^{-6} BM leukocytes.

Overall, standardization, quality control and validation of HTS in a multicentre and scientifically independent setting is required, but still lacking (Table 6.2). Therefore, the scientific consortia EuroClonality (www.EuroClonality.org) and EuroMRD are now collaborating to standardize the HTS methods before implementation in routine practice (Fig. 6.5). This includes the pre-analytical, analytical (e.g. new primers with sample-specific barcodes) and post-analytical phases (e.g. a novel bioinformatics pipeline) as well as the generation of large databases to determine background in different clinical settings, and validation of the technology via large-scale, multi-laboratory testing of clinical samples in the context of clinical trials.

6.6 Conclusions

In ALL, MRD diagnostics has become part of routine patient care. Consequently, standardized MRD diagnostics should be available for assessment of treatment response in each individual ALL patient, to be used for personalized medicine such as accurate risk-group assignment with risk-adapted treatment. This also includes the evaluation of new treatment modalities, where MRD measurements can demonstrate the effectiveness of the novel treatment and be used as surrogate endpoint.

Most standard MRD techniques are not sufficiently standardized or contain patient-specific elements that make in vitro diagnostics (IVD) approval complex. The two new high-throughput MRD technologies can solve these problems, but they have to fulfill a series of requirements for acceptation, such as broad availability, easy implementation, applicability in the vast majority of patients (≥95%), sufficient sensitivity (quantitative range preferably down to 10^{-5}), fast (short turn-around time, particularly for follow-up samples), affordable, and standardized with external QA programs. This requires international (world-wide) collaboration with

interactive workshops and educational meetings for exchange of technologies and tools, as well as agreements on the definition of MRD cut-off levels for risk-group assignment. In the forthcoming years, it will become clear whether HTS-MRD and NGF-MRD can meet these requirements.

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Conflict-of-Interest The authors are members of EuroMRD (JJMvD, VHJvdV, and MB), EuroFlow (JJMvD, AO, and VHJvdV) and EuroClonality (JJMvD and MB). These consortia are scientifically independent organizations, which collectively own intellectual property (IP), including patents. Revenues from licensed IP and patents are collectively owned by the three above mentioned consortia and are fully used for sustainability of these consortia, such as for covering costs for scientific meetings, reagents, and management support as well as for educational materials, which are distributed upon request free-of-charge. BD Biosciences provides support for part of the external EuroFlow educational meetings and workshops, including part of the travelling costs (JJMvD and AO).

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Chapter 7
First Line Treatment: Current Approach

Ajay Vora

7.1 Introduction

Studies conducted by collaborative groups in many different countries have contributed to the current generally gratifying outcome (Fig. 7.1) of children and adolescents with Acute Lymphoblastic Leukaemia (ALL). The first attempt at cure was pioneered in the mid-sixties by the St. Jude's group in Memphis, USA who showed that durable remissions could be achieved in roughly 50% of patients with a combination chemotherapy protocol, which they called total therapy, containing remission induction, pre-emptive Central Nervous System (CNS) therapy and prolonged continuation therapy [1]. While other groups tried to optimise this basic template in randomised studies of various components within it, the Berlin-Frankfurt-Munster (BFM) group took the second major step forward in the late 1970s, documenting that long-term remission rates could be improved to 70% by intensified induction and consolidation therapy [2, 3]. Others, including the MRC in the UK, subsequently confirmed the benefits of intensified therapy even using different combinations and schedules of intensification from the original BFM model [4]. Since then efforts have focused on identifying groups of patients at high risk of relapse to direct further intensification of treatment towards them, and reducing the risk of long-term toxicity for the remainder who achieve high rates of event-free survival with current therapy.

A. Vora
Great Ormond Street Hospital, London, UK

University College London, London, UK
e-mail: ajayjvora@gmail.com

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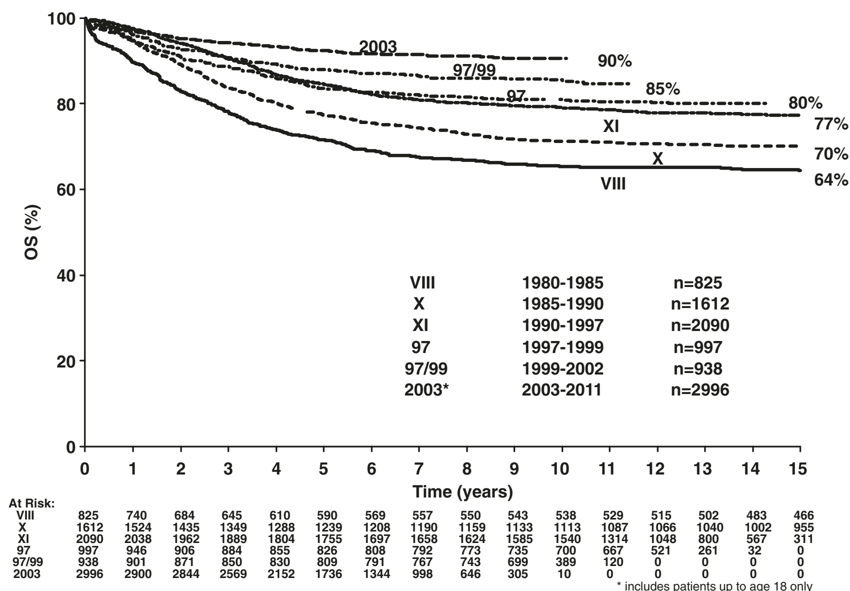


Fig. 7.1 Improvements in overall survival for childhood/adolescent ALL

7.1.1 *Drugs and Protocols*

Eight categories of chemotherapeutic drugs form the mainstay of childhood ALL therapy (Table 7.1). Current front-line treatment protocols contain varying combinations of these eight drugs given over a 2–3 year period to a universal template consisting of Induction, pre-emptive CNS-directed, Intensification and Maintenance (also called Continuing) therapy phases. Induction and intensification therapy is intended to de-bulk tumour load during the first few months of therapy, while maintenance therapy, administered over the remaining 18–30 months, is aimed at clearing Minimal Residual Disease (MRD) (see Chap. 6) of quiescent leukaemia stem cells that are more resistant to intensive therapy. Systemic therapy has variable penetration across the blood brain barrier and, therefore, is insufficient to prevent isolated CNS relapse, the risk of which is substantially reduced by pre-emptive CNS-directed therapy.

7.1.2 *Historical Background*

By the late 1970s, all the drugs currently employed for treatment of ALL had been discovered and most of the important components of childhood ALL treatment were in place. Four weeks of treatment with a three or four-drug induction regimen of vincristine, prednisolone, L-asparaginase and anthracycline achieved complete

Table 7.1 Drugs used in the treatment of childhood ALL

Drug class	Mechanism of cytotoxicity	Route(s) of administration	Part in treatment protocol	Significant toxicities	
				Acute	Late
Vinca alkaloids	Disruption of mitotic spindle	Intravenous bolus	Throughout	Neurotoxicity Constipation Vesicant	Neurotoxicity
Glucocorticoids	Apoptosis by binding to intracellular steroid receptors	Oral and intravenous	Throughout	Hyperglycaemia Weight gain Mood alteration Hypertension	Osteoporosis Obesity Avascular necrosis of bone
L-Asparaginase	Depletion of L-Asparagine	Sub-cutaneous or intra-muscular	Induction and intensification	Hypersensitivity Thrombosis Pancreatitis Hyperlipidaemia Neurotoxicity	None?
Anthracyclines	DNA intercalation Inhibition of topo-isomerase II function	Intravenous infusion	Induction and intensification	Vesicant Myelosuppression	Cardiotoxicity
Alkylating agents (Cyclophosphamide)	Interstrand DNA cross-links	Intravenous bolus or infusion	Intensification	Myelosuppression Haemorrhagic cystitis	Secondary leukaemia Infertility
Methotrexate	Anti-folate	Oral, intravenous and intra-thecal	Maintenance CNS-directed therapy	Mucositis Nephrotoxicity (High Dose) Hepatotoxicity	Osteopenia Neurotoxicity

(continued)

Table 7.1 (continued)

Drug class	Mechanism of cytotoxicity	Route(s) of administration	Part in treatment protocol	Significant toxicities	
				Acute	Late
Thiopurines (Mercaptopurine and Thioguanine)	Incorporation of thioguanine nucleotides into newly synthesised DNA	Oral	Maintenance	Hepatotoxicity Myelosuppression	Hepatic?
Epipodophyllotoxins (Etoposide and Teniposide)	Interferes with topo II religation of double-stranded DNA breaks	Intravenous infusion	Intensification	Myelosuppression	Secondary leukaemia
Cytarabine	Inhibits DNA polymerase	Intravenous, Sub-cutaneous or intra-thecal	Intensification	Hypersensitivity Myelosuppression Central neurotoxicity	Neurotoxicity

remission in over 95% of untreated cases. Following this, long-term remission could be maintained in around 50% of patients with pre-emptive CNS-directed therapy and prolonged continuation therapy [1, 5]. However, the optimum form of CNS-directed therapy and duration of continuing therapy (which was used for periods ranging from 2 to 5 years) were uncertain. Randomised studies revealed no substantial advantage for prolonging treatment beyond 3 years [6] and control of CNS disease was found to be better with combined intrathecal chemotherapy and cranial radiation than the latter alone [7]. Although ‘prophylactic’ testicular radiation, given concurrent with CNS-directed therapy, reduced the incidence of isolated testicular relapse, disease-free survival was not improved by this intervention [8].

7.1.2.1 Intensification Therapy

In the 1980s, the German BFM group reported a 65% event-free survival (EFS) from a relatively large single arm non-randomised study using intensified induction and post-remission therapy [9]. The BFM strategy gained wide acceptance internationally and most other investigators were able to reproduce their results, but almost without exception the original model required modification because its toxicity did not allow delivery as in Germany. A major obstacle to realising the full curative potential of chemotherapy in those early days was infection related deaths, primarily due to gram negative sepsis, pneumocystis pneumonia [10], measles and chicken pox [11]. Availability of drugs [12] and vaccinations for prevention and treatment of these infectious complications, introduction of supportive care measures such as use of allopurinol and hydration for prevention of tumour lysis and establishment of transfusion services providing rapid access to blood components [13] were as important in improving outcomes in that period as availability of new chemotherapy agents and schedules.

In the UK, UKALL X [4] accrued patients between 1985 and 1990, investigated the role of intensification therapy and the best timing for its delivery after achieving remission. Two 5 day intensification courses (unlike the prolonged 8 week BFM courses) achieved a 5 year EFS (71%) similar to that reported by the BFM. The additional treatment was of benefit to all patients, even those within low risk sub-groups.

Despite improvements in supportive care, intensification therapy is associated with significant risk of mortality and morbidity especially when viewed against a low relapse risk in recent trials. Hence, recent trials are testing whether treatment intensity can be de-escalated for sub-groups of patients predicted to have a low risk of relapse (see below).

7.1.2.2 CNS Directed Therapy: Is Cranial Radiotherapy Essential?

Isolated CNS relapse is often associated with minimal residual disease in the marrow and, as such, may be a herald for a systemic relapse [14]. Treatment that only prevents CNS relapse may be associated with increased rate of systemic relapses as occurred in the CCG 1952 trial that compared triple-agent (methotrexate,

hydrocortisone and cytarabine) with single-agent (methotrexate) intra-thecal therapy. Although triple intrathecal therapy reduced CNS relapses, there was an increase in systemic relapses, particularly in those with T-ALL, which were more difficult to salvage, resulting in an inferior OS for the triple intra-thecal group [15].

Hence, having been standard practice for prevention of central nervous system (CNS) relapse in older treatment protocols for children with acute lymphoblastic leukaemia (ALL) [4], pre-emptive cranial radiotherapy (CRT) has increasingly been replaced by other treatment strategies [16–18] due to its associated high risk of late neurocognitive sequaelae [19, 20], endocrinopathy [21] and secondary cancers [22, 23]. A systematic review and meta-analysis of 47 randomized trials of central-nervous-system (CNS)-directed therapy conducted between the 1970s and 1990s showed that CRT can generally be replaced by intrathecal therapy [24]. This observation has been confirmed in single group studies [16, 17, 25–27] and in a more recent meta-analysis of T-lineage ALL only [28]. In parallel, all major collaborative ALL study groups have decreased the percentage of patients that receive CRT. Those that employ CRT now generally restrict this treatment modality to patients presumed to be at increased risk of relapse in the CNS or at other sites [29], typically including subgroups such as those with overt CNS disease present at initial diagnosis, T-cell immunophenotype, high initial white cell count (WCC), or slow early response. Another recent meta-analysis demonstrated that CRT is of no benefit in prevention of relapse after contemporary first line therapy except for a small sub-group of patients with overt CNS disease at diagnosis for whom CRT reduced isolated CNS relapse, but did not affect overall survival which was poor, with or without CRT [30].

7.1.2.3 Intravenous Methotrexate

The vast majority of contemporary treatment protocols include two to four infusions of high-dose (HD-MTX >5 g/m^2) or intermediate-dose methotrexate (1–5 g/m^2) with leucovorin rescue given after recovery from consolidation therapy. Despite this, there is uncertainty whether HD-MTX is essential for treatment of all (or any) sub-group of patients. A recent randomised US COG study demonstrated that HD-MTX was superior to escalating low dose intra-venous methotrexate without folinic acid rescue (Capizzi schedule) for NCI high risk B-lineage patients [31] but found the reverse in a parallel T-ALL study [32]. In UKALL XI, HD MTX was associated with a reduced risk of isolated and combined CNS relapse compared with intra-thecal (IT) MTX but with similar marrow relapse rate and event-free survival (EFS) [16]. Despite not administering HD MTX, overall and CNS outcomes in a recent UK trial, UKALL 2003, were similar or better for all sub-groups of patients compared with contemporary protocols that treat all patients with HD MTX [27].

7.1.2.4 Steroid, Asparaginase and Thiopurine Formulation

Several large randomised clinical trials have demonstrated that dexamethasone is better at preventing systemic and CNS relapse compared with prednisolone particularly in patients with T-ALL [33–35]. Pegylated asparaginase has better

pharmaco-kinetic [36, 37] and pharmaco-dynamic properties [38] than the native formulation and a lower risk of hypersensitivity reactions on re-exposure [39]. Hence, most contemporary protocols use Pegylated asparaginase throughout treatment with a switch to Erwinina asparaginase in patients who develop hypersensitivity reactions to the pegylated product. The dose and intensity of pegylated asparaginase varies with protocol but several groups administer 15–20 doses of 2000–2500 units/m^2 post-consolidation as asparaginase intensification therapy [40, 41]. Some also adjust the dose based on therapeutic drug monitoring (TDM) and switch to Erwinase in patients showing "silent" inactivation [42]. There is no evidence that asparaginase intensification or TDM improve EFS or reduce relapse risk [40]. Intensification is associated with excess toxicity and cost [43], and TDM adds to the latter by increasing the proportion of patients switching to the more expensive Erwinase formulation.

Thioguanine is more effective than mercaptopurine at preventing CNS relapses, especially in younger boys [44, 45], but is associated with an increased risk of death in remission and veno-occlusive disease (VOD) of the liver [44]. A proportion of patients with the latter toxicity have chronic portal hypertension due to peri-portal liver fibrosis [44].

7.1.2.5 Purine Analogues and Proteasome Inhibitors

Nelarabine is a purine nucleoside analogue prodrug of AraG, which is cytotoxic to T-lymphoblasts at micromolar concentrations and has demonstrated single agent activity in refractory/relapse T-ALL with a 55% response rate in a phase 2 study in children and young adults [46]. The COG AALL0434 study is testing whether six 5-day courses of nelarabine in combination with an augmented BFM regimen is safe and effective. Although the efficacy randomisations results are yet to be reported, there was no increased risk of neurological toxicity during the safety phase, [47].

Clofarabine (2-chloro-2-fluoro-deoxy-9-b-D-arabinofuranosyladenine) is a second-generation purine nucleoside analogue which interferes with DNA synthesis by inhibition of ribonucleotide reductase and DNA polymerase and has been shown in vitro to be up to 50 times more potent than Fludarabine [48]. In small numbers of patients with relapsed and refractory childhood ALL, treatment with single agent clofarabine produced a 30% overall response rate (ORR) with an improvement to 55–64% when combined with cyclophosphamide and etoposide. Whether these responses are an improvement on those obtained with standard therapy requires testing in a randomised study. A COG study of clofarabine in first line consolidation treatment of high risk B-lineage ALL was closed early due to excess infection related toxicity [49], although the German CoALL group did not observe excess toxicity when it was given in combination with pegylated asparaginase [50].

Based on genomic and pre-clinical data, targeting the proteasome has been of considerable interest in T-ALL. A number of proteasome inhibitors are in different stages of preclinical and clinical development. The best-studied agent is bortezomib, which has been shown to have single agent activity in T-ALL, to synergize with conventional cytotoxics, and to reverse corticosteroid resistance [51]. Having

been found to be effective in early phase clinical trials in relapsed B and T-ALL [52], it's being tested in a randomized phase III COG trial in *de novo* T-ALL.

7.1.2.6 Haemopoietic Stem Cell Transplant (HSCT)

The proportion of patients transplanted in first remission varies by study group from <5 to 15%. Indications for HSCT include high risk cytogenetic abnormalities, induction failure and persistent MRD post-consolidation therapy. Although some groups have reported a benefit of matched related donor HSCT compared with chemotherapy in these risk groups [53], a transplant related mortality (TRM) of 5–20% associated with unrelated and mismatched donor transplant limits the benefit of HSCT. Although TRM has improved with the incorporation of standardised donor matching and conditioning therapy [54], it remains a significant concern as does acute and late HSCT-related toxicity especially that associated with total body irradiation (TBI) based conditioning. An ongoing randomised international study (FORUM) is testing whether radiation free conditioning is associated with reduced toxicity without compromising efficacy.

7.1.3 Current UK Strategy

7.1.3.1 Risk Stratification

The risk stratification approach currently used in the UK is shown in Fig. 7.2. At diagnosis, patients <10 years old with WCC $<50\times10^9$/l are classified as clinical standard risk; patients ≥10 years of age and those with WCC $\geq50\times10^9$/l as clinical intermediate risk and patients with a cytogenetic abnormality involving rearrangement of the *MLL* gene or hypodiploidy <40 chromosomes or *iAMP21* or *E2A/HLF* abnormality or failure to remit at day 29 of induction are classified as clinical high risk (HR). The clinically defined standard and intermediate risk groups are stratified by measurement of minimal residual disease (MRD) at the end of induction (time point 1, TP1) and recovery from consolidation (prior to start of interim maintenance, time point 2, TP2). Patients with an MRD level <0.005% at TP1 are classified as MRD low risk whereas patients with MRD above that level at TP1 but which has fallen to below 0.5% at TP2 are classified as MRD intermediate risk. Patients with persistent MRD ≥0.5% at TP2 are classified as MRD high risk and receive nelarabine or clofarabine based therapy depending on phenotype to reduce MRD prior to a first remission allogeneic stem cell transplant.

7.1.3.2 Treatment

Patients receive one of three escalating intensity treatment regimens depending on their clinical and MRD risk group (Fig. 7.3). Initial treatment allocation is on the basis of clinical risk criteria with treatment post-induction being determined by MRD response. The treatment regimens described below are the standard treatment arms of the current randomised UK trial, UKALL 2011.

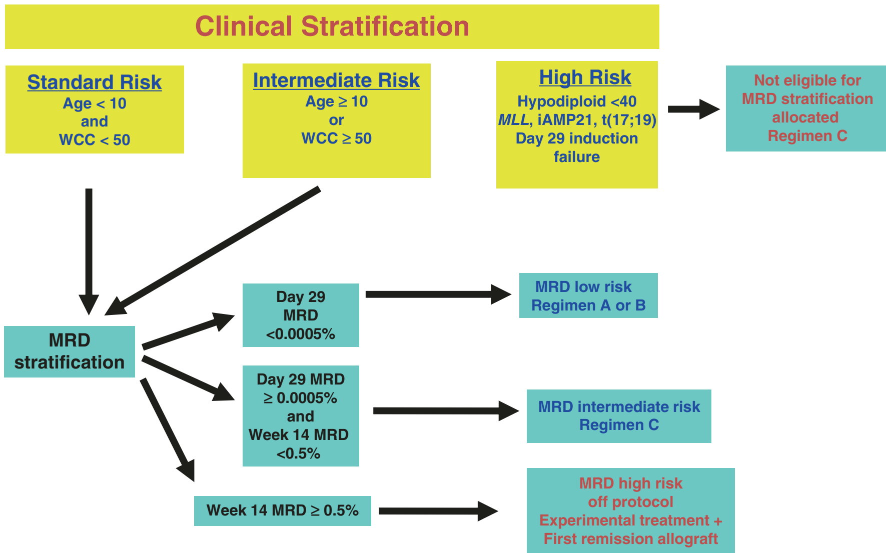
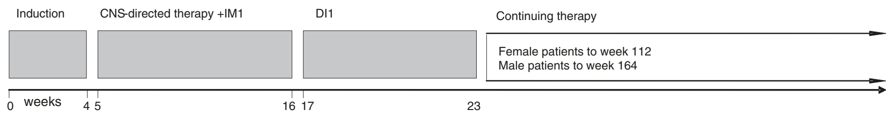
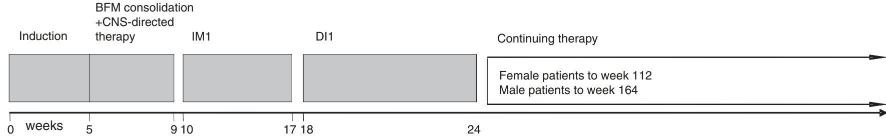


Fig. 7.2 UKALL risk stratification algorithm





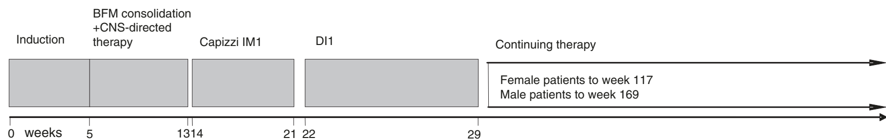


Fig. 7.3 Outline of current UK treatment regimens

7.1.3.3 Induction and Consolidation

Clinical standard risk patients receive a three drug induction containing vincristine, steroids and asparaginase for 4 weeks and intermediate and high risk patients receive in addition Daunorubicin. All patients receive three doses of intra-thecal methotrexate (IT MTX) in induction with patients who have blasts in their cerebro-spinal fluid at diagnosis receiving an additional two doses.

For consolidation, clinical standard risk patients who are MRD low risk receive daily oral mercaptopurine and three doses of weekly intra-thecal methotrexate. Patients who are clinical intermediate risk patients and MRD low risk receive in addition 4 weeks of cyclophosphamide and cytarabine (Berlin Frankfurt Munster (BFM) consolidation). Clinical high risk patients and MRD intermediate risk patients receive an additional four doses of vincristine and two doses of pegylated asparaginase during the BFM consolidation course.

7.1.3.4 Interim Maintenance and Delayed Intensification

Following consolidation, all patients receive 2 months of interim maintenance prior to a single delayed intensification course. MRD low risk patients receive oral mercaptopurine and methotrexate with monthly vincristine and steroid pulses during interim maintenance. Interim maintenance for clinical high risk and MRD intermediate risk patients consists of escalating doses of intra-venous methotrexate without folinic acid rescue, vincristine and pegylated asparaginase.

All patients receive a single Delayed intensification. For MRD low risk patients this consists of a single dose of pegylated asparaginase at day 4 and vincristine, dexamethasone, doxorubicin for 3 weeks followed by cyclophosphamide and cytarabine as given during the BFM consolidation course. MRD intermediate and clinical high risk patients receive in addition two doses of vincristine and one dose of pegylated asparaginase.

7.1.3.5 Continuation Therapy

Regardless of clinical and MRD risk group, all patients receive oral mercaptopurine and methotrexate, monthly vincristine and steroid pulses and three monthly intra-thecal methotrexate. Boys receive treatment for 3 years and girls for 2 years from the start of interim maintenance.

7.1.3.6 Steroid and Asparaginase Formulations, Doses and Schedules

All patients receive dexamethasone 6 mg/m^2 with a 10 mg ceiling dose during induction and maintenance courses. In delayed intensification courses, all patients receive dexamethasone at 10 mg/m^2 (without a cap) for 14 days in a week on, week off schedule. All patient receive pegylated asparaginase 1000 units/m^2/dose given intra-muscularly throughout treatment. MRD low risk patients receive three doses (two in induction and one in delayed intensification course) whilst clinical high risk and MRD intermediate risk patients receive nine doses (additional two in consolidation, interim maintenance and delayed intensification courses).

7.1.3.7 Central Nervous System (CNS) Directed Therapy

Patients with ≥5 leucocytes/microlitre and blasts in a diagnostic cerebro-spinal fluid (CSF) sample with <10/microl red cells (CNS-3) receive an extra two IT MTXs in induction. Patients with traumatic lumbar puncture and blasts in the CSF as well as those with <5 leukocytes/microlitre which were blasts (CNS-2) also receive an extra two IT MTXs during induction. All other patients received intra-thecal methotrexate as described above. High risk patients receive Capizzi intravenous methotrexate at doses <500 mg/m^2 without folinic acid rescue. Thus no patients receive cranial radiotherapy or high dose methotrexate as standard therapy.

7.1.3.8 Allogeneic Haemopoietic Stem Cell Transplantation (HSCT)

Around 2% of patients are eligible for an allogeneic transplant in first remission. These include patients with >25% blasts in their marrow at day 29 of induction or with a high risk karyotype and ≥5% blasts at that time-point. In addition, patients with MRD >0.5% at TP2 are eligible for first remission allogeneic SCT after experimental therapy to reduce the MRD level. Autologous stem cell transplantation is of no benefit in ALL.

7.2 Current Outcomes

Given the above treatment, the 5 year the event-free survival (EFS) of over 3000 children and young people (ages 1–25 years) recruited to the recently concluded UK trial, UKALL 2003 (2003–2011) was 86% [27] with an overall survival of 91% (Fig. 7.4), which compares favourably with outcomes reported from other contemporary trials (Table 7.2). A low incidence of isolated CNS relapse (1.9%) was observed, equivalent to that observed in studies in which a significantly higher proportion of patients received cranial irradiation. Minimal Residual Disease (MRD) response at end of induction was highly discriminatory for relapse risk with the MRD low risk group having an excellent 5 year-EFS of 94% regardless of other prognostic factors (Fig. 7.5). Randomised interventions within the trial demonstrated that treatment can be de-escalated without compromising survival in MRD low risk patients [27], and augmentation of post-remission therapy reduces relapse risk for patients with detectable MRD at the end of induction [55]. While historically outcomes for T-ALL were inferior to B lineage ALL, with recent advances in therapy, event-free survival (EFS) rates have been steadily improving and now exceed 80% in many contemporary clinical trials [17, 27, 40, 56], approaching those observed in B-ALL. Unlike previous reports [57] which suggested patients with an Early T-precursor phenotype had a very poor outcome, their EFS in UKALL

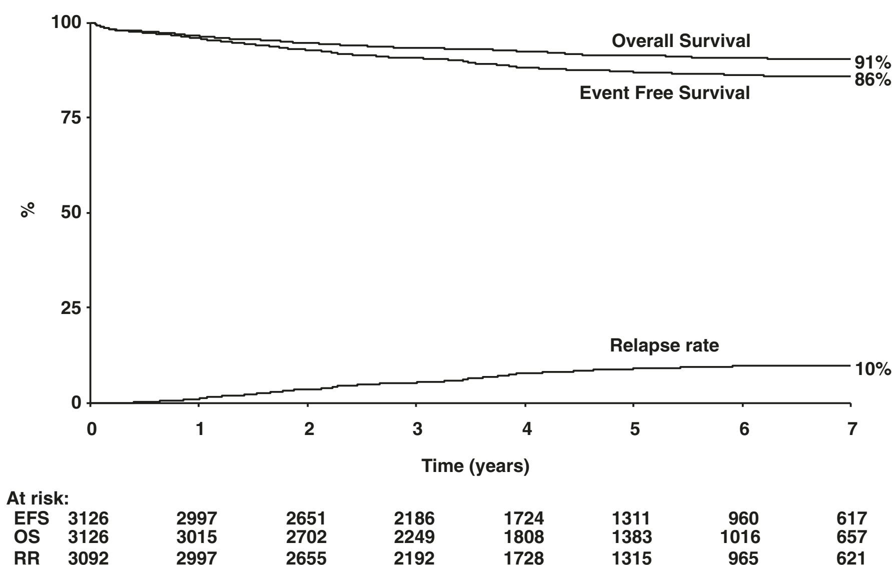


Fig. 7.4 UKALL2003: Event Free Survival EFS), Overall Survival and Relapse Rate (RR)

2003 was 76.7% despite a slow response to induction [58]. This finding has since been confirmed by other groups [59, 60].

The improved outcomes reported in UKALL 2003 and other recent trials have been obtained in the absence of new drugs for the treatment of ALL for over 40 years. The use of dexamethasone and pegylated asparaginase throughout treatment is likely to be an important contributor to the improvement seen in UKALL 2003.

7.3 Treatment of Distinct Sub-groups

7.3.1 Young People (Age 16–25 Years)

A decade ago, retrospective comparisons demonstrated a consistent and large EFS or OS advantage for young people with ALL treated according to paediatric protocols compared with adult protocols. The reasons for this were unclear, but possibly included physician experience and compliance, patient compliance, supportive care, and specific aspects of protocol design. In particular, early dose intensification of chemotherapy, higher cumulative doses of steroids, vincristine and L-asparaginase and less frequent use of alkylating agents, anthracyclines, high dose cytarabine and allogeneic stem cell transplant (with the associated higher treatment related mortality) in paediatric protocols. The results of these retrospective comparisons have been validated in prospective trials that recruited 16–25 year old patients to a paediatric protocol, such as UKALL 2003, in which that age group had a 5 year EFS of 75% without excess toxicity compared with the 10–15 year age group [61].

Table 7.2 Outcomes with contemporary childhood ALL protocols[a]

Trial	Group	Region	Years	Subgroup (n)	EFS (years)	OS (years)
Several	COG	US, Canada, Australia, New Zealand	2000–2005	All patients (6994)	N/A	91.3% (5-year)
				B-ALL (5845)	N/A	92.0% (5-year)
				T-ALL (457)	N/A	81.5% (5-year)
Total XV	SJCRH	US	2000–2007	All patients (498)	85.6% (5-year)	93.5% (5-year)
				B-ALL (422)	86.9% (5-year)	94.6% (5-year)
				T-ALL (76)	78.4% (5-year)	87.6% (5-year)
00-01	DFCI	US, Canada	2000–2004	All patients (492)	80.0% (5-year)	91.0% (5-year)
				B-ALL (443)	82.0% (5-year)	N/A
				T-ALL (49)	69.0% (5-year)	N/A
AIEOP-BFM 2000	BFM	Western Europe	2000–2006	All patients	N/A	N/A
				B-ALL (4016)	80.4% (7-year)	91.8% (7-year)
				T-ALL (464)	75.9% (7-year)	80.7% (7-year)
ALL-9	DCOG	Netherlands	1997–2004	All patients (859)	81% (5-year)	86% (5-year)
				B-ALL (701)	82% (5-year)	
				T-ALL (90)	72% (5-year)	
UKALL 2003 (age 1–25)	MRC/NCRI	UK	2003–2011	All	87.3% (5-year)	91.6%
				B	88% (5-year)	92.3%
				T	82% (5-year)	86.4%

AIEOP-BFM Association of Italian Paediatric Oncology and Berlin Frankfurt-Munster, *COG* Children's Oncology Group, *SJCRH* St. Jude Children's Research Hospital, *DFCI* Dana Farber Cancer Institute Consortium, *MRC/NCRI* Medical Research Council/National Cancer Research Institute, *NCRI* National Cancer Research Institute

[a]Infants <1 year old excluded

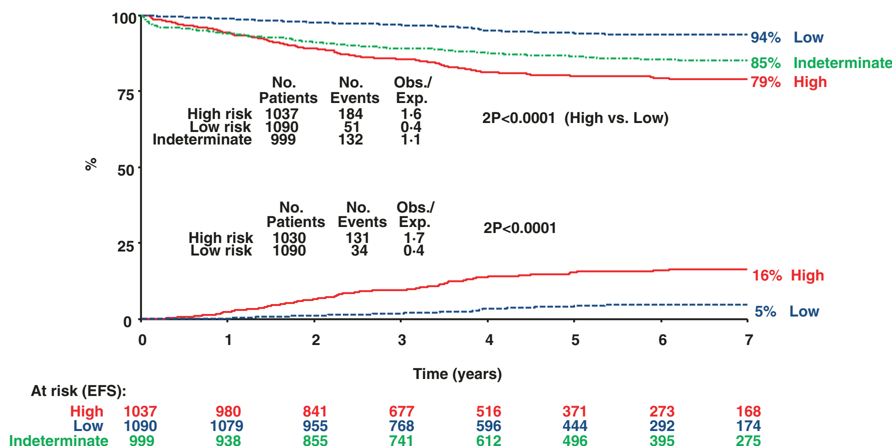


Fig. 7.5 UKALL2003: EFS and RR by MRD risk group

7.3.2 *Infants*

Acute lymphoblastic leukemia (ALL) in infants under 1 year of age is rare and biologically different from ALL in childhood. Infant ALL is characterized by a high frequency of MLL gene rearrangements, a very immature B-cell phenotype (proB ALL), expression of myeloid markers, lack of CD10 expression, presentation with a high tumor load and a poor outcome. MLL rearrangement status, presenting white blood cell count (WBC), age at diagnosis and prednisone response are independent prognostic factors and a model comprising age, MLL status and WBC stratifies patients into three risk groups with distinct 4 year EFS (Low: MLL germ line, EFS 74%; Intermediate: MLL re-arranged, WBC <300 or age <6 months, EFS 43% and High: MLL rearranged and age <6 months and WBC >300, EFS 18%). The current treatment approach within an international trial, Interfant 06, combines this risk stratification algorithm and MRD response to select patients for first remission allogeneic transplant. Unfortunately, despite these international efforts, the outcome for this sub-group remains poor [62]. Molecular investigations and pre-clinical studies indicate that epigenetic modifiers and immune based approaches might be effective in MLL rearranged cases and are to be tested in future trials.

7.3.3 *Down Syndrome*

Children and young people with DS have a 10 to 20-fold increased risk of developing ALL compared to those without DS. This increased risk is limited to the first three decades of life but with a notable absence of ALL in those under a year of age.

T cell ALL is rare in DS, as is the presence of CNS disease at diagnosis. ALL associated with DS has a distinct frequency of genetic changes from ALL seen in the non-DS population. Recurring cytogenetic abnormalities conferring either a favourable or poor prognosis are less common. Favourable risk cytogenetics; high hyperdiploidy and ETV6/RUNX1 fusions, occur in 50% of children with non-DS-ALL compared to 10–20% in DS-ALL. Activating CRLF2 and JAK2 and IZKF1 deletions are found more commonly in DS-ALL. DS-ALL has an inferior survival due to a combination of increased relapse risk and high treatment related mortality (TRM) [63]. Many groups treat DS-ALL with reduced intensity treatment and recommend additional supportive care measures. As in non-DS patients, good risk karyotype and MRD low risk status are associated with a significantly lower risk of relapse in DS-ALL [64]. These sub-groups may benefit from treatment de-escalation to reduce the risk of treatment related mortality and morbidity.

7.4 Future Strategies and Conclusions

Given the good results of current protocols, future studies of childhood ALL therapy face the law of diminishing returns. However, there remains a substantial minority of patients with primary refractory disease (around 2%) or early relapse (5%) who cannot be cured with current treatment, including Haemopoietic Stem Cell Transplant. These patients may be identified in first CR as having persistent high level MRD during the first 20 weeks of treatment. Intervention with novel agents (monoclonal antibodies, tyrosine kinase inhibitors, Nelarabine, Clofarabine and autologous CAR T cells, see Chaps. 8, 9, and 10) followed by HSCT early in first CR (between weeks 12 and 20) might offer some of these patients a cure. In the UK, we plan to test novel agents during the week 12–20 window in these patients using MRD as a surrogate marker.

As cure rates improve, greater attention should focus on reducing treatment related deaths which make up an increasing proportion of treatment failures (see Chap. 12). Identification of groups at high risk of toxicity (e.g. Down syndrome) and pharmacogenomic expression profiling (see Chap. 5) will guide targeted supportive care and individualised drug dosing to reduce toxic deaths. There is evidence that gene expression profiling of leukaemic blasts can predict in-vitro and in-vivo chemosensitivity and treatment in future could be customised to a patient's pharmacogenomic and leukaemia gene expression profiles. In future, new drugs designed to target leukaemia specific receptors and proteins could replace elements of conventional chemotherapy regimens responsible for some of the major toxicities, thereby reducing toxicity whilst retaining overall efficacy of treatment. Translation of recent advances in understanding of the molecular biology of ALL (see Chap. 4) and its influence on phenotype and clinical outcome will help define specific sub-groups that might benefit from such an approach (see Chap. 8). Lastly, international collaboration, as highlighted by the Interfant protocol, will need to increase so as to properly investigate new

treatment strategies and the biological determinants of treatment response in rare sub-group of patients such as those with the Philadelphia chromosome and near haploid abnormalities.

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Chapter 8
Targeted Therapy and Precision Medicine

Sarah K. Tasian and Stephen P. Hunger

8.1 Introduction

Despite successful cure of most children with ALL via modern chemotherapy regimens, relapsed ALL remains a major source of childhood cancer-associated mortality. Current multi-agent drug regimens required to eradicate leukaemia are intensive and relatively non-targeted, often also inducing deleterious short- and longer-term toxicities due to concomitant effects upon normal tissues. To date, treatment success in childhood ALL has largely been achieved via biology- and response-based risk stratification of patients with appropriate modulation of the intensity of standard cytotoxic chemotherapy agents. Recent studies by childhood cancer cooperative groups and associated research laboratories have identified new leukaemia-associated germline genetic variants and somatic alterations in *de novo* and relapsed ALL (described in detail in Chap. 5). Many of these alterations have proven to be of prognostic significance with respect to clinical outcomes and have led to further refinement of ALL risk classification [1]. Efforts are now ongoing to characterize the epigenetic, biochemical, and other functional sequelae resulting from these mutations that may provide therapeutic vulnerabilities within leukaemia cells. Finally, tremendous effort is being directed towards development of small molecule inhibitors and other tailored therapeutic agents to target ALL-associated driver lesions and pathways. The goals of such precision medicine approaches are to increase anti-leukaemia efficacy and decrease relapse, thereby leading to higher cure rates, and to reduce toxicity from off target effects of cytotoxic agents.

Successful implementation of precision medicine approaches for childhood ALL will require clinical development of validated genomic testing platforms capable of

S.K. Tasian (✉) • S.P. Hunger
Department of Pediatrics and the Center for Childhood Cancer Research, Children's Hospital of Philadelphia and the Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA
e-mail: tasians@email.chop.edu; hungers@email.chop.edu

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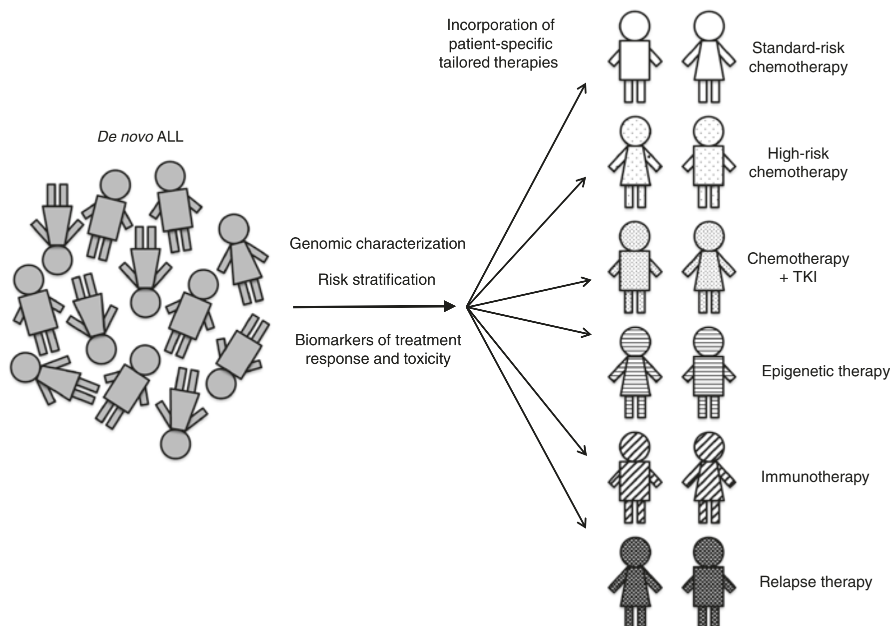

Fig. 8.1 Schema of precision medicine approaches for childhood ALL

identifying a complex milieu of leukaemia-associated alterations, as well as clinical availability and pediatric-appropriate dosing of biologically-relevant drugs (Fig. 8.1). While cases of "exceptional responders" (single patients with dramatic responses to treatment not otherwise effective for most patients) have been reported, well-designed and appropriately-powered clinical trials will ultimately be needed to determine the potential enhanced efficacy or lack of efficacy of new tailored therapeutic strategies for childhood ALL. Given the rarity of some patient subsets, novel trial designs and international collaboration will be essential.

8.2 Precision Medicine for B-ALL

8.2.1 Kinase Inhibition for BCR-ABL1-Rearranged (Ph$^+$) ALL

One of the first major advances in precision medicine for human cancer occurred during the 1960s, 1970s, and 1980s with identification of the *BCR-ABL1* fusion (Philadelphia chromosome, Ph) within leukaemia cells that results in formation of a chimeric BCR-ABL1 protein [2–5]. Somatic *BCR-ABL1* rearrangement was subsequently determined to occur in nearly all patients with chronic myeloid leukaemia (CML) and in a subset of adults (25–30%) and children (3–5%) with B-ALL [6–8].

The molecular consequence of *BCR–ABL1* fusion in both CML and Ph⁺ ALL is constitutive activation of ABL1 kinase signaling, which leads to aberrant downstream signaling via SRC family kinases and PI3K/Akt/mTOR, JAK/STAT, Ras/MAPK pathways. In addition, the majority of Ph⁺ ALL is associated with deletions in transcription factors that regulate B-cell development, including *IKZF1* and *PAX5* [9], although the therapeutic implications of these concomitant alterations remain unknown.

Previously, patients with Ph⁺ ALL had dismal treatment responses and high rates of relapse despite very intensive multi-agent chemotherapy and often hematopoietic stem cell transplantation (HSCT) in first complete remission (CR1) [10]. Indeed, event-free and overall survival (EFS and OS) for children with Ph+ ALL treated in the 1980s–2000s was reliably <50% despite maximal intensity of conventional chemotherapy and frequent employment of HSCT in CR1 [11, 12]. Landmark studies conducted in the early 2000s demonstrated remarkable clinical efficacy of the ABL-targeting tyrosine kinase inhibitor (TKI) imatinib in adults with CML or Ph⁺ ALL, resulting in major cytogenetic remissions and markedly improved OS [13, 14]. Similarly, trials conducted by the international BFM study group and the Children's Oncology Group (COG) demonstrated the safety of combining imatinib with multi-agent chemotherapy in children with Ph⁺ ALL. More importantly, these studies demonstrated improvements in EFS and OS with addition of TKI therapy [15, 16]. Mature clinical trial data have now established that a majority of patients with Ph⁺ ALL can be successfully treated with TKIs and chemotherapy without need for HSCT in CR1 [15]. Ten-year OS for children with Ph+ ALL treated with imatinib and chemotherapy now approaches 80% [17, 18]. The remarkable clinical responses with TKI-based therapies further corroborate *BCR-ABL1* as a driver oncogene in Ph⁺ ALL and establishes a firm paradigm for successful precision medicine therapies in childhood ALL.

However, selective pressure of imatinib therapy over time, particularly as monotherapy for CML, can lead to acquisition of ABL tyrosine kinase domain (TKD) mutations that confer reduced TKI sensitivity or overt therapeutic resistance [19–21]. Various point mutations associated with imatinib resistance have now been reported, and some genotype-phenotype correlation exists with specific mutations (e.g., *ABL1* T315I) leading to particular TKI resistance due to conformational changes in the BCR-ABL1 fusion protein that prevent imatinib binding in the ATP pocket [22]. Over 80% of adults with relapsed Ph⁺ ALL treated with imatinib have evidence of *ABL1* TKD mutations. Controversy remains whether such resistance mutations develop *de novo* during TKI therapy, as pre-existent TKD-mutant subclones have been detected at very low levels in up to 40% of adults with CML prior to TKI initiation. Interestingly, TKD mutations may occur less commonly in patients treated with both TKI and chemotherapy, perhaps due to reduced selective pressure upon imatinib-resistant mutant subclones. Only limited published data are available regarding the true incidence of TKD mutations in children with Ph⁺ ALL (at diagnosis, during therapy, and at relapse). Available data suggest that *BCR-ABL1* TKD mutations occur in only a small minority of children that relapse following intensive

chemotherapy plus imatinib, presumably because combination therapy significantly overcomes the selective pressure of TKI monotherapy [23, 24].

Given frequent emergence of *ABL1* TKD mutations during imatinib therapy for CML, second- and third-generation ABL kinase inhibitors (e.g., nilotinib, dasatinib, bosutinib, ponatinib, bafetinib) have been developed to overcome therapeutic resistance [25]. Many of these TKIs are structurally similar to, but sufficiently distinct from, imatinib that they can bind to and stabilize the inactive conformation of the BCR-ABL1 fusion protein in the context of some TKD mutations to restore sensitivity to ABL inhibition [26]. Several of these newer TKIs also inhibit SRC kinases (e.g., dasatinib, bosutinib, ponatinib) and have improved central nervous system penetration (e.g., dasatinib), which may provide theoretical therapeutic advantages [27, 28], although superiority of specific TKI therapy has not been definitively proven to date in Ph$^+$ ALL [29]. Large scale clinical trials have now demonstrated comparably excellent or superior outcomes in Ph$^+$ leukemias with dasatinib or nilotinib treatment [28, 30, 31], including combined dasatinib and intensive multi-agent chemotherapy treatment of children with Ph$^+$ ALL [32]. Additional studies of ABL1-targeting TKIs in children with Ph$^+$ ALL are ongoing or in development in Europe and North America (NCT01460160). Some trials will also investigate whether therapy with TKIs and less intensive chemotherapy backbones can preserve favorable outcomes while potentially minimizing toxicities.

Ph$^+$ leukemia cells harboring specific ABL TKD mutations may be differentially sensitive or resistant to individual TKIs (Table 8.1). One example is the ABL1 "gatekeeper" T315I mutation resistant to imatinib, dasatinib, and nilotinib, but sensitive to ponatinib and the vascular endothelial growth factor receptor inhibitor axitinib [33, 34]. Tailoring kinase inhibitor therapy to specific mutational profiles is thus likely critical for long-term therapeutic success. Such an approach may also require careful resistance mutation surveillance during therapy and TKI switching if necessary [8, 22, 35]. Furthermore, it remains unknown whether concomitant chemotherapy administered with TKIs for patients with Ph$^+$ ALL diminishes emergence of resistance mutations or if such therapy simply masks low-level mutations that are subsequently detectable at relapse [8, 23, 35]. Of note, pediatric Ph$^+$ ALL trials generally stop TKIs after 2–2.5 years with conclusion of cytotoxic chemotherapy.

While the optimal ABL TKI(s) and chemotherapy backbone combination for childhood Ph+ ALL has not yet been defined, results from trials such as COG AALL0031 and AALL0622 and EsPhALL highlight key concepts in precision medicine with tremendous therapeutic relevance for other high risk genetic subsets of childhood ALL. Successful development of tailored therapies for such patients will similarly require identification of leukemia-associated driver lesions that are critical for oncogenesis and to which the leukemia is "addicted", as well as access to relevant molecularly-targeted drugs that will be tolerable and efficacious in combination with anti-ALL chemotherapy [1, 36].

Table 8.1 Reported *BCR-ABL1* kinase domain mutations in Ph+ ALL, predicted tyrosine kinase inhibitor (TKI) sensitivity, and clinical testing status

ABL1 domain mutation	Location	Sensitivity to clinically-available TKIs						
		Imatinib	Nilotinib	Dasatinib	Bosutinib	Ponatinib	Bafetinib	Danusertib
Y253H	P-loop	N	I	Y	Y	Y	Y	Y
E255K	P-loop	N	I	Y	Y	Y	Y	Y
T315I	SH3-SH2	N	N	N	N	Y	N	Y

Y = yes, I = intermediate, N = no

TKI	Adult indication or clinical trial status	Pediatric trials
Imatinib	First-line therapy for Ph+ CML and ALL	NCT00022737, NCT00287105
Nilotinib	First-line therapy, second-line therapy for imatinib-resistant or -intolerant CML	NCT01077544
Dasatinib	First-line therapy, second-line therapy for imatinib-resistant or -intolerant CML	NCT00777036, NCT01460160
Bosutinib	Second-line therapy for imatinib-resistant or -intolerant CML	Not available
Ponatinib	Second-line therapy for imatinib-resistant or -intolerant CML	Available via IND access
Bafetinib	Phase 2 trial (NCT00352677)	Not available
Danusertib	Phase 1 trials (EudraCT 2007–004070-18, NCT00335868)	Not available

8.2.2 Kinase Inhibition for BCR-ABL1-Like (Ph-Like) ALL

Ph-like ALL is a recently-described subset of B-ALL defined by a kinase active gene expression profile similar to that of Ph+ ALL and associated with a diverse range of genetic alterations that activate kinase signaling pathways [9, 37–39] and is reviewed in detail in Chap. 5. Approximately 50% of Ph-like ALL has rearrangement of the cytokine receptor like factor 2 gene (*CRLF2*), and concomitant *JAK2* or *JAK1* point mutations occur in about half of these cases [40]. An additional 15–20% of Ph-like ALL has translocations or fusions involving *ABL1*, *ABL2*, *CSF1R*, or *PDGFRB* (ABL-class lesions), while another 10–15% harbours fusions involving *JAK2* or *EPOR* genes [39, 41, 42]. As in Ph+ ALL, deletions of *IKZF1* and other lymphoid-associated transcription factors are common in Ph-like ALL [9, 43].

Patients with Ph-like ALL have high rates of treatment failure and relapse when treated with conventional chemotherapy [37, 38, 44]. Given these poor outcomes and the spectrum of identified kinase pathway genomic alterations, Ph-like ALL is an ideal candidate for the development of precision medicine approaches with kinase inhibitors that are already clinically available. Numerous preclinical studies have demonstrated activated ABL, JAK/STAT, and/or PI3K pathway signaling in Ph-like ALL [39, 41, 45–47]. Other studies using Ph-like ALL preclinical models have also reported *in vitro* and/or *in vivo* sensitivity of ABL-class fusions to imatinib and dasatinib and of *CRLF2* rearrangements, JAK2 fusions, and leukemias with *EPOR* rearrangements and truncations (JAK-class alterations) to the JAK1/2 inhibitor ruxolitinib [41, 42, 48]. Preclinical studies have also demonstrated sensitivity of Ph-like ALL models to PI3K pathway inhibitors [48, 49]. These findings strongly suggest potential clinical efficacy of kinase inhibition in genomically-defined subsets of Ph-like ALL and raise the possibility of combination therapy with inhibitors of different signaling pathways. Numerous anecdotal reports have been published of Ph-like ALL patients with ABL-class fusions and poor early responses to chemotherapy who had subsequent dramatic clinical responses with addition of imatinib or dasatinib to chemotherapy [50–52]. Of note, *PDGFRB* fusions appears to occur frequently in patients with induction failure (>25% residual leukemia after one month of induction chemotherapy), and testing for such alterations should be particularly considered for patients in this situation [51, 52].

Cooperative groups are now actively developing clinical trials to test the efficacy of addition of imatinib/dasatinib or ruxolitinib to chemotherapy for patients with Ph-like ALL harboring ABL-class fusions or JAK pathway alterations, respectively (Table 8.2). These efforts have required development of genetic testing capable of identifying the diverse milieu of Ph-like ALL lesions in relative real time, which to date has involved a complex combination of gene expression analyses, fluorescence in situ hybridization, and specific polymerase chain reaction-based molecular testing with Sanger sequencing confirmation [53]. New next-generation sequencing platforms capable of more comprehensive gene fusion detection, including newly discovered alterations, have also demonstrated exciting early clinical promise in identifying Ph-like ALL alterations. Ideally, patients with Ph-like ALL should be

Table 8.2 Clinical trials of TKI therapies for children, adolescents, and adults with Ph-like ALL

Patient population	Age	Disease status	TKI	Trial
Ph-like with ABL class alterations	≥18 years	Relapsed	Dasatinib	NCT02420717
Ph-like with CRLF2/JAK pathway alterations	≥18 years	Relapsed	Ruxolitinib	NCT02420717
Ph-like with ABL class alterations	1–30 years	*De novo*	Dasatinib	NCT01406756
Ph-like with CRLF2/JAK pathway alterations	1–21 years	*De novo*	Ruxolitinib	NCT02723994

rapidly identified via streamlined, comprehensive diagnostic testing, then allocated to clinical trials testing the efficacy of chemotherapy with relevant TKIs added during induction therapy.

8.2.3 FLT3 Receptor Kinase Inhibition for KMT2A (MLL)-Rearranged ALL

Somatic rearrangement of *KMT2A* (formerly *MLL*) occurs in about 75% of infants with B-ALL, particularly in those less than 6 months of age, as well as in a smaller percentage of older children, adolescents, and adults [54, 55]. More than 100 translocation partners have been reported to date. Disease phenotype and prognoses appear to vary somewhat based upon specific *KMT2A* fusions, although clinical outcomes are generally inferior to those of patients without *KMT2A* rearrangement [56, 57]. Infants with *KMT2A*-rearranged ALL frequently present with hyperleukocytosis and central nervous system leukaemia involvement and have particularly poor overall survival despite intensive multi-agent chemotherapy (<50% at 4 years), as demonstrated by multiple childhood cancer cooperative groups [58, 59]. Interestingly, a remarkable paucity of other somatic mutations occurs in infant ALL [60], and the near-universal ALL concordance rate in monozygotic twin infants further demonstrates that *KMT2A* fusions can be fundamental drivers of leukemogenesis [61]. Research characterizing dysregulated pathways in infant ALL that may be ideal targets for precision medicine therapies is therefore a priority.

The fms-related tyrosine kinase 3 receptor (FLT3) is commonly overexpressed in *KMT2A*-rearranged infant ALL [62]. FLT3 appears critical for hematopoietic progenitor cell differentiation and proliferation, and FLT3 ligand binding to its receptor activates numerous downstream signaling networks, including Ras/MAPK, PI3K/mTOR, and STAT5. Preclinical studies in *KMT2A*-rearranged ALL models have demonstrated selective anti-leukemia cytotoxicity of several FLT3-targeting TKIs, including lestaurtinib (formerly CEP-701) and midostaurin (formerly PKC412) [62–66]. Unfortunately, clinical testing of FLT3 inhibitor lestaurtinib in combination with intensive post-induction chemotherapy did not improve EFS in infants

with newly-diagnosed *KMT2A*-rearranged ALL [67]. Similarly, a pilot study of the more selective FLT3 inhibitor quizartinib and chemotherapy in children with relapsed leukemias demonstrated no significant clinical responses in a small number of infants with relapsed *KMT2A*-rearranged ALL [68]. Further studies are needed (perhaps with more potent or more selective FLT3 inhibitors such as gilteritinib, crenolanib, others) to determine if FLT3-targeted therapies can actually improve outcomes in infant and other *KMT2A*-rearranged ALL. Even if efficacy can be demonstrated, acquisition of FLT3 mutations during therapy could be a potential mechanism of resistance, as has been reported in adults with acute myeloid leukemia treated with FLT3 inhibitors [69, 70].

Another potential precision medicine approach for infant ALL relies upon the discovery of frequent epigenetic dysregulation reported in *KMT2A*-rearranged leukemias [71, 72]. Several DNA methylation, histone acetylation, and histone methylation regulators have been implicated in particular. As such, demethylating/hypomethylating agents (e.g., decitabine, 5-azacytidine, disruptor of telomeric silencing 1-like histone H3K79 methyltransferase [DOT1L] inhibitors) and histone deacetylation inhibitors (e.g., vorinostat, panobinostat, bromodomain inhibitors) are also under active preclinical and early clinical evaluation specifically in children with *KMT2A*-rearranged leukemias (NCT02141828, NCT01483690, NCT01321346).

8.2.4 MAP Kinase Inhibition for RAS Pathway-Mutant ALL

Activating mutations in Ras pathway-associated genes (e.g., *KRAS*, *NRAS*, *HRAS*, *PTPN11*, *CBL*, *FLT3*) occur in up to 30% of human cancers [73], including ALL [74–77]. Prevalent Ras point mutations have been reported in children with *de novo* ALL with favourable-prognosis high hyperdiploidy [78, 79], in unfavourable-prognosis hypodiploid ALL [80], and in a subset of Ph-like ALL [39]. Ras mutations have also been reported in one-third of Down Syndrome-associated B-ALL and in nearly 40% of relapsed B-ALL cases [81]. In some studies, *NRAS* and *KRAS* mutations were associated with early risk of relapse and poor clinical outcomes [82, 83]. However, other studies have not identified inferior outcomes of children with Ras-mutant ALL, perhaps due to success of modern intensive chemotherapy regimens [75, 78, 84]. Ras and PI3K pathway mutations have also been described in infant ALL and may be associated with inferior survival [85], although they appear to often occur in subclonal populations that are frequently diminished or lost at relapse [60]. The driver versus passenger nature of Ras mutations in childhood ALL thus remains unknown, and additional studies are required to clarify the prognostic significance of these alterations.

Nonetheless, targeting dysregulated Ras pathway activation in human cancer remains of great therapeutic interest. Inhibition of the “broken switch” Ras protein itself has proven quite challenging, however [73, 86, 87]. While promising *in vitro* activity of drugs targeting post-translational effectors of Ras, such as inhibitors of farnesyltransferases or geranylgernanyltransferases [88, 89], was initially

demonstrated, minimal clinical activity of such agents (e.g., tipifarnib) has been observed to date in small pilot trials in patients with relapsed or refractory leukemias [90, 91]. More recent efforts have instead focused upon targeting associated aberrant MAPK and PI3K signaling in Ras-mutant leukemias. *In vitro* and *in vivo* studies have demonstrated enhanced sensitivity of Ras-mutant ALL cells to MEK inhibition (e.g., selumetinib, trametinib, cobimetinib, binimetinib) or PI3K pathway inhibition (e.g., GDC-0941, BEZ235) [74, 80, 82]. Simultaneous MEK and PI3K inhibition may further have additive or synergistic activity in Ras-mutant ALL and other leukemias [92–94]. Given the clinical efficacy of small molecule inhibitors targeting the Ras/Raf/MEK pathway to date in adults with Ras-mutant solid tumors [95, 96], as well as ongoing testing of MEK inhibition in children with solid tumors (NCT01089101, NCT02124772, NCT02285439), early phase trials of MEK inhibitors in pediatric patients with relapsed/refractory Ras pathway-mutant leukemias are also planned.

8.3 Precision Medicine for T-ALL

While progress has been made to date with identifying potential therapeutic targets in B-ALL and testing small molecule inhibitor therapies, development of precision medicine approaches for T-ALL has proven more challenging. The prognostic impact of recurrent genetic and epigenetic alterations in T-ALL remains incompletely understood, although identification of recurrent kinase mutations and fusions in T-ALL suggest that TKI-based therapies may also warrant evaluation in some T-ALL subsets [97–99].

8.3.1 Kinase Inhibition for T-ALL

Constitutive activation of PI3K pathway signaling has been reported in T-ALL, particularly in leukemias harboring *NOTCH1* mutations and *PTEN* deletions [100–103]. Common cooperating lesions in *NOTCH1*-mutant ALL, such as deletion of the tumor suppressor *CDKN2A* or mutations in the ubiquitin protein ligase *FBXW7*, may further lead to hyperactive PI3K signaling by attenuating physiologic degradation of the NOTCH1 protein [104]. Various studies have demonstrated a key role of PI3K/Akt/mTOR signaling in initiation and maintenance of lymphoid and myeloid leukemogenesis [105], which may provide an opportunity for targeted therapeutics in T-ALL. Additional preclinical studies have demonstrated activity of PI3K pathway inhibitors in ALL models, including mTOR inhibitors (e.g., rapamycin and derivative “rapalogs”) and newer PI3K isoform-selective inhibitors, Akt inhibitors, PI3K/mTOR inhibitors, and TORC1/TORC2 inhibitors [100, 106]. While these agents are under evaluation in numerous trials for adults with relapsed/refractory solid tumors, clinical translation of PI3K pathway inhibition in ALL is currently in

its infancy. Safety, but limited efficacy, was recently reported in small phase 1 trial testing a PI3K/mTOR inhibitor in adults with relapsed/refractory B-ALL [107]. Efforts in childhood leukemias to date have primarily focused upon phase 1 testing of mTOR inhibitors in children with relapsed ALL (NCT01523977, NCT01614197, NCT01403415).

NUP214-ABL1 rearrangements resulting from t(9;9) occur in a subset of T-ALL and are associated with activated ABL1 pathway signaling [97, 108], suggesting potential for improved outcomes with incorporation of ABL kinase inhibitors into therapy. Indeed, preclinical studies have demonstrated inhibition of the ABL1 target phosphoproteins CrkL and STAT5 and leukemia cytotoxicity in dasatinib- or nilotinib-treated *NUP214-ABL1* T-ALL cells [108, 109]. Clinical efficacy has been reported in a small number of patients with refractory *NUP214-ABL1* ALL treated with imatinib or dasatinib [39, 109–111].

JAK/STAT inhibition (e.g., ruxolitinib, tofacitinib, momelotinib) may also have therapeutic relevance in *ABL1*-mutant and other subsets of T-ALL. *IL7R*, *JAK1*, *JAK3*, and *SH2B3* mutations occur frequently in T-ALL, particularly in the early thymic precursor (ETP) subtype [112–114]. Preclinical efficacy of JAK inhibition was recently reported in models of childhood T-ALL [115, 116]. *FLT3* mutations have also been described in a subset of ETP ALL and may be amenable to FLT3 inhibitor therapies [117].

8.3.2 Gamma Secretase Inhibition for Notch1-Mutant T-ALL

In addition to potential therapeutic relevance of PI3K inhibition in *NOTCH1*-mutant T-ALL, anti-NOTCH1 antibody immunotherapies and gamma secretase inhibitors (GSIs) that block NOTCH1 degradation have been investigated [101]. While preclinical studies of GSIs have demonstrated remarkable leukemia cytotoxicity [118–120], on target/off tumor gastrointestinal toxicity induced by first-generation GSIs has limited their clinical efficacy to date in patients with T-ALL [121, 122]. Newer GSIs with more favorable toxicity profiles are currently under study in adults with relapsed T-ALL [123–125].

8.4 Future Strategies/Conclusions

The complete genomic, epigenomic, and transcriptomic landscape of childhood ALL remains incompletely characterized. Continued improvements in understanding the biology of this genetically heterogeneous disease and its associated biochemical, immunologic, and transcriptional sequelae will continue to inform risk stratification of patients and application of appropriately intensive therapies to achieve cure. Clinical development of tailored therapies for childhood ALL will continue to rely upon accurate, swift identification of genetic subtypes and will

require access to biologically relevant inhibitors. One major hurdle in successful clinical realization of precision medicine approaches in childhood ALL is that new agents are commonly first tested as monotherapy in patients with multiply relapsed disease to assess safety and tolerability, lack of efficacy in this setting does not necessarily predict response in patients with newly-diagnosed ALL or in combination with chemotherapy. In addition, it is becoming increasingly apparent that combination inhibitor therapies may be required to achieve long-term anti-leukaemia efficacy while minimizing development of resistance mutations and escape pathways. Finally, clinical trials for evaluation of targeted inhibitors in increasingly smaller, genetically- or epigenetically-defined "boutique" subsets of childhood ALL will likely require (a) comparison to rigorous historic control data of patients treated with chemotherapy and (b) robust correlative biomarker studies to most accurately assess potential improvement in clinical response and to prioritize further drug development. Such efforts will also likely require innovative trial designs and international collaboration to maximize patient accrual in an efficient study timeline. While significant advances in the genomic characterization of childhood ALL have identified new potential therapeutic vulnerabilities, additional studies are necessary to decipher more fully the molecular dependencies of childhood ALL and to bring targeted therapies to fullest fruition. Ultimately, it is expected that the paradigm of imatinib for Ph^+ ALL will continue to inspire successful precision medicine treatment approaches for other subsets of childhood ALL that will improve outcomes and perhaps also minimize toxicities.

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Chapter 9
Monoclonal Antibodies in Pediatric Acute Lymphoblastic Leukemia

Christiane Chen-Santel

9.1 Monoclonal Antibodies

9.1.1 Introduction

Köhler and Milstein developed the first monoclonal antibody (moAb) by fusion of murine myeloma cells with B cells [1]. The first mouse, chimeric and humanized immunoglobulin (IgG) antibodies reached the market in the late 1990s [2]. In the past decades over 30 immunoglobulins have been approved for different indications [3, 4]. The majority belongs to the IgG1 class [5], but the antibody structures have been considerably extended, e.g. to other IgG isotypes [3, 5], and IgG-related products [6] have been developed. There are two main approaches to select antigens for antibody selection. One approach further develops antibodies against 'validated targets' by targeting other epitopes and/or triggering different mechanisms of action e.g. to reduce immunogenicity or increase affinity and avidity for the antigens [7]. The other 'functional approach' identifies new or less well studied target proteins. This method is riskier, because other factors may also play a role in antibody development and extensive validation is necessary [2].

9.1.2 Structure of Monoclonal Antibodies (moAbs)

MoAbs are large molecules consisting of 1300 amino acids and built from two heavy chains with 50 kDa each and two light chains with 25 kDa each. The heavy chains (γ1, γ2, γ3, γ4) divide the IgGs into subclasses, the light chains are κ− and

C. Chen-Santel
Department of Pediatric Oncology/Hematology/BMT, Charité University, Medicine Berlin, Augustenburger Platz 1, 13353 Berlin, Germany
e-mail: christiane.chen-santel@charite.de

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λ−type. They are connected by disulfide bridges giving the antibody its Y-shaped structure and stabilizing the folding [8].The variable domains variable light (VL) and variable heavy (VH) have hypervariable regions responsible for antigen binding. The other domains are conserved sequences called constant domains constant light (CL) and constant heavy (CH1–3) [9]. The size and structure of IgGs allow for a vast amount of different alterations and modifications which may have an influence on structure, pharmacokinetics and function of moAbs [8]. Most monoclonal antibodies are chimeric, humanized or human IgGs with similar constant domains. Improvement of pharmaceutical properties such as microvariants [10], amino acid sequences [11] and of conjugation of drugs to the antibodies (e.g. choice of linker and conjugation position) [12] as well as improvement of simultaneous targeting (bispecific antibodies) [13], the development of oligoclonal/polyclonal antibodies against the same or different targets [14, 15] and alternative antibody or drug formats (protein scaffolds) [16, 17] are needed and are in process in order to improve the function and cytotoxicity of these antibodies.

9.1.3 Clinical Use of moAbs

Monoclonal antibodies are effective through three different mechanisms (1) directly (2) via antibody-dependent cell cytotoxicity (ADCC) or (3) via complement-mediated cytolysis (CMC). Older moAbs were of murine or chimeric origin, whereas recent moAbs are humanized or fully human, which decreases antigenicity and enhances their efficacy in the circulation [18]. Type I antibodies are effective via CMC and ADCC, but weaker on direct cytotoxicity, whereas type II antibodies work through ADCC and direct cytotoxicity, but have low CMC activity [19]. It has been shown that moAbs against immune cells as well as tumor cells lead to responses in patients with solid tumors [20] and hematological malignancies [21].

9.2 Target Antigens in Acute Lymphoblastic Leukemia

In cancer patients antigens are attractive for moAb therapy, when they are expressed densely and consistently by malignant cells, but limited on benign cells and in the soluble form [22, 23] and not physiologically important for normal cells [24]. Other antigens of interest are not expressed on the cell surface, but processed by the human leukocyte antigen (HLA) and presented to the effector cells [25]. Therefore such moAbs are HLA-restricted [24]. Some of the moAb can induce cytotoxcitiy by direct transmembrane signalling [26]. Other mechanisms of action are complement-mediated cytotoxicity [27, 28] and antibody-dependent cellular cytotoxicity (ADCC) [29]. Most of the therapeutic effects of moAbs in hematological malignancies are possibly due to CMC [30]. On the other hand, the whole antigen-moAb complex may be shed from the surface of the malignant cells and transfer part of the cell membrane

via trogocytosis [31] or are internalized. As a result the circulating malignant cells might evade the specific moAbs due to the lack of the antigen on the tumor cell surface [32]. Sometimes crosslinking to a secondary antibody, e.g. a specific IgG, is necessary for cytotoxicity [33]. That crosslinking occurs partly through the binding of the constant region of the IgG to the Fc receptors on the immune effector cells in the tumor microenvironment [34, 35] and could result in a more robust signal [24]. Eighty percent of ALL is of precursor B-cell phenotype. Cell surface antigens include CD19, CD20, CD22 and CD52 [36], which have important physiological functions in cell survival, proliferation and development. CD19 is a B-cell receptor and involved in B cell activation and development [36]. It is expressed in on all stages of B-lineage ALL [37] including expression on leukemic stem cells with self-renewing potential [38] in more than 90% of these ALL patients, whereas it is down-regulated on healthy plasma cells and hematopoetic stem cells [39].

CD22 is a multifunctional regulator of B-cell functions including B lymphocyte survival, signal transduction, cellular adhesion [40] and B-cell homing [41]. CD22 is expressed in more than 80% of ALL patients [42], but not expressed by stem cell precursors [43] or other tissue cells [44]. CD20 promotes B cell differentiation, activation and cell cycle progression [45]. Only about 50% of the precursor B-cell ALL express CD20 [38] which is not expressed on plasma cells and hematopoetic stem cells [46]. In adult patients with ALL CD20 expression is associated with a decreased remission duration and worse overall survival (OS) [47]. On the other hand, CD20 expression is up-regulated by chemotherapy and might therefore be an interesting target for combination therapy [48]. In pediatric precursor B-cell ALL there are conflicting data [49–52]. A meta-analyis on six cohort studies have not shown a prognostic relevance, although patient populations have shown heterogeneity [52]. CD52 is a costimulatory molecule for regulatory T cell induction from normal CD4+ T cells [53] and is expressed on most lymphoproliferative malignant cells, mature B and T cells, but also on monocytes and macrophages [36].

MoAbs against inhibitory signals that limit T cell activation, so called checkpoint blockade moAbs, have been developed and enhance antitumor T cell responses [54]. These include moAbs e.g. against CTLA-4 and PD-1. One caveat is the toxicity, which includes autoimmunity, since these moAbs are general checkpoint inhibitors [55].

9.3 Naked moAbs

9.3.1 CD52

9.3.1.1 Alemtuzumab

CD52 is expressed by 70–80% of T-cell and precursor B-cell ALL [56]. Alemtuzumab, also known as MabCampath, is a humanized anti-CD52 moAb, which induces ADCC in lymphocytes [57]. It has demonstrated activity in CLL. As a single agent it has limited activity in ALL [58, 59]. In 2004 three patients were

treated after hematopoetic stem cell transplantation (HSCT) with several doses of alemtuzumab and showed clinical response without achieving complete remission (CR) [60]. In a phase I study in newly diagnosed CD52+ adult ALL patients alemtuzumab administration post remission showed a one log decrease of MRD in the patient group [61]. In a phase II trial in children with refractory/relapsed ALL only one patient achieved CR and three patients had stable disease [59]. Based on single case reports [62, 63] a phase II trial in adult patients with refractory/relapsed precursor B-cell or T-cell ALL was conducted in combination with G-CSF to boost ADCC [63]. About 30% of patients have shown CR and one patient had clearance of blasts in peripheral blood, but no CR. Interestingly, the expression of CD52 did not correlate with clinical response. In addition CD52 negative cells/clones, which might be the result of defective glycophosphatidylinositol anchoring, might escape therapy and therefore account for the low response rates [64]. Most common AEs were viral infections such as CMV reactivation and infusion related reactions [63].

9.3.2 CD20

9.3.2.1 Rituximab

The chimeric human-mouse moAb rituximab has been approved in 1997 by the FDA for the treatment of B-cell malignancies [65], namely indolent lymphoma [66, 67] and became an integral part of treatment in non-Hodgkin lymphoma (NHL) [37]. Rituximab is a type I antibody [67] and kills the target cells via ADCC and CMC as well as induction of apoptosis [68]. It influences cell cycle progression and differentiation via downstream signalling pathways that modulate level of pro-apoptotic proteins such as Bax, Bak, NFκB and ERK1/ERK2 [45, 69]. In a study of the adult GMALL group in 133 patients with Standard Risk (SR) CD20 positive precursor B-cell ALL rituximab was used in combination with chemotherapy. Molecular remission (MRD negativity) and OS were significantly better than those of historical control [70]. These data were supported by Thomas et al. [71] who have shown that in patients <60 years of age CR, EFS and OS were significantly better, when rituximab was added to hyper-CVAD (hyperfractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone). In a pediatric cohort of precursor B-cell ALL with resistance to triple intrathecal therapy, administration of intrathecal rituximab was well tolerated [72]. Intrathecal (i.th.) or intraventricular (i.vent.) administration of rituximab has also been investigated in a large retrospective trial in children and young adults <21 years with B-cell malignancies. It has been administered as single agent therapy, in combination with methotrexate or triple therapy consisting of methotrexate, prednisone and cytarabine and has only shown limited toxicity in the majority of children with CNS involvement of Burkitt NHL, mature B-cell and precursor B-cell ALL and post-transplantation lymphoproliferative disorder (PTLD) [73]. Data in adult patients confirmed these results. I.th. or i.vent. administration of rituximab in B-cell NHL patients has not shown significant side effects [74, 75] and has a rapid distribution in the cranio-spinal axis [76]. A mechanism for rituximab resistance

might be the loss of CD20 expression due to internalization of the complex with FcγIIb, which might be overcome by FcγIIb inhibition [77].

9.3.2.2 Ofatumomab

Side effects of rituximab due to the mouse origin lead to humanized antibodies such as ofatumomab being developed. Ofatumomab is a humanized, next generation anti-CD20 antibody and binds to an epitope of CD20 that is different from the binding site of rituximab [78]. Ofatumomab binds CD20 with greater avidity than rituximab, which might be the reason for superior ADCC [79]. A phase II study in adult patients with CD20+ ALL combined ofatumomab to standard induction therapy hyper-CVAD. One patient has died of septic shock. All other patients achieved CR after the first cycle of ofatumomab and MRD negativity at 8 months of follow-up. The most common adverse events (AEs) were febrile neutropenia, SAEs included thrombotic events and neuropathy in 5% of patients [80].

9.3.2.3 Other Anti-CD20 moAbs

Newer generation anti-CD20 antibodies include obinutuzumab, veltuzumab and AME-133. Obinutuzumab is a humanized type II antibody [81, 82] and exhibits cell lysis without cross-linking with other antibodies. It has enhanced ADCC via a glycol-engineered fragment crystallisable region [83]. It has shown enhanced B cell depletion in normal volunteers as well as patients within CLL [84]. Its efficacy has been shown in clinical trials in patients with NHL [85, 86]. In vitro studies with NHL and precursor B-cell ALL cell lines obinutuzumab significantly increased cell death compared to rituximab [87, 88]. At equal doses obinutuzumab has demonstrated significantly decreased tumor burden and survival advantage over rituximab in xenograft models for precursor B-ALL [89], which is promising for ALL patients. It has also shown higher affinity for NK FcγRIIIa with enhanced anti-tumor activity [90]. It might be useful in rituximab resistant patients or as firstline therapy and needs to be confirmed in precursor B-cell ALL.

Veltuzumab is a humanized anti-CD20 moAb, which has been investigated as a single agent [91] as well as in combination with milatuzumab, a humanized anti-CD74 moAb, in patients with refractory/relapsed B-NHL with moderate responses [92]. It might be investigated in precursor B-cell ALL in the future.

9.3.3 CD22

9.3.3.1 Epratuzumab

Epratuzumab is a humanized anti-CD22 moAb. CD22 is a member of the sialic-acid-binding immunoglobulin-like family of adhesion molecules, that regulates B-cell activation and the interaction of B cells with T cells and APC [56, 93] It is

rapidly internalized upon binding [94], not shed into the extracellular environment and is effective through modulation of B-cell activation and signalling [95], not through direct cytotoxicity. It has shown modest activity as a single agent in NHL patients [96]. In a Children Oncology Group (COG) trial in children with precursor B-cell ALL combination of epratuzumab with induction therapy has demonstrated the same rate of CR (about 60%) as induction therapy alone, but a higher rate of MRD negativity (40% vs 25%) with acceptable toxicity. The most common AEs were infusion reactions (fever, rigors, nausea) [97]. The study was later amended with administration twice weekly compared to weekly doses in combination with chemotherapy.

These results were confirmed in a follow-up phase II trial in children and young adults (age 2–30 years) with precursor B-cell ALL [98]. One adult trial, SWOG S0919, which added epratuzumab to backbone chemotherapy with clofarabine/cytarabine in adult patients with refractory/relapsed precursor B-cell ALL showed significantly higher CR/CRi rates than historic controls (52% vs 17%) [99]. Children with SR precursor B-cell ALL are currently being recruited in the trial IntReALL SR 2010, a phase III trial randomizing patients to consolidation therapy with or without epratuzumab (NCT01802814).

9.4 MoAb-Drug Conjugates

The idea to link an antibody to a toxin has been exploited by Paul Ehrlich over 100 years ago [100] and has been under development for over 55 years [100, 101]. Through the conjugation with a toxin the moAb can direct the toxin specifically to the tumor cell for internalization and lysis. Potential problems are immunogenicity and the non-specific toxicity [102]. The advantage of conjugation of the moAb with other drugs over the unconjugated moAb is the lower concentration of the moAb to be delivered and the internalization properties of the conjugate [24], thereby maximizing drug delivery [101].

The conjugated drugs are small molecules, which are very potent and therefore reduce immunogenicity [103–105]. A crucial part of the conjugate is the linker that connects the moAb with the drug, since it should not alter the specificity of the moAb, should be nontoxic while bound to the moAb, remain stable in the circulation and release the effect in the right intracellular component [106].

9.4.1 CD22

9.4.1.1 Moxetumomab

The first generation immunotoxin BL22, linked to Pseudomonas aeruginosa exotoxin A [107] demonstrated activity in precursor B-cell ALL in vitro [108] as well as in a phase I trial in patients with hairy cell leukemia [94]. In a phase I trial

including precursor B-cell ALL there was only modest activity of the drug without any patient reaching CR [56]. A small percentage of patients developed neutralizing antibodies, AEs were not observed. In children with precursor B-cell ALL no CRs could be demonstrated [94]. The second generation immunotoxin, CAT-8015 or moxetumomab, was modified with reduction of nonspecific toxicities, increased stability, better affinity, enhanced tissue penetration and improved targeted cellular toxicity [94]. It has shown activity in pediatric precursor B-cell ALL samples in vitro [107] as well as in patients [56]. One phase I trial in children and young adults <25 years of age with precursor B-cell ALL or B-cell NHL have been completed. Two trials in children with precursor B-cell ALL have been terminated in 2015, one including patients with refractory/relapsed ALL, the other patients with positive MRD prior to HSCT (NCT02227108; NCT02338050) due in part to excessive toxicity, specifically haemolytic-uraemic syndrome. Resistance to the immunotoxin has been described in ALL cell lines due to a low level of DPH4 mRNA and protein [109]. The resistant cells were heavily methylated, which could possibly be reversed by the treatment with the hypomethylating agent 5-azacitidine.

9.4.1.2 Inotuzumab Ozogamicin

Inotuzumab ozogamicin, INO or CMC-544, is a humanized anti-CD22 antibody linked to the cytotoxic agent calicheamicin via an acetyl butyrate linker [42, 110]. Calicheamicin is a potent cytotoxic agent, which is derived from the γ-calicheamicin antitumor antibiotic that is produced by the bacterium micromonospora echinospora [101, 111]. A derivative of calicheamicin, N-acetyl-γ-calicheamicin dimethyl hydrazide, is more stable and usually the conjugate to moAbs. Within 30 min of binding to CD22 the complex is internalized via endocytosis [111]. After internalization of inotuzumab CD22 is degraded and calicheamicin released to the nucleus where it exhibits its action [110, 112] without being dependent on cell cycle progression [111]. Calicheamicin binds the minor DNA groove [113] and causes breaks in double-stranded DNA in sequence-specific manner which leads to cellular apoptosis [42, 111] Preclinical results have demonstrated activity in B cell lines [114] and primary ALL cells [113] as well as murine models [115]. Samples from patients with precursor B-ALL have shown that the effect of inotuzumab ozogamicin correlated with the pace of internalization of inotuzumab ozagamicin and the sensitivity of the cells to free calicheamicin. However, efficacy was not dependent on levels of CD22 expression or the ability of the cells to renew their CD22 expression [113, 116]. Preclinical data in B-ALL cell lines [114] and primary ALL cells [113] have shown promising data. Even with low levels of CD22 expression inotuzumab treated ALL cells have shown high intracellular calicheamicin levels [108], so that inotuzumab might also be interesting for patients with low expression of CD22. The first experience was in patients with B-cell NHL [117]. All patients had ≥50% CD22 positive lymphoblasts and most patients were heavily pretreated. Compared to historical controls inotuzumab as a

single-agent is more effective (CR 61% vs 31–44%) as first salvage therapy as well as second salvage therapy (CR 44% vs 10–20%) [118]. The initial clinical trial treated patients with CD22 positive refractory/relapsed precursor B-cell ALL with administration of inotuzumab every 3–4 weeks. It was later amended with increased number of patients and weekly administration of inotuzumab [42]. The response rate was 58% with 72% of the responding patients being MRD negative without a significant difference between three and four weekly-doses and one weekly-doses of inotuzumab. A survival benefit with HSCT after inotuzumab treatment has not been shown [42, 118], although longer term follow-up is needed to evaluate remission [119]. However, division of the total dose of inotuzumab into weekly dosing was less toxic [42, 118]. The initial phase II trial in ALL patients included patients with refractory/relapsed ALL, most patients with two or more prior salvage therapies. Response rate was 57% (CR/CRi) with 63% of the responders being MRD negative. About half of the patients proceeded to allogeneic HSCT, since responses were short-lived without HSCT. In another phase II trial in adult and pediatric patients with precursor B-cell ALL 18% achieved a CR, 39% had a cytologic BM response [120]. Lower intensity chemotherapy in addition to inotuzumab may be less toxic and therefore improve outcome. That was studied with mini-hyper-CVD in newly diagnosed precursor B-cell ALL in patients ≥60 years of age [121, 122]. Eighty percent of patients achieved CR, 17% PR and the 2-year OS tend to be better than standard chemotherapy [121]. The trial was later amended to patients with refractory/relapsed ALL. 59% of these patients achieved CR/CRi, 19% PR. Six patients relapsed, two died in CR/PR, 24 went to allogeneic HSCT, 6 received further chemotherapy and one is being observed [122]. The largest trial to date is an international phase III trial in patients with refractory/relapsed precursor B-cell ALL randomizing standard therapy to inotuzumab. Patients treated with inotuzumab had a better CR/CRi rate (80.7%) than the patients treated with standard chemotherapy (33.3%) and had higher rates of MRD negativity (78.4% vs 28.1%). More patients proceeded to HSCT and had longer durations of remission (4.6 months vs 3.1 months), although durations were short without allogeneic HSCT [123]. The trial is ongoing. Results from another ongoing trial in patients with refactory/relapse CD22 positive precursor B-cell ALL with second or later salvage therapies (NCT01363297) have been shown at ASH 2014 and demonstrated CR/CRi rates in 65.7% of patients with 78% of them being MRD negative [124]. Ninety-seven percent of the 35 patients had to discontinue inotuzumab, half of the patients due to progressive disease. Common AEs include thrombocytopenia and neutropenia, drug-related fever and hypotension and transient elevations of liver enzymes and gastrointestinal side effects [42, 117]. A significant AE is veno-occlusive disease (VOD), the obstruction of small veins in the liver. VOD was more frequent in patients who received alkylating agents as part of the HSCT conditioning regimen [42, 125]. Therefore, the conditioning regimen was changed and the risk of VOD decreased [112]. AEs occurred less often with weekly administration of inotuzumab with the same efficacy [126].

9.4.2 CD19

9.4.2.1 SAR3419

SAR3419, also known as coltuximab ravtansine, is an anti-CD19 humanized moAb linked to the highly potent tubulin inhibitor maytansinoid DM4, which leads to ADCC in the subnanomolar range [127]. After endocytosis the drug is processed and degraded to the active drug, which leads to microtubule disruption, cell-cycle arrest and cytotoxicity [127]. In precursor B-cell ALL and mixed lineage leukemia (MLL) xenografts SAR3419 lead to significantly delayed leukemia progression and improved survival [128]. Administration following induction prevented relapses in all organs but the CNS [128]. Phase I dose escalation studies have been conducted in NHL and have shown a large therapeutic window with minimal toxicity [127]. Response rates were high and reversible corneal toxicity was the dose-limiting toxicity (DLT) reported [129]. A phase II single-arm trial in adults with refractory/relapsed ALL <4 salvage therapy has been conducted. The efficacy was modest with 25% achieving an objective response. It has been well tolerated with AEs being infusion related reactions, diarrhea, nausea and vomiting [130] as described previously [129].

9.4.3 CD19/CD22

9.4.3.1 Combotox

Combotox is a 1:1 mixture of ricin based toxin to moAbs directed against CD22 and CD19 [131]. The toxin inhibits protein synthesis via inactivation of ribosomal RNA [132]. Preclinial studies have demonstrated activity in cell lines, patient samples and in a murine model, when given sequentially [102, 133]. In a phase I dose-escalation study in children with refractory/relapsed precursor B-cell ALL 53% of the 17 patients achieved haematological responses. The DLT was capillary leak syndrome [131]. In an adult study with refractory/relapsed precursor B-cell ALL haematological responses were observed in 31% of the patients. Again, the DLT was capillary leak syndrome, which might be due to a unique amino acid motif in the ricin toxin A chain that damages vascular endothelial cells [131]. Mutation of the toxin or shortening of the half-life are two approaches to prevent capillary leak syndrome [134]. The trials demonstrated activity, but Combotox might not be sufficient as a single agent, since responses in both trials were short-lived [131]. Therefore, this immunotoxin might be given with a lower tumor burden, e.g. MRD positive ALL or prior to HSCT. A murine xenograft model showed superior and synergistic efficacy of a combination therapy with cytarabine over single agent treatment and might hint to synergistic effects of combination therapy of chemotherapy with immunotherapy [132]. This lead to a phase I trial exploring the combination of Combotox with cytarabine in adults with refractory/relapsed precursor B-ALL (NCT01408160).

9.4.3.2 DT2219

DT2219 is a recombinant fusion protein that contains domains of the diphtheria toxin (DT390) that is fused with the antibodies CD19 and CD22 [135]. It has been tested in a phase I trial in adolescent and adults with CD19 and/or CD22 expression refractory B cell lymphoma or leukemia. Most patients received prior antibody therapy (rituximab, ofatumomab, inotuzumab, no blinatumomab) [135]. Out of 25 patients two achieved at least partial remission. The maximum tolerated dose (MTD) has not been reached and neutralizing antibodies were not found, but reversible capillary leak syndrome was the most common toxicity at higher doses [135], which is not uncommon with immunotoxins [136]. Another phase I/II trial in patients >11 years of age with CD19+ and/or CD22+ leukemia or lymphoma is ongoing (NCT02370160).

9.5 Radioimmunotherapy

Radioimmunotherapy combines immunologic and radiological cytotoxicity [137] thereby enhancing antitumor responses [46]. Radioisotopes are delivered directly to the target molecules using various carrier molecules such as moAbs. Hematological diseases are good targets due to their high radiosensitivity, the accessibility of tumor cells to circulating radiolabeled moAbs and the availability of several moAbs [138, 139]. One advantage for that kind of therapy in haematological malignancies might be the possibility of high-dose protocols, since patients can proceed to allogeneic HSCT [138]. Efficacy of radioimmunotherapy has been shown in NHL using CD20 and CD22 radiolabeled moAbs [139–141]. Only one radiolabelled moAb was approved, the murine anti-CD20 immunoglobulin ^{90}Y-ibritumomab tiuxetan® for follicular lymphoma [141]. Despite proven efficacy and durable responses observed with non-ablative activities delivered, it is still underused [138, 140]. The choice of the radionuclide is critical [142, 143], since the path length of penetration of the radioactive emissions is most effective without side effects, when matching the targeted tumor. β (emitters) should be used for microscopic disease, whereas α emitters kill residual cells in a MRD setting [142, 143]. In the context of leukemia, which is highly radiosensitive, but not geographically isolated, α-emitting radioisotopes with a short distance of radiation exposure might be the option of choice [144], but might have the disadvantage of a short half-life [145].

9.5.1 CD20

Anti-CD20 yttrium-90 (^{90}Y) ibritumomab tiuxetan (Zevalin®) or iodine-131 (^{131}I) tositumomab (Bexxar®) are effective in indolent B-cell NHL [146], since lymphoma cells are radiosensitive [46]. Several studies are ongoing in patients with B-cell NHL including conditioning therapy prior to HSCT or PTLD.

9.5.2 CD22

Epratuzumab has been conjugated to the radioisotope yttrium 90 (^{90}Y-epratuzumab tetraxetan) and first studied in two trials in patients with NHL [139, 140]. In a Philadelphia positive precursor B-cell ALL patient with third relapse it was studied as compassionate use. The patient achieved CR and molecular response [146]. Subsequently, the radioisotope was studied in a phase I trial in adults with refractory/relapsed precursor B-cell ALL. Radioimmunotherapy infusion was well tolerated with radiation exposure being minimal for patients and environment [147]. The most common AEs were myelosuppression and infections [148]. Extra-hematological radiation-related toxic effects did not occur. Hoewever, only a small percentage of patients (3/17) responded to therapy [147]. The tetraxetan complex persisted on leukemic blasts for months in patients who did not respond, but also in responding patients. It has been shown in a randomized phase III trial in follicular lymphoma, that incidence of secondary MDS and AML after radioimmunotherapy is significantly higher than without, although the incidence is low [147].

9.6 T Cell Engaging Antibodies/MoAb T Cell Conjugates

It has been shown that T cell cytotoxicity is a key player in cancer immunotherapy, where the contact of the T cells with the tumor cells is crucial. One such approach are the bispecific T cell engaging antibodies (BiTEs) which have already been described in 1985 [149]. BiTEs consist of two single-chain Fv domains of two different antibodies on one polypeptide chain [150], where one arm binds to an antigen on tumor cell and the other to the T cell receptor (T cell surface glycoprotein CD3 ∑-chain (CD3) [151]. The two domains are connected via a glycine-serine linker which allows bending and/or twisting of the two domains [152]. The affinity of the binding for the tumor associated antigen (TAA) can be adapted depending on the TAA expression and/or desired BiTE activity and is independent of MHC expression and specific TCR [152]. BiTEs induce perforin and granzyme B release and killing of the target cells [153, 154]. Furthermore it leads to T cell activation [155] without further costimulation [156] as well as release of pro-inflammatry cytokines which activate and attract other immune cells [157]. BiTE induced target cell lysis depends primarily on memory T cells, while naïve T cells need to be stimulated to be effective [158]. BiTEs can sequentially lyse target cells which is enhanced by synthesis of toxins such as perforin [154]. Bispecific antibodies with intact Fc are non-specific and can result in severe toxicity, whereas those lacking Fc have short half-lives and therefore need to given by continuous infusion, but have less toxicity [24]. The advantage of the short-lived antibodies might also be the short-lived T cell responses which can be stopped anytime or when side effects such as cytokine release are severe [159]. Tribodies which target CD20 and FcgRIIIA (CD16) binding to NK cells have shown higher efficacy compared to rituximab in vitro and in vivo [160].

Manipulation of the structure, valency and stability of the T cell engaging antibodies have generated dual affinity retargeting antibodies (DARTs) and tetravalent tandem diabodies (TandAb). DARTs have two paired polypetides which are connected by a short linker that does not allow for intrachain interaction [161]. A covalent disulfite bond between the poplypeptides stabilizes the complex. Through this manipulation DARTs have been shown to have a longer half-life in vitro, but maintain the same efficacy as the BiTEs [162]. TandAb have four variable domains in one long polypeptide which are connected by linkers of varying lengths. That directs complementary dimerization to form tetravalent bispecific antibodies or diabody folding to from bivalent bispecific antibodies [163]. Through their structure TandAbs offer higher avidity than BiTEs with a lower effector to target ratio but their efficacy depends on the linker length. These new antibodies may provide a longer half-life to avoid continuous administration and increase valency thereby improving affinity and stability of the antibodies. Since these parameters are independent of effective cytotoxity, efficacy may only be evident at later stages of clinical development [164]. A CD19 TandAb has shown efficacy in CLL cells in vitro as well as in xenograft mice in vivo [165]. TandAb against CD30 and CD16A has demonstrated efficacy in a phase I trial in patients with refractory/relapsed Hodgkin lymphoma [166].

9.6.1 CD19

9.6.1.1 Blinatumomab

Blinatumomab, also known as bxcCD19xCD3, MT103, AMG103 (trade name Blincyto©) has been designed in close collaboration between the biotech company Micromet and academic centers in Germany [167]. It is a bispecific T-cell engaging antibody with two arms – one arm binds the cytotoxic T cell (anti-CD3), the other arm binds to the B lymphoblastic tumor cell (anti-CD19) [168]. Blinatumomab establishes tight cytolytic synapses between effector cells and CD19 positive target cells [169, 170]. This leads to activation and proliferation of the engaged T cells and lysis of the tumor cells [150, 171, 172, 173] by perforin-mediated death of the target cells [174]. BiTEs overcome immune escape by direct interaction with cytotoxic T cells [175]. Activated T cells enter the cell cycle, expand and increase the number of T cells present in the target tissue [172]. Blinatumomab administration is given as a continuous infusions, because it has a short half-life of approximately 2 h [176] due to the lack of an Fc domain and its small size [152]. The very first trial with blinatumomab did not show efficacy in NHL and chronic lymphocytic leukemia (CLL) given one to three times per week and was terminated due to toxicity (cytokine release syndrome and neurotoxicity) [177]. A trial in patients with relapsed B-cell NHL has shown efficacy [178]. Based on this trial and an assumption that it would not be effective in relapsed or rapidly progressive B-cell malignancies, the first trial in ALL was performed in MRD positive patients including MLLAF4 and bcr-abl translocations [174]. Eighty percent achieved MRD negativity at the end of cycle one (after 28 days). Relapse free survival at a median follow-up of

33 months was 61%. Six of the nine patients who proceeded to allogeneic HSCT remain in hematologic remission, but even 6 of 11 patients without HSCT remain in remission. Two patients suffered a BM relapse, both of them CD19 negative [171]. In a phase II confirmatory trial 78% (88/113 evaluable patients) were MRD negative [179]. After a child was successfully treated on compassionate use the program in refractory/relapsed precursor B-cell ALL was developed [152]. In adult refractory/relapsed ALL dexamethasone or cyclophosphamide was allowed prior to blinatumomab treatment to decrease tumor burden and to reduce the incidence of cytokine release syndrome. Sixty-nine percent of the patients have demonstrated CR/CRi, and most of these patients (88%) had a MRD response [180]. Ten out of 36 patients were long-term survivors (>30 months OS), 6 of the 25 patients with CR experienced long-term EFS, all of them MRD responders. MRD non-responders have not shown Tem expansion and low absolute T cell counts, whereas responders showed expansion of Tem and Tcm, which is crucial for the response to blinatumomab and survival [181]. In another trial in patients with relapsed/refractory ALL half of the patients who relapsed had CD19 negative relapses, which might be one mechanism of resistance to the drug. In the pediatric posttransplant setting blinatumomab lead to CR and CMR in all patients [182]. Common AEs were cytokine release syndrome [171, 174, 178] with hypotension, fever and dyspnea as well as low immunoglobulin levels. Throughout the infusion B lymphocytes remained depleted [171]. CNS events (up to 20% of cases) were reversible and included seizures, encephalopathy and cerebellar symptoms [171, 174, 178]. A large phase II multicentre confirmatory trial in adult patients with relapsed/refractory precursor B-cell ALL enrolled 189 patients. Almost 2/3 of the patients had a blast count ≥50% in the BM, 1/3 was after allogeneic HSCT and almost 40% had at least two prior salvage therapies. Patients with a high tumor burden were pre-treated with dexamethasone to reduce blast count, which is most likely the reason for the low number of cytokine release syndroms observed [183]. Response rate in this high risk group of patients was 43% (CR or CR with incomplete count recovery (CRi)), which is similar to studies which included both standard risk and high risk patients. Eighty-two percent of the responding patient were MRD negative and 40% proceeded to allogeneic HSCT. This was the largest study in refractory/relapsed ALL at that time point and ultimately led to the approval of blinatumomab by the FDA in this patient population in December 2014 [184]. The EMA approved the drug in November 2015. Another trial in adult patients, the TOWER trial (NCT02013167), in the same patient group randomized patients 2:1 to blinatumomab or salvage therapy with one of four standard chemotherapies (FLA, HD cytarabine-based regimen, HD MTX-based regimen, clofarabine based regimen) and is still accruing patients. Other trials are ongoing, one being the BLAST trial (NCT01207388), which includes patients with cytologic remission, but persistent or relapsed MRD. Preliminary results have been presented at the ASH meeting 2014 and shown engouraging results with 78% of patients achieving MRD negativity [179]. The first experience with blinatumomab in children were reported in three children with refractory/relapsed ALL on a compassionate level [182]. In a phase I/II trial in pediatric patients with refractory/relapsed precursor B-cell ALL 32% patients achieved CR, 10 of the 13 patients with a molecular response [185]. Interestingly no peripheral B cells were documented during the drug-free period of 2 weeks [173, 180]. T cells decreased after initiation of

blinatumomab infusion, returned to baseline by day 8 or 9 and had the maximal expansion by day 15–20 [173]. Cytokine levels peaked after 2 days and then remained low during the infusion time. A correlation of blinatumomab efficacy and cytokine levels have not been shown [173]. Another phase I/II trial in pediatric patients with relapsed/refractory ALL showed 32% CR with 77% of these patients being MRD negative [186]. In relapsed ALL patients after allogeneic HSCT who have received blinatumomab compassionately seven of nine patients achieved CR and five of these seven patients reached MRD negativity [185]. Anti-leukemia responses occur mostly during cycle 1. In case of non-response further treatment have not lead to responses [174]. Relapses after blinatumomab occur, some CD19 negative, but also CD19 positive. There are several clinical trials in children ongoing. One trial is a phase III multinational multicenter trial in children with first relapse in the HR group randomizing blinatumomab vs chemotherapy prior to allogeneic HSCT (NCT02393859). Another trial is a risk-stratified randomized phase III study (NCT02101853) in children with first relapse of precursor B-cell ALL sponsored by the NCI [187]. The most common adverse events (AE) were fever and headache [183] fatigue, tremor, chills, peripheral edema, nausea, vomiting and diarrhea [174, 180, 183] as well as low levels of immunoglobulins [179, 188]. They were transient, and occurred early during cycle 1. Grade 3–4 toxicities included neutropenia and anemia, fatal infection, DIC, cytokine release syndrome and neurological events such as seizures, encephalopathy, confusion and cerebellar symptoms [174, 180, 183, 184]. Adherence of blinatumomab-activated T cells to endothelium is considered as the first step of CNS toxicity [188, 189]. Neurotoxicity usually occurs at onset of treatment and is fully reversible and manageable by withholding infusion and anti-seizure prophylaxis [150, 180, 183, 188]. B cell depletion is rapid [178, 190] and might be sustained up to 1 year after the end of treatment [191]. Blinatumomab clearance with mild or moderate renal impairment is similar to normal renal function [187] and not affected by hepatic dysfunction. A high percentage of Tregs (>12.5%) and high serum LDH levels were found to be independent predictors of lack of response [192]. In addition a higher percentage (73%) of patients with BM blasts <50% achieved CR compared with those (29%) with >50% at start of treatment [183]. Combination therapy with rituximab might be more effective, since in vitro data have shown additive effects of blinatumomab-mediated T cell cytotoxicity with rituximab-mediated NK cell cyototxicity [193].

9.6.2 CD20

An anti-CD20-CD3 bispecific antibody has shown broad activity against normal and malignant B cells expressing CD20 in vitro and in vivo in mice and non-human primates [194, 195]. Even in the presence of rituximab the anti-CD20-CD3 BiTE preserved its activity [194].

The trifunctional heterodimeric bispecific antibody CD20-CD3, Lymphomun, has been used in ten children with HR CD20+ B-cell malignancies. 50% of the treated patients achieved CR, most of them being in CR >2 years after treatment. One child even showed a response after being refractory to rituximab treatment. One child had a

CNS relapse probably due to poor CNS penetration of the antibody [196], which has also been reported for rituximab [197]. Common AEs were infusion related reactions, but no increased incidence of infections. In combination therapy with chemotherapy or donor lymphocyte infusion (DLI) no additional AEs have been observed [196]. There might even be synergistic effects by upregulation of CD20 on the cell surfaces [48]. No CRS and neurotoxicity have been described in small clinical studies with anti-CD20-CD3 BiTE on a compassionate use level [196].

9.7 T Cell Targets

T-cell ALL is characterized by the expression of CD3 together with CD2, CD5, CD7 or CD8. CD3 and CD7 are expressed in all T-cell ALL cases, whereas CD2 and CD5 were expressed by 80% of T-ALL patients [198]. Early T-cell precursor (ETP) ALL is a subgroup with poor prognosis and has low CD5 expression [199].

9.7.1 CD7

CD7 is a cell surface glycoprotein and a member of the immunoglobulin superfamily [200]. CD7 is expressed on most thymocytes, T cells and NK cells [201–203] as well T-cell lymphoma and leukemia [204, 205], but not on a subset of normal T cells [206]. These normal T cells might maintain immune function. The function of CD7 is unknown [207], but it has been shown that CD7 is a ligand for galectin-3 (Gal-3), a β-galactoside-binding lectin [208]. Its binding may lead to activation and proliferation or apoptosis [209, 210]. Loss of CD7 in T-cell leukemia/lymphoma is associated with poor prognosis, possibly by escaping Gal-3 induced apoptosis [207]. Most CD7 conjugates were plant-derived toxins such as ricin, saporin and derivates [211–213]. All these moAb-drug conjugates lacked efficacy and safety (capillary leak syndrome) [212, 214]. A new approach is a nanobody, an antibody fragment containing a single monomeric variable heavy-chain domain derived from camelidae heavy-chain antibodies [215]. Nanobodies are able to deliver conjugates to target cells [216, 217]. One such example is a CD7 nanobody coupled to pseudomonas exotoxin A. It has shown apoptosis in primary AML and T-ALL cells as well as in xenotransplant mice models and might be further investigated in clinical trials [218].

9.7.2 CD5

CD5 is expressed on most T cells and a subset of B cells (15%) [219]. CD5 has been shown to be expressed in B-cell malignancies including lymphoma and acute biphenotypic leukemia and in a few cases of ALL [220]. It has been described as a

defining antigen in early precursor T-cell ALL [199, 221], but has also been detected in cortical/thymic T-ALL [199, 222]. CD5 expression is separated into $CD5^{high}$ and $CD5^{dim}$ expression. $CD5^{dim}$ constitutes 90% of early immature T-cell ALL [222], which is related to AML.

9.7.3 CD2

CD2 is expressed on T cells, NK cells and thymocytes and plays a key role in lymphocyte adhesion and cell signalling through binding to its receptor LFA3 (CD58) [223]. CD2 then mediates cell adhesion to APC with enhancement of antigen recognition and subsequent T cell activation [223]. Anti-CD2 moAbs inhibit T cell responses to various stimuli [224] and is most effective during antigen presentation [225]. CD2 knockout mice have T cells with reduced proliferative responses and reduced IFN-γ release in response to Ag stimulation, but maintain normal cytolytic activity [226].

Siplizumab is a humanized moAb against CD2 and has shown efficacy in an animal model of adult T-cell leukemia/lymphoma [227]. A preliminary clinical trial confirmed these results. In the treated patients NK and T cells were depleted in addition to down-regulation of CD2 on T cells. However, cases of EBV-lymphoproliferative disorder (EBV-LPD) led to the trial being stopped [228]. Another trial with siplizumab also experienced one fatal case of EBV-LPD [229]. Potentially, prior or subsequent immunosuppressive therapy might have a synergistic or additive role for that AE [228]. Siplizumab has also been investigated in adult and pediatric patients with GvHD after allogeneic HSCT under corticosteroid treatment with some partial responses. Infections, especially EBV-LPD, were the most common AEs, but did not occur more frequently than in patients without siplizumab treatment [230]. Mortality with and without siplizumab treatment in the pediatric cohort was high. Therefore EBV-PCR should be monitored [230]. This trial was terminated early because of low accrual, but with the same safety profile in a larger cohort the study might have been closed early as well.

9.8 Inhibitory T Cell Pathways

The concept that the innate and acquired immune system fights cancer with immunotherapeutic strategies has been around for over 100 years, when William Coley observed that cancer patients who developed bacterial infections went into remission [231]. CD28, CTLA-4, PD-1 and ICOS are crucial for the immunological synapse and generate costimulatory or inhibitory signals in T cells upon interaction with antigen [232]. By blocking these checkpoints with moAbs T cell function is stimulated [233]. This stimulation alone can have a therapeutic effect which means that these T cells have been primed before and only needed to be unmasked [233]. CD28 is a stimulatory receptor in the early stages of immune response, whereas CTLA-4 is an inhibitory

receptor [234] following stimulation by CD28 [235], either by setting the threshold of T cell activation above background or by limiting the capacity of T cells to divide [236]. The negative receptor protects tissues by inhibiting T-cell responses. Tumor cells can use these pathways to limit T cell responses [232].

CTLA-4, cytotoxic T-lymphocyte-associated protein 4, also known as CD152, is a CD28 homologue. It is type 1 transmembrane glycoprotein of the immunoglobulin superfamily [237] and mainly expressed by T cells, but also by other immune cells, fibroblasts and embryonic cells, but the role on these cells is not known [238]. Tregs constitutively express CTLA-4 at levels higher than on conventional T cells [239, 240] and use CTLA-4 to supress antitumor immunity [241, 242]. In fact, CTLA-4 is necessary for Tregs to exert maximal immunosuppressive function [242, 243], whereas on naïve T cells it is up-regulated upon activation and reaches a maximum 2–3 days later [239]. It is not detectable in non-activated T cells [244]. Once activated CD4+ T cells express more CTLA-4 than CD8+ T cells [245]. Its ligands are CD80 and CD86, the same as for CD28, but the affinity is 10 times higher for CTLA-4. Furthermore recruitment of CD28 to the immunological synapse can be disrupted by CTLA-4 by competition of the ligands [246]. It has been shown previously, that CTLA-4 blockade reduces interaction time of conventional T cells with Tregs thereby allowing conventional T cells to be adequately primed by APCs [241]. In vitro and in vivo data have shown that CTLA-4 is a negative regulator of T cell mediated immune responses in tumor. A first antibody administered in mice in 1996 has shown efficacy in colon carcinoma [247]. It has been further investigated in highly immunogenic murine tumor models [248] with enhancement of cytotoxic T cell responses, which were dependent on CD4+ T cells [249]. However, as a monotherapy the antibodies failed the rejection of poorly immunogenic tumors which lead to combinatorial approaches [250].

PD-1, programmed cell death protein-1, is a member of the B7 receptor family, which plays a crucial role in the regulation of the immune response. Its receptor is a type I transmembrane protein that is part of the immunoglobulin family [251]. This receptor together with its ligands programmed cell death ligand-1 and 2 (PD-L1 and PD-L2) regulate immune responses by down-regulating signals of the T-cell receptor [252, 253]. It is another inhibitory receptor on progenitor T cells, activated T and B cells, NK cells and myeloid cells [232, 254]. The primary function of PD-1 is regulation of T-cell activation and apoptotic pathways of effector/memory T cells [251]. Its ligands PD-L1 and PD-L2 are expressed on APCs, placental cells and non-hematopoetic cells found in inflammatory and tumor microenvironment, but also on tumor cells such as melanoma, lung carcinoma and glioblastoma [255, 256]. Patients with ovarian cancer had a poor prognosis, when PD-L2 was expressed together with PD-L1 on tumor cells, but not, when PD-L2 was expressed alone [257]. Interaction of PD-1 and PD-L1/PD-L2 controls T cell responses during normal immune responses [258] and could be used by tumor cells to evade host immune response [259–261]. Expression of PD-L1 has been described in haematological malignancies [260, 261]. In leukemia patients PD-1 has been demonstrated at higher levels than in healthy donors [262]. Therefore, up-regulation of both markers might lead to T-cell immunodeficiency via decreased proliferation and activation of T cells, which has been described in adult T-cell leukemia/lymphoma (ATLL) [263].

Durable tumor regression in solid cancer has been induced by antibodies against PD-L1 [264] as well as PD-1 [265]. That has also been confirmed in a phase I trial in patients with haematological malignancies, where about 30% of patients showed a response using a PD-1 moAb [266].

CD137, also known as 4-1BB, was first identified in 1989, is a costimulatory receptor and a member of the TNF receptor superfamily [267]. It is expressed by activated, but not resting T cells and is an activation marker for antigen-specific CD4+ and CD8+ T cells [268, 269]. It is also expressed on NK cells, NK T cells and regulatory T cells (Tregs) as well as other innate immune cells such as DCs, mast cells, monocytes, neutrophils and eosinophils [270–272]. It is also constitutively expressed on T-cell and B-cell leukemia [273]. Its receptors are TNF receptor-associated factors 1 and 2 (TRAF 1 and 2). CD137 induces production and proliferation of IFN-γ and IL-2 and enhances survival of T cells through up-regulation of antiapoptotic pathways [274]. Furthermore, it contributes to maintenance of memory CD8+ T cells and enhances their cytolytic activity upon reactivation [274, 275]. In Tregs it can lead to expansion or suppression [276, 277] thereby enhancing antitumor immunity and abrogating autoimmunity [275].

9.8.1 CTLA-4

9.8.1.1 Ipilimumab

The anti-CTLA-4 moAb ipilimumab has been investigated in melanoma patients as early as 2003 [278] and been approved by the FDA for the treatment of melanoma in 2011 [232]. By modifiying the patient's immune system to control tumor progression sustained immune responses have been observed, even after the end of treatment with ipilimumab [233]. The role of CTLA-4 in leukemia has been described in haematological malignancies such as AML [279], B-NHL [280] as well as after allogeneic HSCT [281]. Interestingly, some of the patients who have benefitted from therapy with ipilimumab often experienced initial tumor growth after start of therapy [282]. Common AEs described were myelosuppression, diarrhea and fatigue, autoimmune pneumonitis, arthritis and enterocolitis [283]. Most of the AEs were reversible, but endocrinopathies such as hypophysitis and thyroiditis frequently required chronic hormone replacement [233]. Effects and degree of autoimmune AEs in solid tumors were higher with ipilimumab than with anti-PD-1 moAbs, but the degree of autoimmune AEs was lower in hematological malignancies [284].

9.8.2 PD-1 Inhibitors

Most of the PD-1 inhibitors have been extensively investigated in melanoma and other solid tumors [285, 286]. Nivolumab has been approved in 2014 by the FDA after it has shown responses in 40% of patients with advanced-stage melanoma. These results

included patients who have not responded to the anti-CTLA-4 antibody ipilimumab [20]. It has also been approved in 2015 for metastatic squamous and nonsquamous non-small cell lung cancer (NSCLC) after progression on platinum-based therapy [287]. Pembrolizumab has been approved in 2015 by the FDA for patients with metastatic refractory NSCLC. Common AEs include rashes, thrombocytopenia, fatigue, pyrexia, diarrhea, nausea and pruritus and are mostly minor [288]. SAEs were myelosuppression with or without infections [284]. High grade toxicities are less common in PD-1 blockade than CTLA-4 blockade [289], but pneumonitis occurs frequently [265]. In 2015 the combination of ipilimumab and nivolumab was approved by the FDA for patients with advanced-stage melanoma based on phase III results with improved response rates and PFS when compared with single agents alone [289]. The approach to inhibit immune regulatory checkpoints has recently also moved to the area of haematological malignancies. It has been shown that the malignant Reed- Sternberg cells in Hodgkin lymphoma expressed large amounts of PD-L1, caused by the chromosomal abnormality 9p24.1 [290], and were surrounded by functionally impaired PD-1 positive T cells [290, 291]. Similar studies were performed in T-cell lymphoma [292] and B-cell NHL [293], where PD-L1 expression varied among histologic subtypes [294, 295]. Nivolumab (87%) and pembrolizumab (53%) have shown efficacy in Hodgkin lymphoma in vitro [296] and in early clinical trials [297] and might be promising for other haematological malignancies.

9.8.2.1 Nivolumab

Nivolumab is a humanized IgG4 moAb which target the PD-1 receptor [298]. It has demonstrated high-affinity binding in vitro. Blockade by nivolumab enhances proliferation of T cells as well as IFN-γ release [299]. Two phase I trials in combination therapy for haematological malignancies as well as two phase II trials in lymphoma patients are ongoing, but none of them include patients with ALL. There is currently no trial in paediatric patients recruiting.

9.8.2.2 Pembrolizumab

Pembrolizumab, formerly known as MK-3475 and lambrolizumab, trade name Keytruda®, is a humanized IgG4 moAb [300] which target PD-1 receptor. Since the IgG4 subtype does not engage Fc receptors or activate complement, cytotoxic effects are avoided. There are currently two phase I trials ongoing including patients with haematological malignancies, but not with acute leukemia.

9.8.2.3 Pidilizumab

Pidilizumab is a humanized IgG1 recombinant moAb with the PD-1 receptor as the target [301]. It attenuates the apoptotic process [251] of T and NK cells. Three clinical trials have investigated pidilizumab in haematological malignancies, one

phase I trial in lymphoma and leukemia including patients with AML, two phase II trials in lymphoma patients. Three other trials in combination with DC vaccines are ongoing.

9.8.3 PD-L1 Inhibitors

9.8.3.1 AMP-224

AMP-224 is a recombinant Fc-fusion protein fusing the extracellular domain of human B7- DC/PDL-2 to IgG1 [302]. It blocks the interaction between PD-1 and its receptor ligands and has shown activity as a single agent as well as in combination with cyclophosphamide [303]. An early phase I trial in advanced cancers has been conducted, results have not been published yet.

9.8.3.2 MPDL3280A

MPDL3280A is a humanized, Fc optimized moAb against PD-L1. The Fc region is modified to avoid induction of ADCC and CMC. There is a phase I multicenter trial recruiting patients with advanced or metastatic solid tumors [254].

9.8.4 CD137

The first studies of anti-CD137 moAb in mice have shown anti-cancer activity. That was dependent on NK cells, since depletion of NK cells lead to abrogation of the antitumor effect [304] while anti-CD137 administration increased NK cell proliferation, degranulation and IFN-γ secretion with enhanced ADCC [305]. Cytotoxic CD8+ T cells were also important. Studies in mice have demonstrated synergistic effects of anti-CD137 moAbs in combination therapy with anti-PD-1 moAbs [306, 307], anti-CTLA-4 [308], anti-CD40 [309], rituximab and trastuzumab [310, 311], IL-12 [312] and adoptive T cell therapy [313]. The anti-human CD137 moAb urelumab [314] and PF-05082566 have been developed for clinical use [315, 316]. The initial phase I study with urelumab was conducted in 2005 in solid cancer patients showing some responses [317]. It has also been investigated in patients with CLL and NHL [284], but results have not been published yet. A phase II study was initiated, but terminated in 2009, because of high hepatic toxicity. Urelumab re-entered the clinic after dose reduction without significant toxicity. The antibody is currently in clinical trial together with rituximab for patients with NHL. Since it has shown activation of T and NK cell, it can potentiate the effect of moAbs by enhancing ADCC [310, 318]. Liver toxicity has been described as AE in mouse models [319] and in clinical trials at high doses [320]. PF-05082566

has been investigated as monotherapy in a phase I clinical trial and showed disease stabilization [321]. It is now being evaluated in combination with rituximab and mogamulizumab (anti-CCR4 moAb).

9.8.5 CCR4

CCR4, the C-C chemokine receptor type 4, is expressed on Tregs and often found on T-cell lymphoma cells [322]. CCR4 positivity is significantly associated with skin involvement in adult T-cell leukemia/lymphoma (ATLL) and an unfavourable prognostic factor [323]. The anti-CCR4 moAb mogamulizumab is a humanized moAb against CCR4. In a clinical trial it has shown efficacy in relapsed ATLL [324]. Skin rashes ≥II° have been associated with better responses [324]. Administration of mogalimuzumab increased CD8+ T cells which might be one of the mechanisms of actions [325].

9.9 Future Strategies/Conclusions

Current strategies and antibodies are directly targeting the tumor associated antigens in ALL patients. They do not necessarily target the leukemic stem cell [326] which might not express the target antigen, but is essential in propagating leukemia by maintaining the generation of the leukemic clones. These clones would persist and eventually lead to relapse. Hence, the goal is to reduce the leukemic burden, but also target the leukemic clone in order to keep the patients in long-term remission. The risk of evasion of leukemic stem cells by down-regulation or no expression of the target antigen can be decreased by combining two antibodies or two modes of therapies, which has been described previously [212]. This approach potentially reduces the risk of resistance. Checkpoint inhibitors are being investigated in haematological malignancies with promising results. They might add a new perspective to the treatment of ALL by strengthening the patients' own immune response to target leukemic cells. Since the patients' own effector cells might have been primed for leukemia before, but their function inhibited by the leukemic cells, the administration of checkpoint inhibitors as single agents or in combination with other targeted therapy may target the leukemic stem cell in addition to the circulating leukemic cells and lead to longer-term remission. They might also be promising for patients with relapsed T-cell ALL who have poor prognosis. For these patients new targets for therapy need to be established without compromising the effector T cell compartment. One caveat of checkpoint inhibition might be autoimmune phenomena, due to immune responses against self-antigens, which might be long-lasting. This problem could be decreased by optimizing the formulation of the antibodies. That approach might help to better understand the mechanisms of response and could qualify patients who will respond [284]. Timing of administration probably is

crucial for synergistic effects and for response to treatment. At diagnosis with high leukemia burden, antibody therapy might not be able to exert its full effect, when given alone. Therefore combination therapy e.g. with standard of care or sequential administration of one or several antibodies or other targeted therapy might be a more effective strategy.

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Chapter 10
Cellular Therapy

Sara Ghorashian and Persis Amrolia

10.1 Introduction

Cellular immunotherapy developed from the observation of durable disease responses following donor leucocyte infusions as sole therapy in patients relapsing post allogeneic stem cell transplantation (SCT) as well as the ability to isolate T cells capable of recognizing tumour-specific antigens from cancer patients. *Ex vivo* expansion of tumour-specific T cells is possible, and such populations can be re-infused with some success e.g. in melanoma therapy [1]. However, many tumours, and ALL in particular [2], rarely express tumour-specific antigens. In this situation, the tumour presents purely self-antigens which are not intrinsically immunogenic. This is because the host T cell repertoire is depleted of cells responding with high avidity to self-proteins during thymic T cell education [3].

Much research interest has focused on redirecting T cells with high avidity receptors against tumour antigens. Polyclonal T cells can be rendered capable of recognising a specified antigen by introducing DNA encoding a receptor with the required antigen-binding properties. T cells were initially redirected in this way using naturally-occurring T cell receptors cloned from T cell lines [4]. More

S. Ghorashian (✉)
Molecular and Cellular Immunology Section, UCL Great Ormond Street Institute of Child Health, London, UK

P. Amrolia
Molecular and Cellular Immunology Section, UCL Great Ormond Street Institute of Child Health, London, UK

Department of Bone Marrow Transplant Great Ormond St Children's Hospital, London, UK
e-mail: persis.amrolia@gosh.nhs.uk

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recently, modular recombinant receptors recognising surface molecules (chimeric antibody receptors, or CARs) have been developed. These consist of an antigen recognition domain often derived from an antibody, fused to one or more T cell signaling domains. CARs effectively weld the specificity of a monoclonal antibody onto the downstream effector machinery and long-lived potential of T cell populations. In recent years, a number of clinical studies of CAR T cell therapy for ALL have been published, reporting approximately 90% complete response rates in patients with advanced ALL [5]. CAR T cell therapy has demonstrated remarkable anti-tumour efficacy against B cell malignancies, particularly ALL. This chapter will mainly focus on the CAR T cell approach, with some consideration of other relevant cellular immunotherapies.

10.2 Non-gene Engineered Cellular Therapies

10.2.1 Donor Leucocyte Infusions

Following initial reports of remissions of chronic myelogenous leukaemia relapsing after allogeneic transplantation [6] induced by infusion of donor leucocytes, this cellular therapy was attempted for a number of haematological malignancies including ALL. Unfortunately, responses in ALL were limited [7], especially in those treated without adjunctive chemotherapy. A number of further small scale studies have reported similar responses, with 1–2 year survival limited to 10–20% [8].

10.2.2 Cytokine Induced Killer Cells

Cytokine induced killer (CIK) cells are a mixed population of T cells (CD3+CD56-), natural killer (NK) cells (CD3-CD56+) and natural killer T (T-NK) cells (CD3+CD56+) demonstrating non MHC-restricted cytotoxicity against leukaemic cells. They are expanded ex vivo from peripheral blood mononuclear cells in the presence of interferon (IFN)-γ, anti-CD3 antibody and interleukin-2 (IL-2), and in some cases in the presence of IL-15 [9]. They have been infused as a bulk cellular immunotherapy to improve graft versus leukaemia (GvL) responses post allogeneic stem cell transplantation [9–13], with the potential advantage over standard DLI of a lower risk of graft versus host disease (GVHD) [9, 13]. Clinical studies are underway, and whilst responses are documented, in general response rates in acute leukaemia have been limited. As a result, there is interest in genetically-manipulating CIK cell populations to express chimeric antigen receptors in order to enhance their anti-leukaemic efficacy [14].

10.3 CAR-Engineered Cellular Therapies

10.3.1 Basic Principles

Seminal studies in the early 1990s demonstrated the modular nature of TCR/CD3 signalling [15] and underpinned the generation of the first chimeric antigen receptors on T cells [16]. All chimeric antigen receptors contain a ligand binding domain, usually, but not exclusively, a single chain variable fragment (scFv) derived in turn from a monoclonal antibody. The nature of the ligand binding domain is important because it determines which antigens can be recognised and the context in which they are recognised. Thus, scFv-based CARs recognise cell surface molecules, either proteins, glycoproteins or lipids, in an MHC-independent fashion, similar to recognition by an antibody. There may be a spacer region which extends the ligand binding domain from the surface of the T cell at an optimal distance and orientation for target antigen binding. Spacer design may affect the degree of T cell activation obtained after binding cognate antigen [17]. Where present, the spacer region may be derived from CD8, IgG or CD28. A transmembrane region anchors the CAR into the cell membrane and is usually derived from the same molecule as the spacer region. The intracellular portion of the CAR contains signalling domains which are derived from CD3ζ alone in first generation CARs, or can be linked *in cis* to one or more domains derived from co-stimulatory molecules involved in T cell activation, such as CD28, 4-1BB or OX-40. Second generation CARs contain a single co-stimulatory domain linked to CD3ζ and third generation CARs contain combinations of co-stimulatory domains as well as CD3ζ.

The incorporation of co-stimulatory domains in CAR design overcame the suboptimal T cell activation seen with first generation CARs in which cytotoxic activity, but not cytokine production or proliferation could be demonstrated [18–22]. Natural TCR signaling is accompanied by co-stimulatory signals provided *in trans* by a wide variety of co-stimulatory molecules such as CD28, 4-1BB (CD137), inducible T cell co-stimulator (ICOS) and OX-40 which bind ligands on professional antigen presenting cells (APCs) at the same time as TCR engages cognate peptide-MHC. CD28 supports a number of key T cell functions including proliferation, survival and cytokine production through enhanced TCR signalling, as well as activation of Akt and B cell lymphoma extra large (BCL-xl) [23]. 4-1BB and OX-40, members of the tumour necrosis factor (TNF) receptor family, which are inducibly expressed on T cells following TCR signaling, and serve to support proliferation for days into an immune response [24, 25].

Second generation CAR design provides co-stimulatory signaling *in cis* through the same linear receptor as that involved in antigenic recognition. T-cells expressing these “second generation” CARs not only kill CD19 expressing targets at lower effector:target ratios [22], but show greater cytokine production and proliferation after antigenic stimulation [26–28]. T cells expressing second generation CARs also

mediate more effective regression of ALL in xenograft models [29]. In a clinical study in which patients were infused with a mixture of CD19-directed CAR T cells of first and second generation, enhanced expansion and persistence was noted in the second generation CAR T cell population [30]. Third generation CARs containing multiple co-stimulatory domains [31] have been tested clinically [32, 33]. However, it remains to be seen if they provide an advantage compared to second generation designs.

10.3.2 CD19 as a Target Antigen for CAR Therapy

In selecting a target antigen for cancer immunotherapy, the differential expression of the antigen upon malignant versus normal tissues is critical, a consequence being 'off-tumour, on-target' damage to healthy tissues; as well as the possibility of cross-reactivity causing 'off-target' effects. CD19 is an ideal tumour antigen for immunotherapy as its expression is maintained on more than 95% of B cell malignancies, including ALL [34], B cell non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukaemia (CLL) [35–37]. It is not expressed on haematopoietic stem cells, myeloid or erythroid populations, T cells or non-haematopoietic cells. CD19 is a 95 kd transmembrane glycoprotein expressed on cells of the B lineage from the early pro-B to mature B cell stages. It is a member of the immunoglobulin superfamily and is part of the B cell surface signal transduction complex. The predictable consequence of immunotherapy targeting CD19 is B cell aplasia, which if prolonged, may lead to hypogammaglobulinaemia, but this is manageable with immunoglobulin replacement [38].

As will be considered below, cases of CD19 negative relapse of ALL have been documented in clinical studies of CD19 CAR T cell therapy, with antigenic escape accounting for up to 50% of relapses [39]. Genetic studies carried out at the University of Pennsylvania suggest that $CD19^{-}$ relapse occurring in patients treated at that centre results from alternative splicing of the CD19 locus, or hemizygous loss of the entire protein. Alternative splicing leads to skipping of exon 2, resulting in loss of the epitope to which the scFv employed at that centre (FMC63) binds [40]. As a result, CAR therapies targeting multiple ALL antigens, e.g. CD19 and CD22, are being developed in the hope of further improving long term outcomes.

10.3.3 T Cell Populations for CD19 CAR Transduction

In naturally-obtained immunity, memory T cells rapidly expand upon antigen re-exposure and provide life-long immunity. Therefore, exploitation of specific memory subtypes may lead to improved anti-tumour effects. Memory T cells are divided into effector and central memory subtypes. The former give rise to the effector T cell pool which are adapted for cytotoxic functions, are excluded from the secondary lymphoid organs due to the chemokine receptors they express and do not persist long term [41, 42]. Central memory T cells, on the other hand have a high

proliferative potential, persist long term and have the ability to repopulate both central and effector memory compartments [43–45]. Further, central memory cells express CCR7, which enables them to penetrate the lymph nodes and bone marrow [41] i.e. classical niches of haematological malignancies.

Data from non-human primate studies of adoptive transfer of antigen-specific $CD8^+$ T cell clones suggest clones derived from central memory T cells (Tcm) but not effector memory T cells (Tem) persist long term in vivo [43]. Further, single cell transfer experiments have confirmed that cells derived from the Tcm compartment are capable of fully reconstituting protective immune responses against bacterial pathogens [46]. These studies suggested cells derived from the central memory compartment may share stem cell-like properties, which may be advantageous in selecting populations for optimal T cell therapies. Indeed, *bona fide* memory stem cells have been identified in human and murine studies [47–49]. These and other studies have suggested naïve or memory stem cells may be optimal for adoptive immunotherapy [48, 50, 51]. Central memory subsets of T cells can be selected by immunomagnetic beads during CAR T cell production [52], however this adds complexity and time to the production method.

An alternative approach to improve persistence of CAR T cells is use of T cells with anti-viral specificity. CAR-bearing virus specific T cells (VSTs) may be primed in vivo by viral antigens**,** promoting persistence and memory formation [53]. Direct evidence of the utility of this approach was in a study of EBV CTLs redirected to a neuroblastoma antigen with a first generation CAR [54]. Patients received two populations of CAR-bearing cells which were distinguishable on a molecular level, one of T cells activated from PBMCs using a standard transduction protocol, the other being EBV-specific. The expansion and persistence of the CAR-transduced EBV CTLs was greater than the standard CAR T cells. Persistence of VSTs can be limited even with use of a second generation CAR design [55]. We have investigated the use of EBV-directed vaccination to improve the persistence of VSTs transduced with a first generation CD19CAR in patients with ALL relapsing post allogeneic-SCT (Rossig et al. submitted). Unfortunately, whilst persistence improved with vaccination, overall, CAR T cell expansion and therapeutic efficacy were limited.

10.3.4 CD19 CAR T Cell Therapy in B-ALL

An initial case report of CD19-directed CAR T cell therapy resulting in an impressive partial response at the US National Cancer Institute [56], led the way for studies of second generation CD19 CAR T cell therapy in B cell-derived ALL. Groups from the Memorial Sloane Kettering Cancer Centre and the University of Pennsylvania first reported findings in 2013 [57, 58], with unprecedented responses in heavily pre-treated patients, but with associated toxicity in the form of cytokine release syndrome.

The group at University of Pennsylvania reported therapy of 30 paediatric or young adult patients with relapsed ALL [39], 18 of whom had relapsed post allo-SCT. They employed a second generation CAR incorporating a 4-1BB co-stimulatory domain and an antigen binding domain from the FMC63 hybridoma

introduced by lentiviral transduction. Of the 30 treated, 27 (90%) achieved complete remission of which 22 were molecular remissions. Durable responses were seen in 50% of patients despite only 3 of the 30 going on to receive allo-SCT following CAR T cells. CAR T cells were detectable for at least 4 months in 70% of evaluable patients, but in some, were detectable at up to 2 years following infusion. B cell aplasia was closely correlated with CAR T cell persistence. Recruitment has continued to 59 patients since the published report. More recent relapse-free survival from this group are reported at 55% at 12 months, with only six patients receiving adjunctive allogeneic transplantation. Similar results were reported in an adult ALL cohort from Davila et al. [59] from the Memorial Sloane Kettering Cancer Centre. Sixteen patients, of which four had relapsed post allo-SCT, were treated this time with a CAR containing a CD28 costimulatory domain and a binding domain derived from the SJ25C1 hybridoma. Retroviral transduction was employed to engineer T cells. Fourteen of 16 patients (88%) achieved a CR, of which 12 were molecular remissions. Persistence of CAR T cells was limited to 2–3 months, and seven of the cohort went on to adjunctive allo-SCT. Thus, CAR therapy was followed by allo-SCT in nearly half the cohort, and updated data presented at the American Society of Haematology Meeting in 2015 reported a 12 month overall survival of 40%.

A later study of 21 paediatric patients from the National Institutes of Health [60] distinguished itself in its intention to treat analysis, allowing the feasibility of providing a CAR T cell product to eligible patients to be assessed, as well as its dose-escalation design. The CAR utilised the FMC63-derived CD19 binder but unlike the CAR used at the University of Pennsylvania, contained a CD28 costimulatory domain. Lee et al. demonstrated a 90% success rate in delivering a CAR T cell product within 3 weeks of study enrolment, and also defined the maximum tolerated dose as 1×10 [6] CAR T cells per kg patient weight. Updated outcomes from this group also reported an overall survival of 40% at the American Society of Haematology meeting in 2015, and as for the group from Memorial Sloane Kettering, this was achieved with approximately half of patients treated as a bridge to subsequent allogeneic transplantation. Most recently, a novel approach was presented by Riddell and colleagues [61] in a study treating 30 adult patients where a unique selection strategy was utilized to obtain a uniform 1:1 CD4:CD8 ratio of CAR transduced cells, in which CD8 T cells had also undergone enrichment for central memory subset T cells prior to CAR transduction. In 18/19 evaluable cases, the CR rate was 93%, but longer term outcomes depended on whether fludarabine had been administered for lymphodepletion, with 1 year disease-free survival of 70% for those who had versus 10% for those who had not received fludarabine. Thirteen patients received allo-SCT following CAR therapy.

10.3.5 Lessons Learnt from ALL Studies

The composite outcomes from the 96 patients treated in the four major studies of second generation CD19 CAR T cell therapy for ALL [39, 59–61] are of a complete response rate of 88%, with 73% being MRD negative (see Table 10.1).

These response rates are unprecedented when one considers the characteristics of the patients being treated, 40/96 had relapsed post allogeneic SCT (see Table 10.1) a significant number were refractory to last chemotherapy [60] and 8/96 cases had CNS leukaemic infiltration. Indeed it seems that CAR T cells efficiently traffic to the CNS and have been documented to contribute to CSF pleocytosis, along with non-transduced T cells, in many following CAR T cell therapy [39]. However, since routine CSF analysis following CAR T cell therapy are only performed for those with prior CNS leukaemia or in those developing neurotoxicity, the overall efficiency of CNS penetration is not clear. Regardless, prior CNS leukaemia does not adversely affect outcomes after CAR T cell therapy, suggesting robust immunosurveillance by CAR T cells at this site.

10.3.5.1 Clinical Outcomes in Relation to CD19 CAR Design

As discussed above, regardless of CAR design (scFv, co-stimulatory domain) or CAR T cell production method (lentiviral vs retroviral, activation method, expansion method), the overall response rates and longer term event free survival in all aforementioned studies of second generation CAR T cell therapy appears broadly similar. However, it is clear that incorporation of a CD28-derived co-stimulatory domain instead of 41BB results in an earlier peak of expansion, earlier CRS, and shorter duration of CAR T cell persistence (approximately 6 weeks persistence with CD28 domain-containing versus 20 weeks or more for 4-1BB domain-containing CD19 CAR T cell studies [5]).

The rates of allogeneic SCT post CAR T cell therapy also differ between the studies. The groups at the Memorial Sloan Kettering Cancer Centre and the National Institutes of Health (NIH) tend to use CAR T cell therapy as a bridge to transplant (with 7/16[59] and 10/20[60] undergoing allogeneic SCT post CAR T cell therapy respectively). As a result, the long term outcomes are a result of composite (CAR + allogeneic SCT) therapy. This has implications for the wider applicability and feasibility of this approach, as well as for the risk of longer term toxicities e.g. graft versus host disease.

10.3.5.2 Factors Associated with Improved Outcomes Following CD19CAR Therapy

The successful outcomes achieved with CD19CAR therapy in general appear to be unaffected by disease burden or the presence of standard prognostic factors such as relapse post allo-SCT, adverse cytogenetics, refractoriness or CNS leukaemia. There are a few exceptions to this. Cases of myeloid leukaemic relapse have occurred in MLL-rearranged ALL following CD19 CAR T cell therapy [62], and the NIH group documented increased disease burden was associated with poorer long term outcomes [60].

Table 10.1. Published findings of second generation CAR T cell therapy for ALL

Reference	Centre	No of patients	Median age, years	Vector (T cell activation method)	CAR design	Adjunctive therapy	Cell dose administered	Persistence of CAR T cells	Adverse events	Outcomes	Best response duration
Davila et al. [59]	Memorial Sloane Kettering Cancer Center	16 (4 post allo-sct)	50	Retrovirus (CD3/CD28 beads, IL-2)	SJ25C1 binder, CD28 domain	Cyclophosphamide 1.5–3 g/m^2	0.14–0.3 × 107 CAR+ T cells/kg	Detected by deep sequencing in BM to 4 months	7 severe CRS	14 CR, 12 MRD-,	Up to 24 months
Lee et al. [68]	National Institutes of Health	21 (8 post allo-sct)	13	Retrovirus (CD3/CD28 beads, IL-2)	FMC63 binder, CD28 domain	Fludarabine 75 mg/m^2 + cyclophosphamide 0.9 g/m^2	0.003–0.3 × 107 CAR+ T cells/kg	Detected by flow cytometry up to 7–8 weeks	4 grade 3–4 CRS, 1 grade 3 dysphasia, 17 B cell aplasia for up to 6 weeks, 1 grade 4 cardiac arrest	14 CR, 13 MRD-	Up to 19 months
Maude et al. [39]	University of Pennsyl-vania	30 (18 post allo-sct)	14	Lentivirus (CD3/CD28 beads)	FMC63 binder, 4-1BB domain	None (3 patients) or various, most commonly Fludarabine 120 mg/m^2 + cyclophosphamide 1 g/m^2	0.076–1.7 3 × 107 CAR+ T cells/kg	Detectable by flow cytometry up to 15 months, and by qPCR for up to 2 years	22 mild-moderate CRS, 8 grade 3–4 CRS, with coagulopathy in 3 of these 8	27 CR, 22 MRD-	Up to 24 months
Turtle et al. [61]	Fred Hutchinson Cancer Research Center	30 (11 post allo-sct)	40	Lentivirus (CD3/CD28 beads)	FMC63 binder, 4-1BB domain	2–4 g/m^2 cyclopho-sphamide or 1–3 g/m^2 cyclophosphamide + 300 mg/m^2 etoposide or 60 mg/kg cyclophosphamide + 75–100 mg/m^2 fludarabine	2 × 10^5–2 × 10^7 1:1 ratio of CD4:CD8 CAR T cells in all cases, in 18 patients 1:1 CD4 to CD8 Tcm cells infused	Up to 10 months where fludarabine lympho-depletion given, otherwise up to 28 days	25 CRS, 7 severe CRS 15 grade 3–5 neurotoxicity	29 CR, 25 MRD-	Up to 21 months

Tcm T central memory cell

The nature of lymphodepletion administered before CAR therapy is important for longer term outcomes. Lymphodepletion may facilitate expansion of adoptively transferred T cells firstly by eliminating regulatory T cells, which can inhibit the activity and expansion of the infused CAR T cells [63] and depletion of the endogenous T cell pool at the time of T cell transfer reduces competition for cytokines supporting lymphocyte survival and function (e.g. IL-2 and IL-7) [64], in turn facilitating CAR T cell engraftment and expansion. Benefit was shown in animal models of CD19 CAR T cell therapy [56] and evidence from early clinical studies of second generation CAR T cell therapy suggested preparatory chemotherapy with cyclophosphamide led to better expansion and persistence of CAR T cells, as well as greater clinical efficacy [65]. A more recent study of ALL CAR T cell therapy showed that CAR T cell expansion and persistence was greater in a cohort receiving both fludarabine and cyclophosphamide compared to cyclophosphamide alone [61]. Early data on event free survival suggests there may also be an improvement in disease outcomes. Further investigation will be required to determine why this is, but its speculated that more stringent depletion of specific immune subsets e.g. regulatory T cells or of immune cells contributing to anti-CAR responses, or manipulation of the tumour microenvironment may contribute.

CAR T cells persistence seems to be associated with better longer term outcomes, particularly in the study from University of Pennsylvania where most patients did not undergo adjunctive allo-SCT [39]. In general, relapses associated with failure of CAR T cell persistence were with $CD19^+$ disease, but about half of relapses were due to tumour escape with $CD19^-$ disease. The mechanisms of tumour escape have been investigated, and as discussed above, appear to be due to exon skipping mechanisms or hemizygous loss of the CD19 locus [40].

10.3.5.3 Toxicity

B Cell Aplasia

This is a predictable 'on-target, off-tumour' effect of CD19CAR T cell therapy, and has been reported with a variable duration in all published studies of CAR T cell therapy. The absence of circulating B cells appears to be a useful surrogate of the persistence of CD19 CAR T cells. Thus, recipients of CD28-containing CAR T cells have B cell aplasia for 2–3 months post CAR T cell therapy [59, 60], but those treated with 41BB containing CARs, which appear to persist longer, may have B cell aplasia for years [39, 66].

A major concern about prolonged B cell aplasia is the risk of infectious complications due to hypogammaglobulinaemia. However, prophylaxis with immune globulin infusions can be instituted where necessary, and, perhaps as a result, no late infectious complications relating to B cell aplasia have been reported to date. Further, there is some evidence that immunoglobulin levels can be maintained by populations of $CD19^-$ plasma cell populations in the absence of B cells and that this seems to preserve humoral immunity after CD19 CAR T cell therapy [66].

Cytokine Release Syndrome (CRS)

This is an immune activation syndrome occurring after CAR T cell therapy with a range of inflammatory manifestations and a spectrum of severity from mild 'flu-like symptoms with fever and myalgia to hypotension, hypoxia and multi-organ failure. CRS has also been seen in patients treated with blinatumomab, a bi-specific recombinant single-chain antibody recognising both CD19 and CD3. Patients with ALL may suffer a higher incidence (94% of patients with ALL treated at the University of Pennsylvania [67]) and severity of CRS (30% severe CRS – see Table 10.1 and [67]) compared to those with other B cell malignancies. The onset of CRS occurs around the peak expansion of CAR T cells, generally within the first week of infusion and persisting for up to 2 weeks after infusion. Its severity has been associated with peak levels of T cell expansion [39, 59–61], as well as pre-existing disease burden [39, 59, 60, 67]. CRS is associated with highly elevated levels of pro-inflammatory cytokines, including interleukin (IL)-6 and interferon gamma (IFNγ). Those developing severe CRS may also have very elevated ferritin levels, akin to those in macrophage activation syndromes [67].

There is much interest in developing biomarkers which can predict which patients are likely to develop severe CRS [59, 67]. This is attractive to allow prioritization of resources such as high dependency care, especially given that in one centre, 20% of patients treated with CD19CAR T cells required intubation for CRS [67]. Prediction of severe CRS also allows timely treatment with tociluzumab, a monoclonal IL-6 receptor blocking antibody. This therapy, initially used empirically to abrogate CRS on the observation that IL-6 levels in particular were highly elevated, appears to be highly successful in mitigating the effects of severe CRS such that fever defervesces within hours and organ support can generally be reduced within 24 h. In managing CRS, timely intervention, both of supportive care e.g. pressor as well as tociluzumab administration can limit severity of toxicity experienced. Pre-emptive therapy is being investigated, predicated upon the accurate and reproducible grading of severity [68]. Further, since the severity of CRS is clearly related to disease burden, development of therapy schedules delivering CAR therapy in the setting of MRD level disease, or for relapsed/refractory patients, incorporating cytoreduction prior to CAR therapy may reduce its incidence. In resistant cases, repeated dosing of tociluzumab may be needed, or adjunctive therapy with corticosteroids or etanercept given. There are concerns that prolonged steroid therapy may abrogate the efficacy of CD19 CAR T cells however, and algorithms for CRS therapy have been devised with the aim of rationally treating more severe CRS with these agents [68].

Neurotoxicity

Transient neurotoxicity arises after second generation CD19 CAR T cell therapy in 30–50% of ALL patients regardless of the specifics of the co-stimulatory domain [39, 59–61]. Neurotoxicity manifests itself from aphasia to obtundation, delirium and seizures [59]. Brain imaging is generally normal, electroencephalograms may

show seizure-like activity and cerebrospinal fluid lymphocytosis is noted, but not exclusively CAR transduced T cells [59], though their levels in the CSF correlated with neurotoxicity in one study [60]. T cell trafficking to the CSF is seen in patients without CNS leukaemia, and similar neurological toxicity has been documented after blinatumomab therapy. Whether neurotoxicity reflects cytokine release affecting the central nervous system is not clear as both syndromes can arise in isolation. In the vast majority of patients, this complication appears to resolve spontaneously after a few days to weeks with supportive therapy alone, though one fatality was noted from the Seattle group [61] and two fatal cases of cerebral oedema were recently reported during a pharmaceutical company-sponsored study.

10.4 Other CAR T Cell Targets in ALL

These include targets such as CD22, which is a B cell differentiation antigen, expressed from an early stage in B cell ontogeny and is involved in the negative regulation of B cell receptor signalling. It appears to be expressed on the vast majority of cases of ALL [69], at reasonably high antigen densities, although the level of expression does appear lower in case of MLL-rearranged ALL. The level of expression does not appear to be reduced following CD22-directed therapy and there is little evidence of soluble CD22 or shedding of CD22 which may otherwise impact efficacy of CD22-targeting therapies. CD22-directed CAR studies are underway in ALL (NCT02315612 and NCT02794961, but the results have not yet been published. Dual targeting of e.g. CD19 and CD22 offers the potential to prevent tumour antigenic escape which is responsible for relapse in 50% of cases [39], though this is still in the preclinical validation phase. Other B cell antigens e.g. Thymic stromal lymphopoietin receptor are expressed on ALL blasts and have been targets for CAR T cell therapy in preclinical testing [70]. Effective cellular immunotherapy for T-ALL is urgently needed and T cell antigens are also the subject of CAR discovery programs.

10.5 Future Directions

Recent trials have established ALL as the model disease for anti-tumour efficacy with CAR T cell therapy. These have put gene-engineered T cell products on the cancer therapeutics map, and provided hope to patients with highly advanced disease for whom traditionally, no other option existed. There is room for improvement, however, and a number of key challenges remain. Currently, up to 30% of ALL patients undergoing CAR T cell therapy develop severe CRS, which by definition requires care in a high dependency setting. It is not clear whether effective immune activation is necessarily associated with some degree of CRS, and it is not clear indeed, if some degree of CRS is required for therapeutic efficacy.

Therapeutic schedules with CD19 CAR T cells have yet to be optimised. It may be that treatment of high risk patients earlier in their disease course is more effective than in the relapsed/refractory or post-transplant setting. A further benefit of treating patients in morphological remission is a reduced risk of CRS because of lower disease burden.

Whilst response rates to CD19 CAR T cell therapy in ALL are impressive, those in other B cell malignancies e.g. lymphoma and CLL are lower. In these disease settings, strategies to improve efficacy may be beneficial, e.g. co-administration of immune check-point inhibitory agents, or further gene-engineering of CAR T cells to enhance their trafficking to, as well as persistence and function within the tumour microenvironment. Such lessons may also benefit those investigating CAR T cell therapy for non-haematological malignancies as response rates in these settings have also been lower. Targeting strategies for other malignancies e.g. AML are more problematic, as leukaemic blasts and their initiating cell populations do not express tumour specific antigens, the antigens they do express are shared with healthy haematopoietic stem cells. Such leukaemic populations are more heterogeneous with subclones expressing different combinations of antigens necessitating multi-antigenic approaches, and adding complexity to their clinical translation.

Investigators are currently working on ways to engineer T cells with multiple antigen-recognition capability and with CAR designs which endow CAR T cells with more 'intelligent' cognate interactions, such that they can act as biologic logic (e.g. AND, OR, NOT) gates. In this way T cells can be programmed to activate in the presence of antigen A AND B only, A OR B, A and NOT B respectively. This combinatorial programming of T cell activation may allow better discrimination of malignant versus healthy host tissues and by targeting more than one expressed antigen, may reduce the risk of relapse due to selection of antigen-negative sub-clones.

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Chapter 11
Relapsed Acute Lymphoblastic Leukemia of Childhood

Su Han Lum, Denise Bonney, and Vaskar Saha

11.1 Introduction

In contrast to improvements in outcome in newly diagnosed patients, survival post relapse remains relatively unchanged over the last three decades [1], in part due to lack of new drugs that may overcome resistance to agents used in frontline therapy and recurrent disease is by definition due to highly resistant leukaemic cell clones In this chapter, we discuss the pathogenesis and treatment of relapse ALL with a focus on future therapeutic options utilizing drugs which have different mechanisms of action to combination chemotherapy and target the physiological pathways that sustain leukemic cell survival.

11.2 Pathogenesis of Relapsed ALL

The patterns of recurrence and outcomes of therapy in second remission appear to be consistent across different study groups, suggesting that failure of therapy is not directly related to resistance to specific chemotherapeutic agents. Two key features of relapse are (i) a predilection for recurrences to involve the CNS and (ii) outcome of after relapse relates to the duration of the first remission.

S.H. Lum • D. Bonney
Royal Manchester Children's Hospital, Central Manchester University Hospitals NHS Trust, Manchester M13 9WL, UK

V. Saha (✉)
Royal Manchester Children's Hospital, Central Manchester University Hospitals NHS Trust, Manchester M13 9WL, UK

Children's Cancer Group, Paterson Building, Institute of Cancer, University of Manchester, Manchester M20 4BX, UK

Tata Translational Cancer Research Centre, Tata Medical Center, Kolkata 700156, India
e-mail: vaskar.saha@manchester.ac.uk

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11.2.1 CNS Relapses

A striking feature of relapse site is the significant increase in CNS involvement (~40%) compared with that at initial presentation (around 2–3%) [2]. This is primarily a leptomeningeal disease without parenchymal involvement. Normal lymphocytes are able to migrate across endothelial barriers. Post mortem analysis [3] and murine models [4] of CNS leukemia show perivascular cuffing of vessels within the brain parenchyma (Virchow-Robin spaces) by lymphoblasts without damage to the vascular endothelium, suggestive of pericellular diapedesis. Current evidence suggests that this ability is a general phenomenon of ALL cells [5], though the expression of integrins and adhesion molecules by subclones may facilitate this process [6]. Blast cells that express interleukin-15 (IL-15) appear to have a predilection for CNS infiltration. IL-15 is a target for Natural Killer (NK) cells. As NK cells cannot pass through an intact blood-brain-csf barrier, they can control disease in the periphery but not within the CNS [7]. In this model, the CNS is a sanctuary site where blasts can survive chemotherapy and evade surveillance. However, the great majority of ALL blast cells (~95%) do not divide and while metabolically active have a short life span [8]. How then do such cells survive for such a long time in the restricted environment of the cerebrospinal space? Most patients with isolated CNS disease have detectable low levels of medullary involvement [9]. As the medullary compartment is considerably larger than the csf compartment, in fact the bulk of the disease lies outside the CNS. In children, the calvarial marrow is an active site of haematopoiesis and shares its circulation with the cerebral venous plexuses as well as the posterior spinal venous complex. These vascular beds also drain cerebral vessels and the choroid plexus. Thus it is possible cells surviving in calvarial marrow niches, or in the recently identified lymphatics associated with dural sinuses [10], may seed both the csf and other distant marrow sites. As the csf compartment is considerably smaller, the earliest symptoms correspond to an obstruction of the csf flow by dying cells or infiltration of the cranial nerves. Both models are compatible with the significantly reduced incidence of CNS recurrence seen in regimens using frequent intrathecal therapy along with drugs that have higher penetration into the csf (e.g. dexamethasone and intravenous methotrexate).

11.2.2 Bone Marrow Relapses

As shown in Fig. 11.1, bone marrow relapses fall into three broad clinical phenotypes. Patients with T-ALL have a poor outcome irrespective of the duration of first remission (CR1) even after allogeneic stem cell transplant (SCT) in second CR. In B-cell precursor (BCP) ALL, outcomes are dependent on whether disease recurred early while still on therapy (or within 6 months of stopping therapy) or late i.e. more than

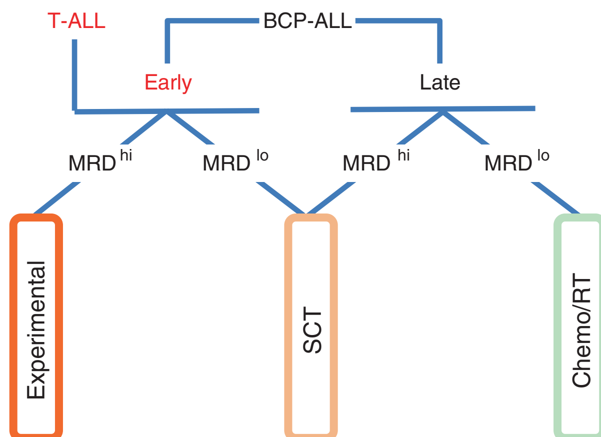

Fig. 11.1 Treatment strategy for bone marrow relapses in childhood ALL. Late BCP-ALL patients who are MRD^{lo} ($<10^{-4}$) at the end of induction mostly do not require allo-SCT to maintain CR2. Late BCP-ALL marrow relapses that are MRD^{hi} ($\geq 10^{-4}$) at the end of induction and early relapses that are MRD^{lo} appear to benefit from allo-SCT. Outcomes of MRDhi early BCP-ALL and all T-ALL bone marrow relapses remains poor even with allo-SCT. Early = within 6 months of stopping therapy; Late = 6 months or more after therapy has been stopped.

6 months after stopping therapy. Early BCP-ALL relapses have outcomes similar to T-ALL and are classified as high risk (HR). Within the HR group, the small minority of patients with low MRD levels at the end of re-induction have a better chance of cure with intensive therapy and allo-SCT [11, 12]. Conversely, in late relapsing BCP-ALL, a low post induction MRD level identifies patients who have a chance of cure without an allo-SCT [13]. These clinical phenotypes thus pose interesting biological conundrums.

Current evidence suggests that ALL cells within protective stromal niches may give rise to disease recurrences. Lymphoblast modulation of the hematopoietic stem cell niche leads to suppression of normal haematopoiesis while sustaining the survival of the ALL clone(s) [14, 15]. ALL subclones adapt to oxidative stress and reprogram the metabolic state within the niche [16]. Potentially these adaptations lead to an epigenetically regulated survival programme that may also protect against cytotoxicity. SCT is thought to both alter the leukaemic bone marrow environment and promote cell-mediated cytotoxicity through a graft versus leukemia effect. Hypothetically, the MRD^{lo} and MRD^{hi} populations in the HR and BCP-ALL late medullary relapses could represent populations of cells that are protected from chemotherapy by the microenvironment but still benefit from SCT. On the other hand, the MRD^{lo} late relapsing group could have evolved to lose epigenetically programmed drug resistance [17]. The HR MRD^{hi} group most closely fits the traditional profile of

intrinsic resistance, though even here >70% of patients achieve CR. This suggests that even at this stage, the tumor is an admixture of sensitive and resistant subclones.

Cytogenetic subtypes with prognostic significance are well described in ALL. These clonal subtypes retain prognosis at relapse [18]. Within these clonal events, subclones carry different secondary mutations. While most are passengers mutations some are of prognostic significance. Clonal diversity is comparable at diagnosis and relapse, with a composition of major and minor clones [19]. A number of reasons may lie in this differential clonal composition. Subclones may appear at different times and a younger clone may dominate over older clones (selective sweep) [20]. Alternatively, rapid clonal expansions may occur in bursts (punctuated equilibrium) [21]. Often at relapse, a minor resistant clone present at diagnosis is established as the dominant one at relapse under selection of first line therapy. Occasionally mutations not identifiable at diagnosis are identified at relapse. It's uncertain whether this is a result of the clone being below the level of detection at diagnosis, an example of peripatric speciation in isolated populations, influenced by the microenvironment or clonal evolution is unclear. As minor resistant subclones at diagnosis become dominant at relapse, mutations that predict for relapse may lose their significance in predicting outcome after relapse. For example, deletions of *IKZF1* associated with higher relapse rates are no longer prognostic in relapsed patients [18]. On the other hand, mutations and deletions of *TP53* that are not prognostic at first diagnosis are associated with poor outcomes when present at relapse [22].

Thus the mechanisms by which blast cells survive chemotherapy and give rise to treatment failure is a combinatorial one. The current hypothesis is that subclonal mutations on a clonal genetic background facilitates cross talk with host cells leading to epigenetic reprogramming of select clones to promote cell survival in a stressful environment (Fig. 11.2). What exactly these prosurvival pathways are and if they can be targeted remain an area of intense investigation.

11.3 Outcomes of Relapse: The Pre MRD Era

In early studies by St Jude's [23] and Great Ormond Street [24, 25] children with bone marrow relapses were treated with the same drugs used in front line therapy. They observed that reinduction with vincristine and steroids with or without an anthracycline achieved a second remission (CR2) in over 65% of the patients but disease recurred in a majority. Both groups noted that outcomes were inferior for patients who relapsed on therapy when compared to later relapses (Tables 11.1 and 11.2). The BFM pioneered the systematic approach to relapsed ALL. In ALL-REZ 83 and REZ 85, they demonstrated that about a quarter of patients could be cured post relapse after intensive therapy including intravenous methotrexate given at a dose of 1 gm/m^2 over 36 h [61]. The ALL-REZ 85 trial first established the risk stratification now commonly used in relapsed ALL (Fig. 11.3). Remission rates were achieved in 92%. The 6-year EFS was 72% ± 11 in those with an

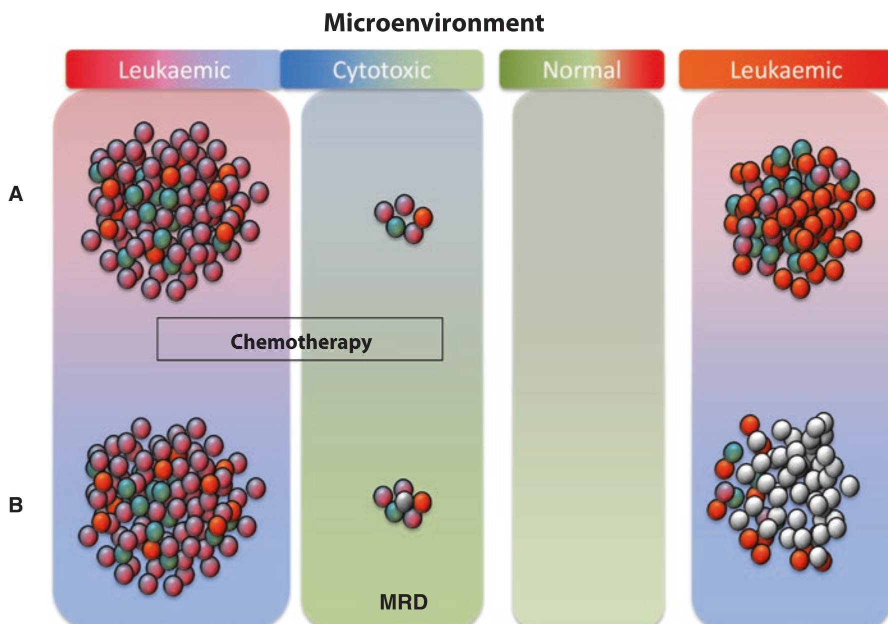

Fig. 11.2 Schematic of postulated model of disease recurrence. (**a**) Minor subclones present at diagnosis persist to give rise to disease recurrence. (**b**) Recurrent disease arises from clonal evolution of subclones (Adapted from [19]. The ALL cell alters the microenvironment to favour leukaemic cell survival (*purple background*). Chemotherapy affects both the microenvironment and leukaemic cell (*blue*) and the environment gradually returns to normal with restoration of haematopoiesis (*green*)

extramedullary; 30% ± 7 in late marrow/combined and 18% ± 5 in early bone marrow/combined relapses [62]. Other groups using various therapies [63, 64], or a designated relapse strategy [52] subsequently verified this risk stratification (Tables 11.3 and 11.4). These reports showed that irrespective of the frontline protocols and subsequent therapy, T-cell patients with bone marrow relapses at any time and BCP-ALL patients with bone marrow relapses within 6 months of stopping therapy had a poor outcome as did relapse at any site occurring within 18 months of first diagnosis. In contrast, BCP-ALL with late bone marrow relapses and late isolated extramedullary relapses could often be cured by the use of more intensive chemotherapy protocols (Table 11.5). The definitions for early versus late relapse differed slightly among study groups. CCLG (UK) and Berlin-Frankfurt-Münster (BFM) study groups have defined early relapses as very early (within 18 months of diagnosis), early (beyond 18 months of diagnosis and up to 6 months after completion of frontline therapy) and late (beyond 6 months after completion of frontline therapy). In Children Oncology Group (COG) trials, early relapse is defined as occurring within 36 months of initial diagnosis while late relapse is defined as occurring after 36 months of initial diagnosis. St Jude's Children's Research Hospital (SJCRH) study has defined early relapse as less than 6 months from completion of

Table 11.1 Number of relapses according to clinical trials for childhood acute lymphoblastic leukaemia

Study group	Years	No of patients		Age range (years)	Relapse according to site, n (%)				5-year OS (%)	5-year EFS (%)	Remarks
		All	T-cell n (%)		All	i-CNSr	c-CNSr	n-CNSr			
Medical Research Council (MRC) childhood ALL trials											
UKALLX [2, 26]	1985–1990	1612	136 (8.4)	0–14	558 [27]	104 [7]	67 [4]	387 [25]	77.4	61.9	Double DI therapy improved EFS
UKALLXI [2, 28]	1991–1997	2090	206 (9.8)	1–14	790 [29]	132 [6]	150 [7]	508 [24]	84.6	63.1	Third DI had no additional benefit Continuing IT could replace CRT or HDMTX
ALL97 [2, 30]	1997–1999	957	118 (12.3)	1–17	238 [24]	44 [5]	43 [4]	151 [15]	83.5	74.0	ALL97 and ALL97/99 First NCI risk stratification in MRC ALL97 carried 3 DI arm of UKALLXI Modified to ALL97/99 with 2 CCG modified BFM DI and the frequency and dosing of ASP were changed
ALL97/99 [2, 30]	1999–2002	938	92 (9.8)	1–17	162 [17]	27 [3]	13 [1]	122 [13]	88.0	80.0	DXM at 6.5 mg/m^2 was superior that PDN at 40 mg/m^2 6-TG at 40 mg/m^2 resulted in fewer relapses but increased incidence of deaths in remission when compared with 6-MP at 75 mg/m^2

UKALL 2003 [31]	2003–2011	3126	386 (12.3)	1–24	192 (6.1)	44 (1.9)	69 (2.2)	123 (5.9)	91.5	87.2	Risk stratification using NCI criteria, cytogenetics, early response to treatment and MRD Treatment reduction was feasible for patients with a low risk of relapse based on rapid MRD clearance by end of induction
International Berlin-Frankfurt-Münster study group (I-BFM-SG)											
ALL-BFM 81 [32]	1981–1983	611	62 (12.0)	0–18	174 (28.5)	30 (5.0)	27 (4.5)	117 (19.1)	80.6	67.5	*Reduction in treatment burden* (reduction of dose intensity in induction; elimination of reintensification) had a major adverse impact on outcome
ALL-BFM 83 [32]	1983–1986	653	87 (13.9)	0–18	220 (33.8)	18 (2.6)	39 (6.1)	163 (25.0)	76.1	64.3	*Presymptomatic CRT* can be safely reduced to 12Gy or eliminated if it is replaced by early intensive systemic and IT MTX.
ALL-BFM 86 [32]	1986–1990	998	127 (13.1)	0–18	257 (25.9)	18 (2.6)	30 (3.0)	209 (21.0)	81.2	72.1	*Maintenance therapy* given a total of 24 months from diagnosis provides a lower rate of relapse
ALL-BFM 90 [32]	1990–1995	2178	284 (13.4)	0–18	428 (19.8)	22 (2.1)	45 (2.1)	361 (16.7)	85.5	78.0	*Poor PDN response* at Day 7 has very high risk of relapse

(continued)

Table 11.1 (continued)

Study group	Years	No of patients		Age range (years)	Relapse according to site, n (%)				5-year OS (%)	5-year EFS (%)	Remarks
		All	T-cell n (%)		All	i-CNSr	c-CNSr	n-CNSr			
BFM-95 [32]	1995–1999	2169	277 (12.7)	0–18	358 (16.9)	39 (1.8)	48 (2.2)	271 (12.9)	87.0	79.6	Dose reduction in anthracyclines did not impact outcome in SR ALL Extension of continuation treatment to 36 months failed to improve outcome for boys Intensification of consolidation/reinduction treatment improved outcome of HR ALL
AIEOP/BFM 2000 [33]	2000–2007	3720	432 (11.9)	1–18	552 (14.8)	56 (1.5)	45 (1.2)	449 (12.1)	DEX: 90.3 PDN: 90.5	DEX: 83.9 PDN: 80.8	Risk stratification based on MRD by PCR of Ig/TCR DXM in induction was associated with significant relapse reduction and increased treatment-related mortality No overall survival benefit was achieved with DXM except in patients with T-ALL and good early treatment response
Italian Association of Pediatric Hematology and Oncology (AIEOP)											
AIEOP 82 [34]	1982–1987	902	110 (12.2)	0–15	346 (38.4)	90 (10.0)	30 (3.2)	226 (25.2)	70.4	56.4	Poor result in HR ALL treated with intensive, short duration therapy without CRT
AIEOP 87 [34]	1987–1991	632	87 (13.8)	0–15	207 (32.7)	51 (8.1)	15 (2.4)	141 (22.2)	78.6	66.8	Benefits were seen in HR patients received intermediate dose of MTX, CRT and protracted L-ASP

AIEOP 88 [34]	1988–1992	396	54 (13.6)	0–15	115 (29.0)	20 (5.1)	14 (3.5)	81 (20.4)	78.2	67.6	BFM therapy was feasible in AIEOP centers
AEIOP 91 [34]	1991–1995	1192	144 (12.1)	0–15	312 (26.2)	19 (1.6)	11 (0.9)	282 (23.7)	79.1	70.9	Extended Erwinia ASP did not improve response in intermediate risk Multiple intensive blocks did not improve outcome in high risk ALL IT chemotherapy could replace CRT
AEIOP 95 [34]	1995–2000	1743	191 (11.0)	0–17	398 (22.8)	21 (1.2)	16 (0.9)	361 (20.7)	85.5	75.9	Double 8-drug reinduction therapy improved outcome of HR ALL compared to multiple intensive blocks DXM and VCR pulses given every 10 weeks during maintenance therapy in BFM-based therapy did not improve outcome in SR ALL
Children's Cancer Group (CCG)/Pediatric Oncology Group (POG)/Children's Oncology Group (COG)											
CCG 100 series [35]	1983–1988	3713	319 (8.6)	0–21	542 (14.6)	106 (2.6)	NA	436 (11.4)	78.7	65.5	Intensive induction/ consolidation improved EFS Cranial RT in children with SR CNS negative could be spared with IT MTX adequate with intensified systemic therapy

(continued)

Table 11.1 (continued)

Study group	Years	No of patients		Age range (years)	Relapse according to site, n (%)				5-year OS (%)	5-year EFS (%)	Remarks
		All	T-cell n (%)		All	i-CNSr	c-CNSr	n-CNSr			
CCG 1800 series [35]	1989–1995	5121	431 (8.4)	0–21	753 (14.7)	144 (2.8)	NA	609 (11.9)	85.0	75.2	Slow early responders had improved outcome with increased post-induction therapy Double delayed intensification improved outcome in IR ALL
CCG 1900 series [35]	1996–2002	4464	522 (11.7)	0–21	687 (15.3)	149 (3.3)	NA	538 (12.0)	86.3	76.0	DXM 6 mg/m^2 was more effective than PDN 40 mg/m^2 for SR IV 6-MP was inferior treatment Triple IT resulted in lower CNS relapse rate but more haematological relapse than IT MTX in SR ALL 6-TG at 60 mg/m^2 yielded better EFS but had unacceptable liver toxicity PEG asparaginase was as effective as *E.coli* asparaginase and with lower immunogenicity

POG [36]	1986–1994	4404	439 (10.0)	1–21	NA	NA	NA	NA	NA	70.9	Intensive treatment blocks did not improve outcome in high risk ALL IV MTX was superior to oral in intensification IV mercaptopurine was not helpful to improve outcome NCI criteria did not predict outcome in T-cell disease
COG [37]	2000–2005	7153	459 (6.4)	0–21	NA	NA	NA	NA	90.4	NA	
St Jude Children's Research Hospital (SJCRH)											
SJCRH 11 [38]	1984–1988	358	62 (17.3)	0–18	78 (21.8)	21 (5.9)	5 (1.4)	52 (14.5)	79.2	72.1	The use of early treatment intensification improved outcome Schedule-dependent leukaemogenic effects of epipodophyllotoxins were identified
SJCRH 12 [38]	1988–1991	188	29 (15.4)	0–18	52 (27.7)	19 (10.1)	8 (4.3)	25 (13.3)	83.5	67.6	Individualized therapy based on pharmacokinetics was proven
SJCRH 13A [38]	1991–1994	165	22 (13.3)	0–18	26 (15.7)	2 (1.2)	6 (3.6)	18 (10.9)	83.0	77.6	Early intensification of IT reduced CNS relapse and allowed reduction of cranial irradiation

(continued)

Table 11.1 (continued)

Study group	Years	No of patients		Age range (years)	Relapse according to site, n (%)				5-year OS (%)	5-year EFS (%)	Remarks
		All	T-cell n (%)		All	i-CNSr	c-CNSr	n-CNSr			
SJCRH 13B [38]	1994–1998	247	44 (17.8)	018	35 (14.2)	7 (2.8)	2 (0.8)	26 (10.5)	85.7	80.1	MRD was an independent prognostic factor Confirmation of the efficacy early intensification of IT Pharmacogenetics affects treatment toxicity and antileukemic outcome First leukaemia trial to apply pharmacogenetics in therapy
SJCRH 14 [38]	1998–1999	53	NA	0–18	7 (13.2)	0	2 (3.8)	5 (9.4)	81.1	77.4	Early termination for excessive toxicities encountered during remission induction
SJCRH 15 [39]	2000–2007	498	66 (13.2)	1–18	33 (6.6)	11 (2.2)	4 (0.8)	18 (3.6)	93.5	85.6	Prophylactic CRT can be safely omitted from the treatment of ALL with effective risk-adapted therapy, irrespective of presenting features
Daba-Farber Cancer Institute Consortium											
DFCI 85–01 [40]	1985–1987	220	20 (9.1)	0–18	37 (16.8)	NA	NA	NA	83.9	78.4	First risk stratification based on age, WBC and IP
DFCI 87–01 [40]	1987–1991	369	38 (10.2)	0–18	72 (19.5)	NA	NA	NA	87.7	76.9	Age, sex, WBC, phenotype and CNS status, male were independent adverse predictors
DFCI 91–01 [40]	1991–1995	377	28 (7.4)	0–18	46 (12.2)	4 (1.1)	8 (2.1)	34 (9.0)	87.8	83.5	No difference in EFS in patients received doxorubin 30 mg/m^2 as either a bolus dose or a 48-h continuous infusion

DFCI 95–01 [40]	1996–2000	491	52 (10.6)	0–18	79 (16.0)	3 (0.6)	12 (2.4)	63 (12.8)	89.6	81.6	Dexrazoxone did not interfere with anti-leukaemic effects of doxorubicin
											CRT was not necessary for SR patients
											Erwinia ASP at 25,000 IU/m^2 was less efficacious than *E.coli* ASP given at the same dose
DFCI 00–01 [41]	2000–2004	492	49 (10.0)	1–18	69 (15.0)	NA	NA	NA	91	80	DEX and individualized asparaginase dosing were associated with improved outcome
DFCI 05–01 [42]	2005–2011	551	69 (12.5)	1–18	NA	NA	NA	NA	NA	86	Intensified consolidation improved outcome for B-ALL with high end-induction MRD
											IV PEG was as effective as and no more toxic than weekly IM *E.coli* ASP
Other clinical trials											
COALL 97 [43]	1997–2003	667	94 (14.1)	0–1	131 (19.6)	14 (2.1)	11 (1.6)	106 (15.9)	85.4	76.7	MRD was a better predictor of outcome than in vitro drug sensitivity testing
CPH-95 [44]	1996–2002	380	56 (14.7)	0–18	72 (18.9)	4 (1.0)	8 (2.1)	60 (15.8)	82.0	73.2	Reduction in prophylactic CRT did not compromise clinical outcome
DCOG-9 [45]	1997–2004	859	90 (10.5)	1–18	133 (15.4)	22 (2.6)	NA	NA	86.4	80.6	DXM based therapy without the use prophylactic CRT improved outcome

(continued)

Table 11.1 (continued)

Study group	Years	No of patients		Age range (years)	Relapse according to site, n (%)				5-year OS (%)	5-year EFS (%)	Remarks
		All	T-cell n (%)		All	i-CNSr	c-CNSr	n-CNSr			
Ma-Spore-ALL2003 [46]	2002–2011	556	49 (8.8)	0–18	36 (6.5)	8 (1.4)	2 (0.4)	26 (4.7)	88.4	80.6	3-drug remission-induction with MRD-based risk stratification is an effective strategy for childhood ALL
NOPHO-2000 [47]	2002–2007	1023	115 (11.2)	1–15	126 (12.3)	24 (2.3)	14 (13.7)	74 (7.2)	89.0	77.0	Prophylactic CRT failed to reduce CNS relapse in HR ALL
TCCSG 95–14 [48]	1995–1999	597	58 (9.7)	1–15	92 (15.4)	10 (1.7)	5 (0.8)	77 (12.9)	82.0	76.8	DXM 8 mg/m^2 and PDN at 60 mg/m^2 yielded comparable results from SR and IR ALL
TPOG2002 [49]	2002–2007	788	76 (9.6)	0–18	116 (14.7)	24 (3.0)	9 (1.2)	83 (10.5)	83.5	77.4	2 reinduction courses did not improve outcome over one course for SR ALL
											Reduction in CRT was not associated with increased rate of CNS relapse

Clinical trials: *AIEOP* Associazione Italiana di Ematologia ed Oncologia Pediatrica, *BFM* Berlin-Frankfurt-Münster ALL Study Group, *CCG* Children's Cancer group, *COALL* Cooperative ALL Study Group, *CPH* Pediatric Hematology in the Czech Republic, *DCOG* Dutch Childhood Oncology Group, *DFCI* Dana-Farber Cancer Institute ALL Consortium, *Ma-Spore* Malaysia-Singapore ALL 2003, *NOPHO* Nordic Sociate of Pediatric Hematology and Oncology, *SJCRH* St: Jude Children's Research Hospital, *TCCSG* Tokyo Children's Cancer Study group, *TPOG* Taiwan Pediatric Oncology group, *UKALL* UK Medical Research Council working Party on Childhood Leukaemia

Relapses: *c-CNSr* combined CNS relapse, *i-CNSr* isolated CNS relapse, *n-CNSr* non CNS relapse

Risk groups: *SR* standard risk, *SR-L* low-standard risk, *IR* intermediate risk, *MR* medium risk, *HR* High risk, *VHR* Very high risk

Lineage: *B* B-lineage *T* T-lineage

Treatment: *ASP* asparaginase, *DI* Delayed intensification, *CRT* cranial radiotherapy, *DXM* dexamethasone, *HD* high dose, *MTX* methotrexate, *IT* Intrathecal therapy, *IV* intravenous, *6-MP* mercaptopurine, *PEG* Pegylated, *PDN* prednisolone, *6-TG* thioguanine, *VCR* Vincristine, *CNS* central nervous system, *EFS* event-free survival, *MRD* minimal residual disease, *OS* overall survival, *IP* immunophenotyping, *NA* Not available

Table 11.2 Treatment outcome of relapsed childhood acute lymphoblastic leukaemia according to clinical trials

Study group	Years	No of patients		Age range, year	Outcome according to site and time of relapse					Remarks
		All	T-cell n (%)		Risk group	Site/Time		No of patients	EFS/DFS (%)	
Medical Research Council (MRC) childhood ALL trials										
UKALL X [50]	1985–1993	489	53 (10.8)	1–14	NA	Isolated marrow (n = 235)	E	83	1	Overall 5-year EFS: 28%
							I	29	14	Early (E): 2 years from diagnosis
							L	123	52	Intermediate (I): 2–2.5 years from diagnosis
						Marrow + CNS (n = 54)	E	18	18	
							I	13	13	Late (L): >2.5 years from diagnosis
							L	23	23	
						Marrow + others (n + 43)	E	43	5	
							I	15	40	
							L	23	50	
						Isolated CNS (n = 98)	E	62	24	
							I	23	35	
							L	13	64	
						Others (n = 59)	E	11	36	
							I	26	36	
							L	22	68	

(continued)

Table 11.2 (continued)

Study group	Years	No of patients: All	No of patients: T-cell n (%)	Age range, year	Outcome according to site and time of relapse: Risk group	Site/Time		No of patients	EFS/DFS (%)	Remarks
UKALL R1 [51]	1991–1995	256	11 (4.3)	1–14	NA	All (n = 256)	E	47	19	Overall 5-year EFS: 46%
							I	72	38	
							L	137	60	
						Isolated marrow (n = 121)	E	22	5	
							I	20	35	
							L	79	51	
						Marrow + CNS (n = 43)	E	5	0	
							I	17	41	
							L	21	81	
						Marrow + others	E	2	50	
							I	12	25	
							L	19	58	
						Isolated CNS	E	13	46	
							I	9	56	
							L	4	100	
						Others	E	5	20	
							I	14	36	
							L	14	70	
UKALL R2 [52]	1995–2002	150	15 (10.0)	1–27	SR	Refer to Table 11.4		13	92	
					IR			105	51	
					HR			32	15	
UKALL R3 [53]	2003–2009	216	24 (11.1)	1–18	216 children were randomized to idarubicin (n = 109) or mitoxantrone (n = 103)					
					3-year EFS: Idarubicin: 35.9% versus Mitoxantrone: 64.6% ($p = 0.007$)					

International Berlin-Frankfurt-Münster study group (I-BFM-SG)									
ALL-REZ BFM 90 [54, 55]	1990–1995	525	73 (13.9)	0–18	A	EIMR/CMER	126	17	OS: 36%; EFS 30%
					B	LIMR/CMER	183	43	Time point, site of relapse, IP and HSCT were significant predictors of EFS
					C	IExMR	64	54	In HR, HSCT should be recommended in CR2
					PPG	VEMR; T-ALL	152	15	No benefit of high dose of MTX (1 g/m^2 versus 5 g/m^2) No advantage of additional R3 block 24-h IV infusion high dose MTX (5 g/m^2) compared with the 36-h IV infusion of intermediate-dose MTX (1 g/m^2) did not improve OS and EFS in children with relapsed ALL
ALL-REZ BFM 2002 Stackelbery, unpublished data	2003–2007	538	NA	1–18	S1	*Refer Table 11.3	34	68	Post-induction therapy with Protocol II-IDA was associated with fewer subsequently relapses compared to R-courses (21% vs 30%, $p = 0.03$)
					S2		359	60	
					S3		70	31	
					S4		128	28	

(continued)

Table 11.2 (continued)

Study group	Years	No of patients		Age range, year	Outcome according to site and time of relapse					Remarks
		All	T-cell n (%)		Risk group	Site/Time		No of patients	EFS/DFS (%)	
Children's Cancer Group (CCG)/Pediatric Oncology Group (POG)/Children's Oncology Group (COG)										
CCG 100 [56]	1983–1989	1144	NA	0–21	NA	Isolated marrow	All	642	16	Early (E): 0–17 months
							E	233	5	Intermediate (I): 18–35 months
							I	194	10	Late (L): ≥36 months
							L	215	33	
						Combined marrow	All	120	29	
							E	34	9	
							I	26	11	
							L	60	48	
						Isolated CNS	All	220	37	
							E	102	24	
							I	84	44	
							L	34	59	
						Isolated testes	All	112	64	
							E	22	48	
							I	24	44	
							L	66	76	
CCG-1941 [57]	1995–1998	214	17 (7.9)	1–21	241 children with early first marrow relapse (within 12 months of the completion of primary therapy					Outcome was poor for children with early marrow relapsed ALL
					Conventional sibling BMT: EFS 29%					BMT did not show superior result in these patients
					Alternative donor BMT: EFS 21%					
					Chemotherapy: EFS 27%					

CCG-1961 [58]	1996–2002	272	48 (16.9)	1–21	5-year EFS: 71.3%	Initial therapy on the CCG 1961 trial had no significant impact on post relapse survival. The emergence of a resistant subclone that had acquired spontaneous mutations was independent of the initial therapy
COG AA01P2 [59]	2003–2005	145 (124 reported)	7 [6]	1–21	1-year EFS Early: 36% Late: 80% MRD negative at the end of block 1: 80% MRD positive at the end of block 1: 58%	AALL01P2 regimen was a tolerable and active reinduction platform
Other clinical trials						
DCOG Rel-ALL 98 [60]	1999–2006	158 (98 reported)	NA	NA	5 year EFS Early: 12% Very late: 58%	Poor prognosis for early relapse

Clinical trials: *BFM* Berlin-Frankfurt-Münster ALL Study Group, *CCG* Children's Cancer group, *UKALL* UK Medical Research Council working Party on Childhood Leukaemia

Risk groups: *SR* standard risk, *IR* intermediate risk, *PPG* poor prognosis group

Treatment: *ASP* asparaginase. *DXM* dexamethasone, *IT* Intrathecal therapy

Site and time of relapses: *IMR* Isolated marrow relapse, *IEMR* Isolated early marrow relapse, *CMExR* Combined marrow and extramedullary relapse, *LIMR* Late isolated marrow relapse, *IExMR* Isolated extramedullary relapse, *LExMR* Late isolated extramedullary relapse, *MR* Marrow relapse, *VEMR* very early marrow relapse, *CNS* central nervnous system, *EFS* event-free survival, *HSCT* Haematopoietic stem cell transplantation, *OS* overall survival, *IP* immunophenotyping, *NA* Not available

Fig. 11.3 Simplified schematic of risk stratification in relapsed ALL. Early = from diagnosis to within 6 months of stopping therapy. Late = 6 months or more after stopping therapy. *iBM* isolated bone marrow, *C* combined, *iEM* isolated extramedullary disease. *Red* allo-SCT not MRD dependent, *orange* allo-SCT for MRD^{hi} patients, *green* no allo-SCT

Table 11.3 Risk group stratification of relapsed childhood ALL according to clinical trials

1.1.Definition of time point of relapse						
Time point	After primary diagnosis			After cessation of primary therapy		
Very early	<18 months		and	<6 months		
Early	≥18 months		and	<6 months		
Late				≥6 months		
1.2 Definition of site of relapse						
Bone marrow (BM)		M1 (<5%)	M2 (≥5% and <25% blasts	M3 (≥25% blasts)		
Extramedullary (EM) relapse	No	No relapse	Requires follow up control	Isolated BM relapse		
	Yes	Isolated EMR	Combined BM and EM relapse			
1.3 BFM ALL relapse risk groups by immunophenotype, time point and site of relapse						
Time	Non-T			(Pre-)T		
	Isolated EM	Combined BM	Isolated BM	Isolated EM	Combined BM	Isolated BM
Very early	S2	S4	S4	S2	S4	S4
Early	S2	S2	S3	S2	S4	S4
Late	S1	S2	S2	S1	S4	S4
1.4 UKALL3 relapse risk stratification						
Time	Non-T			(Pre-)T		
	Isolated EM	Combined BM	Isolated BM	Isolated EM	Combined BM	Isolated BM
Very early	I	H	H	I	H	H
Early	I	I	H	I	H	H
Late	S	I	I	S	H	H
S: Standard; I: Intermediate; H: High risk						
1.5 IntReALL SR/HR 2010 risk groups						
Time	Non-T			(Pre-)T		
	Isolated EM	Combined BM	Isolated BM	Isolated EM	Combined BM	Isolated BM
Very early	HR	HR	HR	HR	HR	HR
Early	SR	SR	HR	SR	HR	HR
Late	SR	SR	SR	SR	HR	HR
SR: Standard risk; HR: High risk						

Table 11.3 (continued)

1.6 Children's oncology group, USA	
Risk group	
Low	Late extramedullary (CR1≥18 months)
Intermediate	B-lineage late marrow
	B-lineage late combined
	Early isolated extramedullary (CR1 duration <18 months)
High	B-lineage early marrow or combined
	T-lineage marrow or combined (early or late)
Early: <36 months from initial diagnosis; Late: ≥36 months from initial diagnosis	
1.7 St Jude Children's Research Hospital, USA	
Risk group	
Standard	Isolated extramedullary
	B-lineage late marrow or combined and end-induction MRD <0.01%
High	Any T-lineage
	B-lineage early marrow or combined
	Any standard-risk with end-induction MRD <0.01%
Early: <6 months from completion of frontline therapy; Late ≥6 months from completion of frontline therapy	

S standard risk, *I* intermediate risk, *H* high risk

frontline therapy while late relapse as 6 months or more from completion of front-line therapy.

During this time an increasing number of patients were also being transplanted, initially with matched related and then with alternative donors [84]. However the benefit of this procedure in relapsed ALL remained unclear. What became apparent was that second remission was short lived in a number of patients and a second relapse often occurred prior to SCT [57, 66]. Nevertheless evidence suggested a benefit of BMT, particularly for early bone marrow relapse [27, 54, 72] as these patients invariably had a second relapse if not transplanted. In late bone marrow relapsing patients, outcomes of those transplanted versus those treated with chemo with or without radiotherapy appeared to be comparable. But the relapse risk was higher in the latter group, suggesting a sub-group could benefit from SCT if transplant related mortality improved. Improvements in transplantation techniques have realized this improvement as well extending the donor pool to alternative donors [29, 72, 82].

Treatment of CNS relapse remains challenging as the combination of local therapy with systemic therapy inevitably leads to long-term sequelae. The dilemma in local therapy is choosing cranial radiotherapy alone or craniospinal irradiation. In the earlier trial by POG in 1985, craniospinal irradiation conferred a lower subsequent CNS relapse rate of 25% compared to cranial irradiation alone with a subsequent CNS relapse rate of 55% [85]. In POG trial 8304, early cranial irradiation at 24 Gy was administered after remission therapy, followed by post remission therapy. Triple intrathecal therapy was given weekly during induction and monthly for remaining of treatment. Although the 5-year overall event-free survival was 42%, the observed rate of leukoencephaloapathy is 17% with substantial acute and chronic neurotoxicity [86].

Table 11.4 Comparison of HSCT versus chemotherapy for childhood ALL in second remission

Year/population	Treatment group	No of patients	EFS, %	*p*-value
Outcome of HSCT versus chemotherapy on all relapses				
NA	Chemotherapy	21	5	0.002 [65]
	HSCT	24	38	
1980–1984	Chemotherapy	40	22	0.39 [66]
	HSCT with MSD	13	35	
NA	Chemotherapy	600	0–23	NA [27]
	HSCT with MSD	168	36	
NA	Chemotherapy	40	9	<0.005 [67]
	HSCT with HLA-matched donor	21	47	
NA	Chemotherapy	280	31	NA [68]
	HSCT with MSD[b]	51	52	
1980–1989	Chemotherapy	230	22	0.006 [69]
	HSCT	57	41	
1983–1993	Chemotherapy	134	47	NA [70]
	HSCT with MSD	17	36	
1980–1987	Chemotherapy	37	26	0.03 [71]
	HSCT with MSD	38	62	
1991–1995	Chemotherapy	125	39	NS [72]
	HSCT with MSD	104	45	
Outcome of HSCT versus chemotherapy in patients with high-risk relapsed ALL in second remission				
1985–1987 166 first relapse <6M off therapy	Chemotherapy	115	22	<0.01 [62]
	HSCT with MSD	51	53	
1983–1991 358 first relapse after remission <36M	Chemotherapy	179	10	<0.001 [73]
	HSCT with MSD	179	35	
1981–1991 189 first BM relapse after remission <6M off therapy	Chemotherapy	126	15	<0.01 [74]
	HSCT with MSD	63	35	
1992–2000 171 first relapse after remission <30M	Chemotherapy	142	16	0.002 [75]
	HSCT with MSD	29	33	
1991–2001 108 first BM relapse after remission after 6M off therapy	Chemotherapy	53	0	<0.001 [76]
	HSCT with URD	53	44	
1991–1997 202 first relapse after remission <36M	Chemotherapy	110	23	<0.001 [77]
	HSCT with MSD	92	41	
1995–1998 72 first BM relapse <51M in boys and <39M in girls from diagnosis	Chemotherapy	35	20	NS [57]
	HSCT with URD	37	21	

Table 11.4 (continued)

Year/population	Treatment group	No of patients	EFS, %	*p*-value
1990–1995 160 first BM relapse <6M after off therapy and all T-ALL BM re	Chemotherapy	76	76	<0.005 [54]
	HSCT with MSD and URD	84	84	
Outcome of HSCT versus chemotherapy in patients with intermediate-risk relapsed ALL in second remission				
1983–1991 152 relapse >36M from Dx	Chemotherapy	76	32.0	<0.001 [73]
	HSCT with MSD	76	53.0	
1985–1987 216 relapse >6M off therapy	Chemotherapy	165	41	NS [62]
	HSCT with MSD	51	52	
1992–2000 116 relapse >6M of Dx	Chemotherapy	88	39.6	NS [75]
	HSCT with MSD	28	54.7	
1991–1997 149 relapse >36M from Dx	Chemotherapy	78	66	NS [77]
	HSCT with MSD	75 (61 TBI; 14 non-TBI)	TBI: 63 Non-TBI: 32	
1990–1995 48 relapse >6M off Dx and IEM	Chemotherapy	33	46	NS [54]
	HSCT with matched donor	25	52	
2002–2009 203 with intermediate risk relapse according to ALL-REZ BFM 2002	Chemotherapy	100	MRD $<10^{-3}$: 66	NA [78]
			MRD $>10^{-3}$: 24	
	HSCT	103	MRD $<10^{-3}$: 80	
			MRD $>10^{-3}$: 64[a]	

HSCT haematopoietic stem cell transplantation, *CR* complete remission, *DFS* disease-free survival, *EFS* event-free survival, *MSD* Matched sibling donor, *USD* unrelated donors, *BM* bone marrow, *CNS* central nervous system, *IMR* Isolated marrow relapse, *CB* cord blood, *Cy* cyclophosphamide, *MSD* matched sibling donor, *NA* not available, *TBI* total body irradiation, *UR* unrelated donor, *Dx* diagnosis, *M* month, *NA* not available, *NS* not significant

[a]EFS of all MRD poor patients

[b]2 patients had one antigen mismatched graft

POG trials have demonstrated successful outcome with EFS of 70–80% using strategy of delaying cranial irradiation for 6–12 months to allow initial intensification of systemic chemotherapy [87, 88]. In POG 9061, craniospinal irradiation with 24Gy cranial and 15Gy spinal was delayed to allow administration of intensive systemic chemotherapy. These patients were given 18 months of maintenance therapy following craniospinal irradiation. The 4-year EFS of 83 patients with first isolated CNS relapse in this trial was 71% with acceptable toxicity [89]. In subsequent COG trials, patients with first isolated CNS relapse occurring at least 18 months from first remission received only 18Gy cranial irradiation at 12 months of treatment. Excellent outcomes of 4-year EFS of 77% were achieved in these patients [54].

Table 11.5 Transplant outcome in relapsed childhood acute lymphoblastic leukaemia

Year	No of patients	Median Age At HSCT (range)	Time/site of relapse/Risk group	Relapsed treatment protocol	Donor and stem cell source	Conditioning	5-year Overall survival, %	DFS/EFS, %	Learning points
NA	51 in CR2	100 months (26–214)	34 IMR	12 ALL-REZ-BFM 83	51 MSD	27 TBI/Cy	NA	Overall: 52	Significant advantage between chemotherapy and BMT for early relapses over chemotherapy [68]
			7 BM +CNS	17 ALL-REZ-BFM 85	(2 with one antigen mismatched)	23 TBI/VP-16		Early: 56	
			10 others	20 ALL-REZ-BFM 87 2 ALL-REZ-BFM 90		1 TBI/Cy/Ara-C		Late: 47	
1990–2007	87 in CR2	MSD: 4.6 years	NA	NA	32 MSD	53 TBI/Cy	NA	MSD: 41	Similar outcomes in MSD recipients versus recipients of unrelated marrow and CB
	9 T-ALL	MUD: 3.4 years			18 MUD	37 TBI/Cy/VP-16		MUD: 57	Outcome was better in children with CR1≥3 years (56% versus 33%, $p=0.02$)
		MMUD: 5.4 years			16 MMUD	3 TBI/Cy/Flu		MMUD: 19	5-years EFS in patients with T-ALL (n=9) was 0% compared to 46% in patients with B-ALL ($p=0.04$) [79]
		CB: 2.2 years			21 CB			CB: 43	

1990–2007	395 in CR2 36 T-ALL	8 years [2–20]	BFM risk S1: 9 S2: 167 S3: 40 S4: 69 Unknown: 110	AIEOP LAL	MFD: 199 UD: 196	357 TBI-based 36 Chemotherapy 2 unknown	All: 57 Children: 57 Adolescents: 55	All: 54 Children: 54 Adolescents: 54	The outcome for adolescents in CR2 had no difference compared to children in CR2 (<14 years old) [80]
1984–2009	83 in CR2	1984-1992: 117 months (9-235) 1992–2002: 97 months (10-206) 2001-2009: 108 (17-221)	NA	NA	NA	15 TBI-based 26 chemotherapy 31 TBI-based 17 chemotherapy 45 TBI-based 2 chemotherapy	35.7 46.8 78.9	49.6 56.6 68.2	EFS and OS have improved significant over the past three decades [81]
2003–2011	411 CR1: 209 CR2: 172 >CR2: 29	10.1 years (0.8–18.5)	IMR: 162 IEM: 9 Combined 31	BFM or COALL trial protocol	MSD: 105 MUD: 306	> 2 years old: TBI/VP-16 < 2 years old: Bu/Cy/VP-16	<u>All patients</u> MSD: 79 MUD: 73 <u>CR2 and CR3</u> MSD: 78 MUD: 67	<u>All patients</u> MSD: 71 MUD: 67 <u>CR2 and CR3</u> MSD: 62 MUD: 62	4-years EFS was similar in recipients of MSD and MUD ($p=0.405$) [82]

(continued)

Table 11.5 (continued)

Year	No of patients	Median Age At HSCT (range)	Time/site of relapse/ Risk group	Relapsed treatment protocol	Donor and stem cell source	Conditioning	5-year Overall survival, %	DFS/EFS, %	Learning points
2003–2011	113	NA	IMR: 90	BFM 96 or 2002 protocol	MSD: 24	>2 years old: TBI/VP-16	NA	All: 55	Marrow MRD was assessed on Day +30, +60, +90, +180 and +365
	CR2: 94 >CR2: 16 Non-CR:3 T-ALL: 17		IEM: 5 Combined: 18		MUD: 68 MMD: 21	<2 years old: Bu/Cy/VP-16		CR2: 60 >CR2: 50 Non-CR: 0	The discriminatory power of MRD detection to predict the probability of relapse after 1, 3, 6 and 9 months was more than 96%, more than 87%, more than 71% and more than 61%, respectively [83]

AIEOP Associazione Italiana di Ematologia ed Oncologia Pediatrica, *BFM* Berlin-Frankfurt-Münster ALL Study Group, *COALL* Cooperative ALL Study Group, *BM* Bone marrow, *CNS* central nervous system, *IEM* Isolated extramedullary relapse, *IMR* Isolated marrow relapse, *CB* Cord blood, *MSD* matched sibling donor, *MMUD* mismatched unrelated donor, *MUD* matched unrelated donor, *Ara-C* cytarabine, *Bu* Busulfan, *Cy* cyclophosphamide, *Flu* fludarabine, *TBI* Total body irradiation, *VP-16* Etoposide, *CR* complere remission, *DFS* disease-free survival, *EFS* event-free survival, *MRD* minimal residual disease, *OS* overall survival

Current strategy for treatment of CNS relapse consists of intensive block of reinduction and maintenance therapy for 12–24 months. Cranial irradiation is usually delayed for about 1 year. The role of cranial and craniospinal irradiation remains unclear, though the latter leads to retarded spinal growth and myelotoxicity and the former is associated with secondary malignancies [90, 91]. As there is little evidence of graft-versus-leukaemia in CNS leukaemia, the role HSCT in isolated CNS relapse is unclear. In COG 1990–2000 trials, Cooprall-97 and Japanese study groups, there were no significant difference in survival in patients treated with chemoradiotherapy alone or HSCT [92–94].

11.4 Outcomes of Relapse in the MRD Era

The previous section illustrates the different outcomes of BCP-ALL patients who relapse on and off therapy and the irrespective poor outcomes of those with T-cell marrow relapses. Evidence across a number of clinical trials suggested that late isolated extramedullary disease was curable with a more intensive chemotherapy regimen, radiotherapy or in some cases orchidectomy. The increasing availability of matched unrelated donors led to an increase in patients with bone marrow relapses receiving an allo-SCT. As the use of MRD became routine for monitoring the therapeutic response a number of groups reported poor outcomes in patients with MRD levels of $\geq 10^{-3}$ prior to allo-SCT.

11.4.1 MRD in BCP-ALL Late Bone Marrow Relapses

As shown in Fig. 11.4, the largest group of relapsed patients are the BCP-ALL patients with a late bone marrow relapse. In a retrospective analysis, the BFM group showed that in this group, treated on the ALL REZ BFM 90, 95 and 96 protocols, patients with a MRD of $<10^{-3}$ (by Ig/TCR PCR) at day 36 had a 86% (95% CI, 77–95) survival compared with 0% in those who had a higher MRD level. None of these patients had received an allo-SCT [95]. The results of the COG AALL01P2 trial were less discriminatory. In patients with late relapse the 12-month EFS was 86% in MRD $<10^{-3}$ versus 77% in the higher MRD group, though here some patients did receive a CR2 transplant. These reports implied that in late bone marrow relapses, MRD estimation at the end of induction identified patients who may not require an allo-SCT to consolidate CR2 [59].

Both the BFM and CCLG (UK) clinical trials in relapsed ALL, REZ 2002 and UKALL R3 stratified these groups by MRD for allo-SCT with a matched donor (sibling or alternative). Both groups reported comparable outcomes in late bone marrow relapses where MRD positive patients were eligible for allo-SCT and MRD negative patients continued with chemotherapy. In the REZ 2002 patients with bone marrow involvement and MRD $<10^{-3}$ after ALL-REZ BFM F1/2 induction therapy achieved EFS (8 years) of 70%; SE, ± 0.05 whereas those MRD $\geq 10^{-3}$ had EFS rates of 64% SE, ± 0.05 [13].

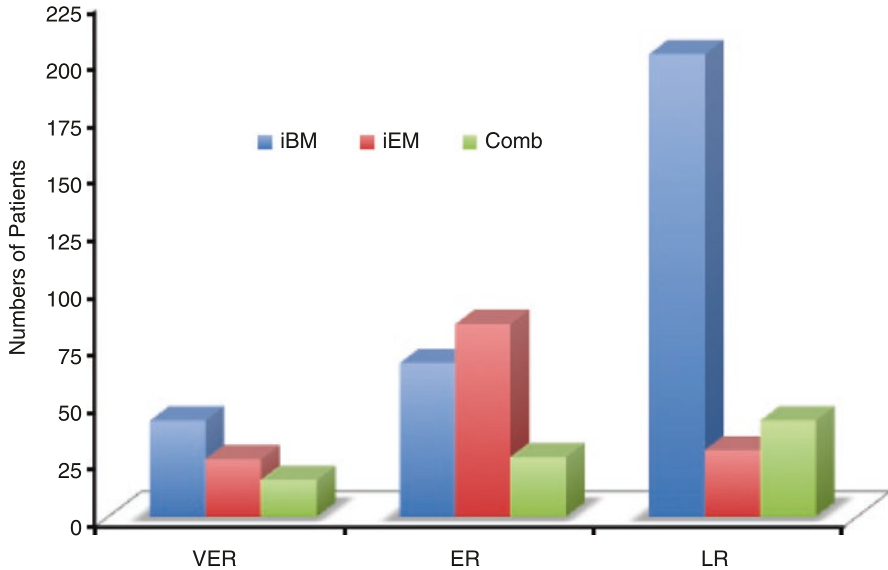

Fig. 11.4 Patterns of relapse in childhood ALL. *iBM* isolated bone marrow, *iEM* isolated extramedullary, *Comb* combined. *VER* very early relapse within 18 months of first diagnosis, *ER* early relapse more than 18 months from diagnosis but within 6 months of stopping therapy, *LR* late relapse 6 months or more after stopping therapy (Personal communication, Dr Catriona Parker)

In UKALL ALLR3 the induction MRD cut-off was set at a level of $\geq 10^{-4}$. Results similar to the BFM were observed. The different end of induction MRD cut off points in R3 and BFM groups have been analysed and outcomes compared. MRD levels achieved after induction with the ALL-R3 mitoxantrone arm were lower when compared to ALL-REZ BFM 2002. This is most likely an effect of the intensive use of anthracycline during induction in ALLR3 and emphasises the important of prior therapy on MRD levels which should be taken into account when comparing across protocols [53].

In the current IntReALL clinical trial (www.intreall-fp7.eu; NCT01802814) randomising between the UK and BFM approaches, the MRD levels have been retained according to the protocol used to stratify for allo-SCT. In this group, targeted therapy is being used to examine whether this will further decrease post induction levels and lead to better outcomes.

11.4.2 MRD in BCP-ALL Early Bone Marrow Relapses

Results in this group of patient have been uniformly poor across all groups with overall survival at around 25% or lower [50, 51, 54, 57, 96, 97]. All such patients are eligible for allo-SCT with any donor. CR2 rates post induction are

lower in this group, at around 70%. A significant proportion of those who attain remission relapse prior to SCT and of those transplanted, around 25–30% will relapse after allo-SCT. Thus overall outcomes in this group have remained poor over the last three-decades. Though MRD is measured post induction in both UKALLR3 and REZ2002, unlike for late bone marrow relapse there is no further stratification of therapy in this group. In the REZ2002 study, around a third of such patients attained MRD levels of $<10^{-3}$ after induction with an EFS of 60% ± 8% compared to 31% ± 6% for those with MRD $\geq 10^{-3}$ [12]. In UKALL R3, a similar proportion of patients achieved CR rates of $<10^{-4}$ at the end of induction and PFS of 63% (95% CI 50, 75), compared to 21% (95% CI 15, 27) for patients with MRD$\geq 10^{-4}$. Thus even in this group, MRD continues to identify a group of patients with a relatively favourable outcome after allo-SCT. The BFM and CCLG are engaged in a comparative analysis to examine if this favourable group can be identified early on to avoid exposure to unnecessary toxicity.

11.4.3 T-Cell Bone Marrow Relapses

T-cell bone marrow relapse at any time, whether on or off therapy continue to have a poor outcome and are classified as HR by the IntReALL group. In UK ALL R3, the addition of clofarabine to induction resulted in some improvement in remission rates but with much greater toxicity that offset any benefit of the change. In the US the TACL group have investigated the use of nelarabine, etoposide and cyclophosphamide in relapsed T-ALL and the results are awaited.

11.4.4 Extramedullary Relapses

Late isolated extramedullary relapses are rare (Fig. 11.4) as the efficacy of frontline protocols have improved. For early and very early isolated extramedullary relapses, UKALL R3 chose to offer allo-SCT with a matched donor. Results improved, with allo-SCT offering a better curative approach over targeted CNS directed therapy for very early and early combined or isolated CNS relapses. In those with early and very early – isolated CNS disease, 71% of those allocated allo-SCT but not transplanted (receiving chemoradiotherapy) suffered a second relapse, while recurrence rates were 21% in those transplanted. Nevertheless, the optimal strategy for this group remains unclear. In IntReALL, combined early and very early isolated and combined extramedullary relapses are eligible for an allo-SCT with a matched donor. The few patients with combined relapse follow the strategy for late bone marrow relapse. Where MRD is non-informative, patients are also eligible for an allo-SCT.

11.5 The Role of Allogeneic Hematopoietic Stem Cell Transplantation

As previously stated, allo-SCT appears to benefit BCP-ALL late bone marrow relapses with persistent MRD post induction. If MRD cannot be quantified after induction, most groups recommend that patients with late BM relapse are eligible for matched donor (MD)-SCT but not for mismatched donor (MMD)-SCT and patients with early combined BM relapse are eligible for both, MD and MMD-SCT. In all other categories of relapse, the evidence for allo-SCT remains at best equivocal (Table 11.5), though a matched pair analysis suggested that allo-SCT benefits high risk relapse [76].

11.5.1 When to Transplant

The timing of transplant in relapsed ALL is usually after induction and consolidation therapy. This allows time for donor work up and further reduction in disease burden. The MRD level prior to allo-SCT is highly predictive. Studies suggest that MRD level of $<10^{-3}$ is associated with better post allo-SCT remission rates [98, 99]. Higher levels are associated with an increased risk of disease recurrence and these patients may benefit from further attempts to reduce disease burden. The use of targeted therapies is attractive in this setting and may lead to refinement in future treatment strategies in allo-SCT.

With the addition of T-cell depletion, matched unrelated donor (MUD) allo-SCT has been shown to consistently have outcomes comparable to matched sibling allografts [82, 84, 100]. The development of cord blood banks has further extended the availability of donor stem cells. A number of studies have now shown comparable outcomes in children with ALL allografted with either allele-matched related/unrelated donors or matched or 1–2 HLA mismatched cord blood sources [29, 101–103]. A major limitation of cord blood is the number of haematopoietic cells available.

Haploidentical SCT from a mismatched family member offers yet further options to those patients who lack an HLA matched family, unrelated or cord blood donor. The main limitations of haploidentical transplants are graft rejection, delayed immune reconstitution, GVHD and susceptibility to infections [104, 105]. Removing T cells from the graft can reduce the risk of GVHD but will also reduce the graft versus leukaemia effect of the graft and thus increase the likelihood of relapse. The antileukemic effect can be potentiated by selection of a HLA-disparate natural killer (NK) cell alloreactive donor [106]. This strategy is currently being used in children with ALL who lack a suitable HLA matched donor [107–109]. In this setting allo-reactive donor-derived NK cells are thought to promote engraftment, reduce GVHD, and decrease leukemic relapse. Subsequently, modified donor selection models

based on the observation that donor NK cells expressing a certain inhibitory KIR would be alloreactive if the respective ligands (C1, C2, or Bw4) in the patient are missing have been described. This model [110] has demonstrated better clinical outcome in haploidentical HSCT for paediatric ALL. These approaches are complex and expensive. An alternative approach pioneered in Baltimore [111] uses a post-transplant combination of cyclophosphamide, MMF and tacrolimus in conjunction with non-myeloablative transplantation either BM or G-CSF-mobilized PBSC grafts and has been reported to produce comparable results [112]. This approach may offer a cheaper more practical approach to haploidentical allograft in resource-limited settings where access to a matched donor is either absent or prohibitively expensive.

11.5.2 Conditioning Therapy

Conditioning therapy is designed to provide a combination of myeloablation and immune suppression. Total body irradiation (TBI) based conditioning regimens have previously been shown to be superior to radiation based conditioning. However TBI based regimens cause considerable mortality and morbidity due to toxicity, delayed immune reconstitution and infection. Changing conditioning strategies from full intensity myeloablative regimens to reduced intensity and reduced toxicity chemotherapy regimens may maximise graft versus leukaemia effect needed to improve DFS for leukaemia and also reduce mortality and morbidity from toxicity by promoting engraftment and rapid immune reconstitution. Fludarabine and treosulfan based conditioning protocols, which are used in many non-malignant transplant indications are well tolerated and are now starting to be used more often in relapsed malignancy, especially when children are deemed to be too unwell to tolerate full myeloablative conditioning regimens. Whether a change in strategy will result in similar or improved outcomes remains to be seen but is currently being tested in the FORUM ALL SCTped study (NCT02670564). This study is the first trial of its kind to directly address the question of whether radiotherapy is required to cure ALL and will directly compare TBI with non TBI based conditioning in children with relapsed ALL.

11.5.3 Novel Approaches

The development of targeted and cellular therapies described in Chaps 8, 9 and 10 offer the opportunity to improve outcome in relapsed ALL. Such agents have been investigated either as a single agent therapy or by integration of targeted agents into established chemotherapy platforms. Table 11.6 provides a summary of novel drugs that have been tested in relapse or refractory ALL.

Table 11.6 Emerging novel therapy for treatment of relapsed childhood ALL

Category	Drug name	Drug properties	Clinical trials in children with relapsed/refractory ALL		
			Study design	Patients	Results
Nucleoside analogs	Clofarabine	Second generation purine analog	Phase I study: clofarabine [113]	25 children	Response rate: 32%
		Inhibit DNA synthesis/repair and induce cell death		ALL: 17; AML:8	CR: 5; PR: 3
			Phase II study: clofarabine [114]	61 children with R/R ALL	Response rate: 30%
					CR: 7; CRp: 5; PR: 6
			clofarabine/Cy/VP-16 [115]	25 children with ALL	Response rate 56%
				Refractory: 17; Multiple relapse: 8	CR: 13 (52%); CRp: 1 (4%)
			Phase I study: clofarabine/Cy/VP-16 [116]	25 children	Response rate 64%
				ALL: 20; AML 5	CR: 10 (ALL: 9; AML 1); CRp: 6 (ALL:2; AML:4)
			Phase II study: clofarabine/Cy/VP-16 [117]	25 children with R/R ALL	Response rate: 64%
				B-ALL: 21; T-ALL: 1; Unknown: 3	CR: 11 (44%); PR: 3 (12%)
			5 had clofarabine alone [118]	23 children with R/R ALL	Response rate: 61%
			18 had clofarabine/Cy/VP-16	B-ALL: 18; T-ALL: 4; Biphenotypic: 1	CR: 12 (52%); PR: 2 (9%)
			clofarabine/Cy/VP-16 [119]	11 children with R/R ALL	Response rate: 64%
					CR: 3; PR: 3
			Phase II study: clofarabine + Topotecan/Vinorelbine/Thiotepa [120]	17 children	Response rate: 69%
				AML: 12; ALL 4; Biphenotypic: 1	AML: 8 (67%); ALL: 2 (67%); Biphenotypic: 1 (100%)
			clofarabine + cytarabine [121]	21 children with R/R ALL	Response rate: 19%
				B-ALL: 15; T-ALL: 1; Unspecified: 5	CR: 3 (14%); PR 1 (5%)
			clofarabine/Cy/VP-16 [122]	13 children with R/R ALL	Response rate: 69%
				B-ALL: 9; T-ALL: 4	CR: 7 (54%); PR: 2 (15%)
			Phase 1 study: Clofarabine + mitoxantrone/VP-16/ASP/DXM [123]	20 children with very early/second/post-transplant relapsed ALL	CR: 11 (57.9%)

	Nelarabine	An inhibitor of purine nucleotide phospharylase	Phase I study: Nelarabine [124]	93 patients with R/R haematological malignancy	Response rate: 31%
				59 adults; 34 children	T-ALL: CR 23%; PR 31% B-ALL: CR 0; PR 20%
			Phase II study: Nelarabine [125]	121 children and young adult with refractory ALL	Overall response (CR + PR): 33% T-ALL, first relapse: CR 45% T-ALL, second relapse: CR 22% CNS relapse: CR 19%
			Nelarabine/VP-16/Cy [126]	7 children with R/R T-ALL/ lymphoma	CR after 1 course: 4 CR after 2 courses: 1
Monoclonal antibodies	Epratuzumab	Anti-CD22 monoclonal antibody	Epratuzumab in combination with chemotherapy [127]	15 children first/late CD22-positive ALL marrow relapse	Response rate: 67%
			Phase II study: Epratuzumab combined with re-induction chemotherapy [128]	114 children with early marrow relapsed B-ALL	Response rate: 66%
	Inotozumab	Anti-CD22 monoclonal antibody bound to calicheamycin	Inotozumab [129]	90 patients (age range 4–84 years)	Response rate: 58%
					CR: 17 (19%); CRp 27 (30%); Marrow CR: 8 (9%)
			Monotherapy with Inotozumab followed by HSCT [130]	26 patients: 24 adults; 2 children	Response rate: 18 in 20 evaluable patients (90%)
					EFS: 6 months: 30%; 1 year: 22%
			Monotherapy with Inotozumab with escalating doses [131]	5 children with relapsed B-ALL	Response rate: 60%
	Blinatu-momab	Anti-CD19 bi-specific T cell engagers	5-6 weeks of continuous intravenous infusion [132]	3 children with post-transplant relapsed B-ALL (1 had Ph+ ALL)	All CR after 4 weeks of therapy
			4 weeks of continuous intravenous infusion [133]	9 children with post transplant relapsed B-ALL	Response rate: 67% 6 had M1 marrow; 5 had MRD negative

(continued)

Table 11.6 (continued)

Category	Drug name	Drug properties	Clinical trials in children with relapsed/refractory ALL		
			Study design	Patients	Results
Proteasome inhibitor	Bortezomib	Induces apoptosis in tumour cells via the intrinsic mitochondrial pathway, the extrinsic death-receptor pathway and the endoplasmic reticulum stress response pathway	Phase I study: Bortezomib with 4-drug induction (VCR, DXM, ASP and doxorubicin) [134]	10 children with relapsed ALL First relapse: 5; Second relapse: 5 B-ALL: 9; T-ALL: 1	Response rate: 67% Marrow CR: 7/9 (78%) Marrow CR with persistent CNS leukaemia: 1
			Phase II study: Bortezomib with 4-drug induction (VCR, DXM, ASP and doxorubicin) [135]	22 children with R/R ALL B-ALL: 20; T-ALL: 2	Response rate: 73% CR: 14; CR without platelet recovery: 2 B-ALL: 16/20 (80%); T-ALL: no response
Target molecules	Quizartinib	FLT3 inhibitor	Phase I study: Quizartinib with 5D of cytarabine/VP-16 [136]	NA	17 evaluable patients: CR: 2; CRp: 1; SD: 10; PD: 3
New formulas of existing agents	Liposomal cytarabine	A sustained-release formula of cytarabine encapsulated into multivesicular lipid-based particles	IT liposomal cytarabine on Day 1 after diagnosis of CNS R/R followed by every 15 days [137]	30 children with CNS R/R disease ALL: 22; AML: 3; NHL: 2	25 (83%) achieved CNS cytological CR

ALL acute lymphoblastic leukaemia, *AML* acute myeloid leukaemia, *R/R* Relapsed/refractory, *CR* complete remission, *CRp* complete remission with platelet recovery, *PD* progressive disease, *PR* partial remission, *SD* stable disease, *ASP* Asparaginase, *Cy* Cyclophosphamide, *DXM* Dexamethasone, *VCR* Vincristine, *VP-16* Etoposide, *D* days, *HSCT* Haematopoietic stem cell transplantation, *IVI* intravenous infusion, *IT* intrathecal

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Chapter 12
Medical Supportive Care for Treatment-Related Toxicity in Childhood ALL

Etan Orgel and Deepa Bhojwani

12.1 Introduction

As discussed elsewhere in this textbook, increased treatment intensity over the past decades for childhood acute lymphoblastic leukemia (ALL) has improved outcomes and 90% of children with newly diagnosed with ALL are now expected to become long-term survivors [1–3]. Concurrently, the burden of intensified therapeutic regimens has become evident; treatment-related toxicity (TRT) not only leads to long-term morbidity and late effects in survivors of childhood ALL [4], but also compromises optimal delivery of otherwise curative chemotherapy [5–9]. Early efforts to reduce TRT focused on refinements in risk-stratification to better identify groups that may benefit from high morbidity treatment modalities. This approach led to significant mitigation in the use of cranial radiation and anthracyclines with reduction of late mortality [10]. However, the prevalence of TRT during therapy has nevertheless remained relatively constant. Despite an increased focus on medical supportive care, severe grade 3 or 4 toxicity is reported in ~40–75% of those treated on recent ALL consortia trials [11–13]. Non-disease related treatment related mortality (TRM) continues to be problematic on current regimens, affecting 1–3% of children treated for pediatric ALL irrespective of the specific regimen or consortia [6, 14–17]. While relapse of disease remains the number one cause of death in children with ALL, improvement in disease response has reduced the number of relapse-related deaths. As a result, the relatively constant rate for TRM over the years now constitutes an increasing proportion of mortality; approximately one in five deaths on therapy are now due to TRM rather than the disease itself [1]. The highest risk for TRM occurs within the initial Induction phase, with up to 50% of non-disease

E. Orgel • D. Bhojwani (✉)
Childrens Hospital Los Angeles, Los Angeles, CA, USA
e-mail: dbhojwani@chla.usc.edu

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related deaths occurring within those first 28 days [6, 16]. The Induction phase of ALL chemotherapy is therefore not only critical for obtaining a remission for eventual cure [18], but also the period of greatest risk for a life-threatening event. In the following chapter, we provide an overview of the key toxicities that occur during ALL treatment with an emphasis on the current states of the science and future directions. The overall goal of this chapter is to provide insight into common supportive care issues impacting therapy and to aid the clinician in providing anticipatory supportive care and prompt recognition and intervention for serious toxicity.

12.2 Hyperleukocytosis

Approximately 15% of patients with ALL present with hyperleukocytosis ($>100\times10^9$/L) at initial diagnosis. The incidence is higher in patients with Philadelphia-chromosome positive , MLL rearranged and T- cell ALL [19, 20]. The resulting hyperviscosity can cause pulmonary leukostasis requiring mechanical ventilation, neurologic complications (e.g. intracranial hemorrhage, seizure and confusion), thrombosis, bleeding, and acute kidney injury. These manifestations are most evident in patients with leukocyte counts $>400\times10^9$/L [21]. The role of leukapheresis in the management of hyperleukocytosis of ALL is not well defined. Recent studies show that enhanced supportive care and careful initiation of cytoreductive, low-dose chemotherapy may mitigate the need for more invasive leukapheresis [20, 22, 23]. Maintaining euvolemia with adequate hydration prior to the initiation of chemotherapy and close monitoring of respiratory and neurologic status (especially in the peri-anesthesia period) are other measures to decrease morbidity and mortality [24]. In patients with hyperleukocytosis, it remains imperative to transfuse platelets to mitigate the risk of bleeding, but to avoid packed red cell transfusions as increased blood viscosity can increase risk for intracranial hemorrhage [20, 25].

12.3 Tumor Lysis Syndrome

Though hyperleukocytosis and other indicators of high tumor burden such as hepatosplenomegaly and elevated LDH are risk factors for tumor lysis, all children with ALL can be considered to have bulky disease as the volume of the bone marrow is approximately 20 ml/kg of body weight [26]. Spontaneous or chemotherapy-induced blast cell lysis and massive release of intracellular metabolites are responsible for the clinical and laboratory consequences of TLS [27]. The Cairo-Bishop definition of laboratory TLS requires elevation of two or more values of serum uric acid, potassium, phosphate or decrease in serum calcium (the latter may fall secondary to hyperphosphatemia) at presentation or a change in these values by 25% in the period of 3 days prior to 7 days post initiation of chemotherapy [28]. Clinical TLS

is defined as renal insufficiency, cardiac arrhythmia or seizure in addition to one or more laboratory parameters.

Close monitoring and prophylactic measures are essential to minimize adverse outcomes of TLS. These include aggressive hydration to maintain urine output of 80–100 ml/m^2/h, judicious use of diuretics (only in the absence of hypovolemia and obstructive uropathy), and rapid intervention to normalize electrolyte imbalance (e.g. hyperkalemia, hyperphosphatemia and hyperuricemia). Though alkalinizing of urine increases the solubility of uric acid, it enhances calcium- phosphate precipitation in the kidneys, and is no longer recommended, particularly in the presence of hyperphosphatemia.

Allopurinol inhibits the formation of uric acid by blocking xanthine oxidase, however the accumulated precursors of uric acid (hypoxanthine and xanthine) can also precipitate in the renal tubules compromising renal function further. Rasburicase (recombinant urate oxidase) is highly effective in converting uric acid to allantoin, a highly soluble compound readily excreted in the urine. The use of rasburicase has significantly decreased the need for dialysis in patients with TLS and hyperuricemia [29].

12.4 Infectious Morbidity and Mortality

Serious infectious complications during ALL therapy are common, with children at risk for all three major categories – bacterial, fungal, and viral. As discussed above, an increasing proportion of deaths in ALL are now due to TRM and not disease; of these, infectious mortality is the single greatest contributor to TRM. UKALL2003 is the largest trial to date to provide detailed descriptions of infectious morbidity and mortality; infectious mortality contributed to a third of all study deaths on the trial and caused nearly two-thirds of all TRM. Bacterial infections were the most prevalent (68%) followed by fungal (20%) and viral infections (12%) [30]. A 20 year history of infections on the ALL Total Therapy Studies (XI, XII, XIIIA, XIIIB, XIV) conducted at St Jude Research Hospital (SJCRH) found close to 80% of TRM was due to infection, with a similar preponderance of bacteremia [16]. While detailed infectious data is not typically included in reporting of primary outcomes for many ALL regimens, surveying retrospective cohort studies across multiple consortia reinforces the high prevalence of infectious mortality (Table 12.1). A clear trend is evident on examination of these reports from two decades of ALL therapy. While the overall incidence of TRM generally remains less than 5%, infection remains a major contributor to TRM for patients irrespective of treatment era.

Both host and regimen factors contribute to infectious morbidity and mortality. Lymphoid-directed therapy for treating ALL is inherently immunosuppressive [31] and the addition of myelosuppresive chemotherapies introduces additional at-risk periods for severe infection [32]. Despite therapeutic backbones incorporating many of the same chemotherapy agents, the timing and specific combination of agents for pediatric ALL has been associated with marked variation in rates of treatment-associated infection. Even substitutions of medicines within the same class can have

Table 12.1 Infectious mortality on frontline ALL consortia trials

Consortia (location)	Regimen	Years	Study population[a]	TRM[b] (%)	IRM/TRM[c] (%)	Reference
Medical Research Counsel (United Kingdom)	UK ALL 2003	2003–2011	SR-ALL, HR-ALL, T-ALL	3.7	64	O'Connor et al. [30]
St Jude Children's Research Hospital (United States)	ALL Total Therapy XI, XII, XIIIA, XIIIB, XIV	1984–1999	Infant ALL SR-ALL, HR-ALL, T-ALL	3.6	56	Rubnitz et al. [16, 63]
Children's Oncology Group (United States, Canada, Australia, New Zealand, Europe)	AALL0232	1996–2002	HR-ALL	3.3	58[d, e]	Larsen et al. [6, 41]
Dana Farber Cancer Institute (United States, Canada)	95-01	1996–2000	Infant ALL SR-ALL, HR-ALL, T-ALL	1.2	86[e]	Moghrabi et al. [170]
Nordic Society of Pediatric Haematology and Oncology (Norway, Sweden, Denmark, Finland, Iceland)	ALL-92 and ALL-2000	1992–2008	SR-ALL, HR-ALL, T-ALL	2.7	76[e]	Lund et al. [14]
Dutch Childhood Oncology Group (Netherlands)	ALL-6, ALL-7, ALL-8	1984–1996	Infant ALL SR-ALL, HR-ALL, T-ALL	1.7	53[e]	Slats et al. [17]
International Berlin-Frankfurt-Munster Study Group (International consortium)	ALL IC-BFM 2002	2002–2007	Infant ALL, SR-ALL, HR-ALL, T-ALL	7.2	60[e]	Stary et al. [13]

[a]B-ALL risk group as per NCI/Rome criteria
[b]*TRM* treatment-related mortality; defined as deaths following start of therapy not due to disease progression or relapse
[c]*IRM* Infection related mortality; defined as percent treatment related deaths due to infection
[d]Post-remission only
[e]Calculated from reported data

serious unintended consequences. For instance, glucocorticoids are an integral component for induction chemotherapy, but the combination of glucocorticoid selection, dose, and duration has contributed to significant infectious mortality in some trials [33–36] but not others [6, 30]. Similarly, different permutations even within chemotherapy class can dramatically affect infectious mortality, as recently found in COG AALL01P2 for relapsed ALL and COG AALL0631 for infant ALL. Both studies were substantially amended for "unacceptable" toxicity [37, 38]. This concept of "unacceptable toxicity" from infection is a challenging concept that incorporates subjective balancing of risk and reward in the context of disease prognosis. For instance, while the recent frontline ALL trial within the COG closed one experimental arm for "unacceptable" infectious toxicity [39], modified versions of UK ALL R3 for relapsed ALL have been widely adopted as best therapy with a "manageable" severe infection rate exceeding 90% [40]. In addition to regimen considerations, certain host factors contribute to infectious mortality. Some are not amenable to intervention, such as the vulnerability of infants and adolescents [38, 41] or the genetic susceptibility associated with Down Syndrome [30, 42] or certain genotypes [43]. Others, however, are potential targets. For instance, both body mass index [44, 45] and hyperglycemia [30, 46, 47] are potentially modifiable and have been independently associated with greater severe infectious toxicity during therapy. Determining a priori thresholds for allowable infectious toxicity and identifying risk factors is instrumental to personalizing appropriate monitoring, early empiric interventions, and/or incorporation of anti-infectious prophylaxis.

Debate continues, however, on how best to develop practical stratagems to reduce infectious morbidity. The introduction of detailed supportive care guidelines has reduced toxicity despite increasing treatment intensity [15, 38, 48], but broad consensus remains lacking. Anti-infectious prophylaxis is appealing in concept, but the risk for therapeutic pressure selecting resistant organisms is of significant concern [49, 50]. To date, only prophylaxis for the fungus *pneumocystis jiroveci* is widely accepted as standard of care during ALL chemotherapy due to a documented reduction in associated mortality [51]. This is demonstrative of a successful prophylaxis paradigm, but the risk versus benefit ratio for other forms of anti-infectious prophylaxis is less clear. The use of anti-mold and anti-candida prophylaxis during ALL therapy varies among centers, primarily due to the low overall prevalence of invasive fungal disease in this population [52] and the additive concerns of azole drugs potentiating toxicity from vincristine and other agents [53]. The role for antibacterial prophylaxis is likely limited only to the highest risk treatment phases, but even then remains controversial. Fluoroquinolone prophylaxis in adults is increasing, and some early data supports incorporation into pediatric ALL regimen, but literature from children receiving more myelosuppresive AML-type therapy demonstrates uncertain benefit [48, 49, 54]. Until data from frontline ALL regimens is available, however, the mainstay of preventing severe infectious morbidity or mortality remains early intervention for suspected infections. Prompt, broad spectrum antibiotic administration during febrile neutropenic periods is the primary intervention. Multiple evidence-based guidelines have therefore been published to guide practitioners in risk-stratifying management of fever and neutropenia [55, 56].

While antibiotic selection is an institutional decision based on local prevalence and resistance of organisms, appropriate antimicrobial stewardship is imperative to guide care and limit the development of resistance. The largest randomized studies to date for antimicrobial prophylaxis are currently underway within the COG [57] but focus instead on higher risk patient populations. Extrapolation from these studies will nonetheless provide potential guidance for anti-infectious prophylaxis during high-risk phases in ALL therapy. Pending future studies in ALL, a combination of risk-stratified anti-infectious prophylaxis, close monitoring, and early intervention with broad spectrum agents are the backbone of infectious precautions to help mitigate TRT and TRM during increasingly intensive ALL therapy.

12.5 Neurotoxicity

Central nervous system (CNS) neurotoxicity during ALL therapy can have varied manifestations such as headache, seizures, encephalopathy, ataxia and psychosis. Though the pathogenic mechanisms may be distinct, specific etiologies are often difficult to discern as multiple neurotoxic agents are administered concurrently. The timing of symptoms in relationship to various therapies, clinical course, time to resolution as well as neuroimaging findings are helpful in attributing the toxicity to a specific drug. In general, 4–10% of children treated on contemporary ALL regimens develop symptomatic CNS neurotoxicity [58, 59]. Long-term neurocognitive toxicities are beyond the scope of this discussion. However, it is important to note that although decreases in intelligence quotient (IQ) and severe neurocognitive deficits are not frequent in the current era since the omission of prophylactic cranial irradiation, up to 50% of ALL survivors treated with chemotherapy-alone perform below average in measures of attention and other higher neurocognitive functions. [60].

12.5.1 Methotrexate-Related Neurotoxicity

Methotrexate is essential for leukemia control in the CNS; its use has enabled contemporary therapy regimens to eliminate highly neurotoxic cranial irradiation in most patients. However, methotrexate is also known for its acute, subacute and long-term neurotoxicities. Acute toxicities are most consistent with chemical meningitis (e.g. headache, vomiting and fever) [61], while subacute toxicity is more varied and occurs in 3–4% of children with ALL [58, 62, 63]. The latter typically presents a few days to up to 3 weeks after intrathecal or high-dose intravenous methotrexate exposure, with symptoms that can wax and wane over several days. Seizures, aphasia, dysarthria and ataxia are some manifestations of subacute methotrexate toxicity, but most alarming is methotrexate stroke-like syndrome (SLS). Hemiparesis and altered mental status require close monitoring in the intensive care unit and occasionally ventilator support to maintain the airway. Aminophylline and/

or dextromethorphan have been used during the acute phase and for secondary prophylaxis with reports of benefit, however even severe symptoms of methotrexate neurotoxicity are transient in the majority of patients, with or without the use of these agents [64, 65]. Various studies have demonstrated the safety of re-challenge with methotrexate after the complete resolution of symptoms, without a high chance of recurrence of neurotoxicity. Therefore holding potentially curative therapy after a first episode of methotrexate-related neurotoxicity is not recommended [62, 66]. In a report from the UKALL 2003 study group, of 28 patients with SLS re-challenged with intrathecal methotrexate, 5 developed a second episode of neurotoxicity [66]. Four of these five patients were re-challenged a second time, of whom three patients were able to complete all scheduled doses of intrathecal methotrexate. One patient continued to have persistent neurological deficits.

Characteristic white matter changes (leukoencephalopathy) in the frontal or parietal areas, best seen on diffusion weighted MRI often accompany clinical methotrexate related neurotoxicity [67]. However, 20% of children with ALL without any neurologic signs or symptoms are also noted to develop leukoencephalopathy on therapy [62]. The clinical significance of these white matter changes is currently unknown, but studies investigating association of leukoencephalopathy to long-term neurocognition are underway [68]. Other than appropriate leucovorin rescue, and possibly older age, risk factors for methotrexate related neurotoxicities have not been identified [63, 69]. Interestingly, results from a genome-wide association study indicate that genes and pathways involved in neurogenesis previously implicated in other neuropsychiatric disorders such as attention deficit hyperactivity and Alzheimer's may also play a role in individual susceptibility to methotrexate-related neurotoxicity [62].

12.5.2 Posterior Reversible Encephalopathy Syndrome (PRES)

PRES is a clinico-radiographic diagnosis with a reported incidence of 1.6% in children with ALL [70]. The pathogenesis of PRES in ALL is twofold. Most frequently, it is a consequence of hypertension leading to disruption of autoregulation of cerebral blood vessels with resultant vasogenic edema [71]. Direct cytotoxicity of chemotherapy on cerebral blood vessel endothelial cells has also been implicated in PRES not associated with hypertension [72]. Almost all patients present with seizures with or without additional symptoms such as headache, depressed consciousness or cortical blindness [70, 73]. Abdominal symptoms and constipation are frequently noted; one hypothesis is that increased vascular tone from straining during bowel movements in the setting of existing steroid-induced hypertension places a child with ALL at greater risk for PRES. [74] Characteristic radiographic findings are seen typically in bilateral occipital and parietal regions. These include decreased attenuation on computerized tomography (CT) and bright signal on T2-weighted MRI that are best noted on fluid-attenuated inversion recovery (FLAIR) sequences [71]. Therapy for PRES is directed to the precipitating cause, in particular hypertension, and

concurrent anti-epileptic drugs to reduce risk for seizure recurrence. As the name suggests, the clinical and radiographic signs are reversible in the majority of cases (80%), however a subset of patients develop long-term neurologic compromise such as epilepsy and/or persistent electroencephalogram and MRI changes [70, 73, 75].

12.5.3 Sinus Venous Thrombosis (SVT)

Asparaginase and steroids are responsible for the prothrombotic state during ALL therapy. SVT can involve the superficial sagittal and transverse sinuses or the deep sigmoid sinus, often leading to ischemic stroke [76, 77]. The cumulative incidence of SVT during ALL therapy is approximately 2% and the majority of cases occur during remission induction therapy [76]. Available data does not demonstrate consistent association with inherited thrombophilia [77]. Headache, altered consciousness, seizures and focal neurologic signs are the common presenting features. SVT is best diagnosed by MRI, ideally with concomitant magnetic resonance venography (MRV). Management includes anticoagulation with low molecular weight or unfractionated heparin and intensive supportive care as mortality related to SVT can be as high as 10% [76]. Anticoagulation is generally well tolerated without bleeding complications, and is continued for 3–6 months [77, 78]. The majority of patients make full neurologic recovery and can be safely re-exposed to asparaginase [59, 79]. Acute management is challenging when SVT presents with co-existent large intracerebral hemorrhage and in such cases initiation of anticoagulation may need to be delayed. A localized hemorrhage confined to an area of venous infarction is not a contraindication to anticoagulation [78].

12.5.4 Steroid-Induced Psychosis

Corticosteroids are known to cause psychologic symptoms that range from mild mood swings to frank psychosis [80, 81]. Examples are agitation, aggressive behavior, hallucinations, flat affect, homicidal and suicidal ideation and sleep disturbances. A survey of parents of children with ALL revealed that the impact of corticosteroids was the most stressful aspect of the maintenance phase of therapy [82]. The mechanisms of these toxicities are not well defined, but involve effects on neurotransmitters and deregulation of the hypothalamic-pituitary-adrenal axis [83]. Inherited genetic predisposition and environmental stressors likely play a role in individual susceptibility to steroid-induced psychosis. Younger children and those exposed to dexamethasone (versus prednisone) seem to be at higher risk [84, 85], however systematic studies investigating the incidence, risk factors, and impact on long-term neurocognitive functioning are lacking. Since the type and severity of symptoms are extremely heterogeneous, future refinement of the definition and grading of this particular toxicity is necessary for consistent capture. Uniform

guidelines on management of steroid-induced psychosis are not available, but practices include cessation or reduction in dose of the corticosteroid in very severe cases, switching from dexamethasone to prednisone, psychological counselling of patient and family members, and/or the use of anti-psychotic medications such as risperidone [86, 87].

12.5.5 Peripheral Neuropathy

Peripheral neuropathy is a dose limiting toxicity of vincristine. It can manifest with motor (e.g. weakness, gait disturbance), sensory (e.g. pain, numbness, tingling) or autonomic (constipation) symptoms. Reported incidence varies widely and is dependent on the tools and grading scales utilized for assessment. The Common Terminology Criteria for Adverse Events (CTCAE) underestimates severity of vincristine-induced peripheral neuropathy [88, 89], thus the Balis Pediatric Scale of Peripheral Neuropathies and the Total Neuropathy Score-Pediatric Vincristine (TNS©-PV) are recommended for monitoring in prospective studies. In a recent study of 128 children with ALL assessed with TNS©-PV which includes objective and subjective measures, 78% of patients were noted to develop vincristine-neuropathy within the first year of therapy [89]. Severe peripheral neuropathy from vincristine causes significant acute morbidity, and a recent study showed long-term survivors continue to suffer from peripheral sensorimotor impairments, especially those who received a cumulative vincristine dose of >39 mg/m^2 [90]. As there is no known treatment to reverse peripheral neuropathy, early symptom identification and prompt initiation of physical therapy can optimize strength and minimize the necessity of dose reductions. All azoles inhibit CYP3A4, the enzyme that metabolizes vincristine, thus concomitant use of azole antifungals significantly increases vincristine neurotoxicity and is associated with syndrome of inappropriate diuretic hormone (SIADH) and should be avoided when possible [91, 92]. Older children may be more sensitive to vincristine's neurotoxic effects, although this does not appear to be due to pharmacokinetic differences [93]. Caucasian children develop vincristine neuropathy more frequently than African Americans, presumably due to more rapid metabolism of vincristine by the latter [94]. The search for additional inherited risk factors is ongoing; a polymorphism in *CEP72*, a gene that encodes a centrosomal protein essential for microtubule formation, was recently identified to be associated with vincristine neuropathy in children with ALL [95].

12.6 Pancreatitis

Multiple studies have investigated asparaginase-associated pancreatitis (AAP) in childhood ALL with reported incidence between 1% and 18% [96–100]. On occasion, other components of ALL therapy such as thiopurines, glucocorticoids and

trimethoprim-sulfamethoxazole have been implicated in the development of pancreatitis [101, 102]. Diagnostic criteria include clinical symptoms (i.e. abdominal pain), laboratory markers (i.e. elevation of the pancreatic enzymes lipase and/or amylase more than threefold above normal), and supportive radiographic findings. Risk factors for AAP are older age, Native-American ancestry, and a higher cumulative dose of asparaginase [103]. The formulation of asparaginase has not been reported to influence the incidence of AAP [103], but it is unclear if the severity of pancreatitis is greater with extended duration pegylated formulations. Several highly penetrant inherited variants in the *CPA2* gene were associated with early pancreatitis in a large cohort of children with ALL, however, these variants are very rare in the general population [103]. Analyses of common variants with weaker penetrance identified in the same study point to pathways of purine metabolism and cytoskeletal function as likely contributors to the pathogenesis of AAP. Further study is therefore needed into gene-environment/asparaginase interactions to develop appropriate risk-stratification for asparaginase use.

In severe cases, the patient can develop hemorrhagic or necrotizing pancreatitis and associated hypovolemic shock. A third of patients require admission to the intensive care unit [99]. Subacute and chronic complications include pseudocyst formation and dependence on total parental nutrition for many months [98, 104]. In general, acute management of AAP entails aggressive supportive care for pancreatitis associated systemic inflammatory immune response (SIRS), fluid resuscitation, and close monitoring of end-organ perfusion. Large pseudocysts can lead to chronic pain and decreased appetite, but are best managed conservatively to avoid complications of surgical intervention, as most pseudocysts self-resolve [104]. Evidence-based recommendations for management of pancreatitis in adults note the benefit of early feeding, either by oral route or nasogastric/nasojejunal tube; these guidelines are also likely applicable for AAP in children with ALL [104, 105].

As recommendations for severe AAP include no further asparaginase exposure, limited studies have explored AAP's impact on survival. While one earlier study indicated that patients in whom asparaginase was prematurely discontinued due to intolerance had a higher risk of relapse of their leukemia [8], more recent studies have not confirmed this finding [99, 100]. Re-challenge of asparaginase after an episode of mild AAP (defined as symptom resolution with 72 h) appears to be safe [98, 100], though uniform guidelines for re-challenge and monitoring have not been developed yet.

12.7 Endocrine and Metabolic Disturbances

Disruptions of metabolism and of the endocrine system from ALL treatment are due to a combination of chemotherapy and behavior changes that occur in children on therapy. While certain endocrine toxicities have an immediate impact on health during therapy, others begin to exert an impact during treatment but primarily adversely affect long-term health. These long-term complications include such important late effects as growth, hormonal development, and gonadal function, but are beyond the

scope of this chapter focusing on acute metabolic toxicities that occur during therapy. Endocrine toxicities during therapy principally revolve around insulin resistance, obesity, and cardiovascular morbidity.

Hyperglycemia is one of the most common metabolic abnormalities encountered during therapy and occurs primarily during the Induction phase due to insulin resistance from the combination of asparaginase and prolonged glucocorticoid steroids. Transient hyperglycemia during induction occurs in more than 20% of newly diagnosed patients with ALL [47], with adolescents particularly at risk [47, 106–108]. Diabetic ketoacidosis is a dangerous but fortunately rare phenomenon during ALL therapy [109]. However, insulin resistance alone adversely impacts quality of life from dietary restrictions and often a requirement for exogenous insulin. Moreover, insulin resistance contributes to immune-mediated risks for muscle wasting [110], infection [30, 46, 47] and, potentially, even relapse [108, 111, 112]. Older age, Hispanic ethnicity, and obesity have all been reported as demographic predictors of hyperglycemia [107, 108, 113] and are important to help identify patients for close monitoring. While genome-wide association studies (GWAS) have demonstrated a genotype risk profile for steroid-induced hypertension [114], future research is necessary to similarly clarify genetic predisposition for hyperglycemia to further guide dose-intensity and monitoring for this common high-morbidity toxicity.

Obesity is another prevalent and particularly intransigent toxicity during therapy. On one recent COG study, a quarter of newly diagnosed patients began therapy overweight or obese with nearly half meeting those criteria by the end [115]. Obesity has been associated with multiple acute and long-term adverse health outcomes in otherwise healthy populations [116], but is particularly problematic for children with ALL. Obesity is strongly associated with poorer disease response to Induction therapy on modern ALL regimens [117] and also confers a greater risk for relapse [44, 118]. While the causal link with poorer survival has yet to be fully elucidated, ongoing research implicates a direct effect of adipocytes in the microenvironment on chemotherapy pharmacokinetics and/or leukemia resistance [119]. Moreover, obesity during therapy is associated with greater incidence of typically dose-limiting toxicities such as pancreatitis, hepatitis, and muscle weakness [44]. Obesity during therapy also confers significant cardiovascular risk factors during active treatment, including hypertension, metabolic syndrome, and lipid dysregulation [120–123]. Research focused on targeting this intersection of obesity and cancer has therefore become an area of emphasis for the American Society of Clinical Oncology and the American Cancer Society [124, 125] and will provide future avenues of intervention to reduce obesity-associated toxicities.

12.8 Bone Health

Bone health is a specific endocrine toxicity of central importance to ALL therapy. While the toll of ALL treatments on long-term bone structure heavily impacts the quality of life in survivors [4, 126], it is evident these changes begin early in therapy.

Osteotoxicity during ALL therapy manifests as either osteonecrosis ("avascular necrosis") or osteopenia. Although corticosteroids are frequently implicated as the precipitating agent for bone toxicity, other common ALL chemotherapies contribute as well, including asparaginase, methotrexate, and alkylating agents [127]. Nonetheless, corticosteroids are strongly associated with the risk for osteonecrosis (ON) and while the hypothesized if not delineated pathology of ON is reviewed elsewhere [127], it is important to examine the specific risk factors for development of ON. Age appears to be a central determinant, as children younger than 10 years only rarely develop ON irrespective of regimen (incidence ≤1% [128, 129]) but ~10–20% of adolescents will suffer from severe ON during or after therapy [6, 128–130]. Use and exposure to different corticosteroids have been consistently shown to predict ON, with dexamethasone at high and prolonged exposures associated with the greatest rates of ON [130–132]. While dexamethasone was well tolerated on MRC UK ALL2003 trial, the recently concluded COG frontline trial in ALL was amended due to an age-corticosteroid interaction resulting in high rates of ON [6, 129]. Exploration of dexamethasone's effect on ON in cohort studies is complicated by significant pharmacokinetic variability, and particularly by alteration in steroid exposure from concurrent use of asparaginase [133]. Other host factors such as obesity and sex have also been found to predict risk for ON [132, 134]. Ongoing research using GWAS has shed some light on the genetic underpinnings for this common toxicity [130, 135] with future research aimed at developing validated risk-stratifications incorporating genotype and demographic variables to titrate steroid exposure for precision in balancing cure with toxicity [136].

Although the morbidity from ON is potentially more severe, ALL-associated osteopenia is more prevalent. Severe osteopenia resulting in radiographic or symptomatic fracture of the bone during therapy occurs in approximately 10–30% of children [137–142]. Osteopenia, however, is nearly ubiquitous with a wealth of literature showing bone mineral density during therapy is both lower than healthy controls and declines with time [137, 139, 142–145]. Contrary to historical views, new evidence shows osteopenia is often present at diagnosis and progresses early during therapy. Severe early declines in bone density are already present during the Induction phase alone [138, 146]. Treatment for pediatric ALL occurs during the period of bone accretion and not maintenance (<25 years of age); debate continues whether the declines in bone density during treatment are of long-term significance [147, 148] or whether sufficient "recovery" of density post-therapy occurs [149–152]. Regardless, osteopenia at diagnosis and during therapy is closely associated with fracture risk during therapy [137, 138]. The etiology of treatment-associated osteopenia is likely multifactorial [153], resulting from a direct effect of chemotherapy on osteoblasts [154, 155], Vitamin D insufficiency [146], obesity [156], and inactivity during therapy [157]. Prior ON was also shown to be a specific risk factor for osteopenia during ALL therapy [158], although it is unclear whether this was due to resultant inactivity as the authors suggest and not sensitivity toward osteotoxicity. Similar to ON, osteopenia is more likely to be present in adolescents, females [140, 145], and those receiving dexamethasone-based chemotherapy regimens [145, 159]. Genetic predisposition toward osteopenia has not been explored as widely as

in ON, but several single nucleotide polymorphisms have been identified [160]. Due to the established benefit from osteotoxic ALL regimens, future studies will focus on mitigating the high rates of both osteopenia and ON in older patients to provide optimal therapy and maximize quality of life.

12.9 Toxicities Associated with Novel Immunotherapies

Promising results of efficacy from early-phase clinical trials are driving rapid clinical development of immunotherapeutic strategies such as immunotoxins, bispecific antibodies, and chimeric antigen receptor (CAR) modified T-cells. These therapeutics bring along unique toxicities not commonly noted with conventional cytotoxic drugs and it is important for the pediatric hematologist-oncologist to appropriately recognize and manage them appropriately.

12.9.1 Cytokine Release Syndrome (CRS)

CRS is defined as a systemic disorder characterized by nausea, headache, tachycardia, fever, hypotension, rash and shortness of breath, caused by a rapid release of cytokines from cells. Symptoms manifest approximately 1–5 days after T-cell infusion and vary in severity, ranging from fever alone to significant shock requiring cardiorespiratory and renal support for multi-system organ failure. The incidence of CRS is reported to be 15–30% with CD19-directed CAR T-cell therapy [161, 162] and 8% with blinatumumab [163] and is strongly correlated with pre-treatment disease burden [164]. A variety of cytokines are implicated, in particular interleukin 6 (IL6), interleukin10 and tumor necrosis factor (TNF) [161]. Cytokine muting therapies like steroids are effective in mitigating CRS; however they are also lymphotoxic and are avoided to preserve efficacy of anti-leukemia therapy. Newer agents such as tocilizumab (an anti-IL6 receptor antibody) and etanercept (a TNF inhibitor) are also very effective in rapid attenuation of symptoms while preserving lymphocyte function [162, 165]. Only in rare situations is CRS fatal and most patients make full recovery with aggressive supportive measures and anti-cytokine therapies. Concerted efforts have been made in developing uniform grading systems and algorithms for management of CRS [165, 166].

12.9.2 Central Nervous System (CNS) Toxicities

Though neurologic symptoms often accompany CRS, unlike CRS itself, their incidence and severity are not related to pre-treatment disease burden, suggesting they may be distinct pathogenic entities [161, 162]. The mechanisms of CNS toxicities

from novel immunotherapies are not yet clearly elucidated, but are thought to be secondary to a generalized T-cell mediated hyper-inflammatory state [167]. Blinatumumab does not cross the blood brain barrier thus direct toxicity is unlikely; however CAR T-cells infiltrate the CSF and are known to provide provide CNS leukemia control. The presence and degree of CNS leukemia however, does not correlate with the occurrence of CNS toxicity [162], but a correlation was present between CNS toxicity and the absolute number of CAR T-cells that trafficked to the CNS [161]. Twenty to 50% of patients develop CNS symptoms of various grades with CD19-directed immunotherapies [161, 162, 168]. Symptoms vary from mild confusion, to word finding difficulties, aphasia, delirium, seizures, significant encephalopathy and obtundation requiring intubation for airway protection. Fortunately, these symptoms are transient, and most patients recover fully with intensive supportive care. As the field moves forward with wider applicability of immunotherapy in ALL, it is crucial to gain a deep understanding of pathophysiology of CNS toxicities to enable targeted preventative approaches.

12.10 Conclusion

Medical supportive care is now recognized as an integral component of providing increasingly intensive therapies for childhood ALL. Not only survival, but also quality of life in survival, has been improved through reducing treatment-related early deaths and minimizing dose-limiting or treatment-interrupting toxicities. We would acknowledge that the vast scope of treatment-related toxicities in this review prevented inclusion of toxicities with principally late effects, such as cardiac toxicity or gonadal dysfunction, but these are important late effects that would benefit from preventive strategies during therapy. While disparities in toxicity grading systems often challenges our ability to compare directly between consortia and regimens, general themes and lessons as described here can be extracted from the literature to help guide both the development of new regimens and personalized care for patients. To better understand toxic outcomes, greater uniformity in reporting is needed. To this end, a recently formed international taskforce led by the Ponte de Legno (PdL) childhood ALL group comprehensively reviewed current practices of 15 ALL study groups worldwide and critically evaluated available literature to develop consensus definitions for various severe acute toxicities during ALL therapy [169]. The goal for this task force is to unify the capturing and reporting of toxicity to enable comparison of incidence, risk factors, and explore genotype-phenotype correlations. Recognition of at-risk populations offers new opportunities to intervene early; the unambiguous severe morbidity suffered by ALL populations highlights the need and opportunity for new collaborations and approaches as embodied by the PdL toxicities task force. Expanding our understanding of the complex interactions of host, treatment regimen, pharmacogenomics, and pharmacokinetics, will provide the building block for the next large step forward to reduce treatment-related toxicity through effective prevention and management strategies.

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Chapter 13
Developing World Perspective

Allen Yeoh

Childhood ALL (cALL), the most common form of childhood cancer, is curable with ~90% becoming long term survivors using contemporary multi-agent chemotherapy. But there is a great disparity in treatment outcome; this high success rate is limited to high income countries (HIC) with little improvements in low and middle income countries (LMIC) over the last 50 years. This inequity is unfortunate because cALL is curable with cheap generic drugs using an appropriately designed treatment protocol, good diagnostic and supportive care, and socio-economic support for the family. Even in the most deprived settings, 30% of cALL can be cured.

However, simply copying intensive treatment protocols from HIC when supportive care is inadequate, exposes children to severe treatment-related morbidity and mortality, increasing costs, and abandonment. To succeed, LMICs need to study the reasons for failure, plan what resources and funding are available, train healthcare professionals on how to deliver appropriate treatment, manage complications using standardized protocols and collect comprehensive data including toxicity and reasons for abandonment. Twining with an aspirant or mentor institution enables training and weekly online conference calls to monitor and discuss patients and problems. Above all, the cure of cALL in LMICs can be achieved through strong partnerships between supportive governments, strong charity, dedicated mentoring institution and a passionate team led by inspiring and visionary leadership. I will explore and summarise practical areas where LMICs may find useful in managing cALL with limited resources.

A. Yeoh
Viva-University Children's Medical Institute, Singapore, Singapore

Khoo Teck Puat-National University Children's Medical Centre,
National University Hospital, Singapore, Singapore

Yong Loo Lin School of Medicine, National University of Singapore, Singapore

National University Health System, Singapore, Singapore
e-mail: allen.yeoh@nus.edu.sg

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13.1 Why the Developing World?

Eighty percent of children with ALL reside in LMICs but account for only for 6.2% cancer expenditures worldwide [1]. ALL is essentially a childhood cancer with peak incidence between 2 and 6 years old. The population in LMICs is young and rapidly growing. With death from diarrheal and infectious diseases decreasing and poverty alleviation programs pulling millions out of extreme poverty, LMICs are ready to tackle cALL. Except for Africa and war-torn south-central Asia, the rising incidence of childhood cancer, falling under-5 year old mortality rates and childhood cancer mortality in LMIC provide the unique opportunity to tackle cALL (Fig. 13.1).

Curing cALL is cost effective. The most effective chemotherapy drugs are generic, available and affordable. Eighty percent of treatment costs are consumed by supportive care especially antibiotics and safe blood components. Resource stratified care appropriate for each LMIC can optimise the treatment outcome and keep costs affordable. Survivors of cALL have normal intelligence and life span, their potential economic returns over the next 60 years of their life easily justify treatment costs.

13.2 Resource-Stratified Care

We know more about cALL than any other cancer. We know the biology of this disease, what is needed to cure and when good supportive care is most critical. Low income countries can cure ~30% of children with ALL with simple protocols that

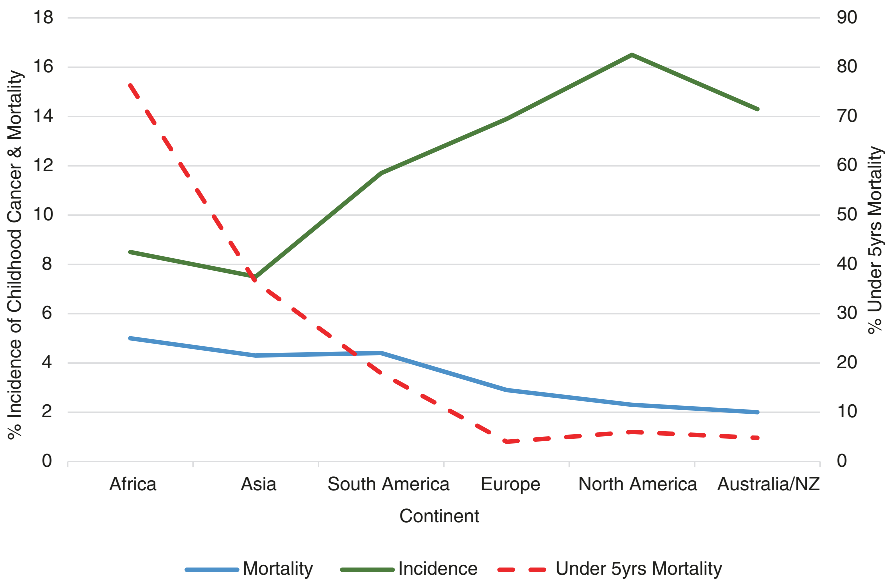

Fig. 13.1 Incidence of childhood cancer and cancer mortality and Under 5-years mortality in 6 inhabited continents in the world. With improved health care, more childhood cancers are diagnosed and fewer cases missed. With lowered under 5-years mortality, most LMICs are poised to improve treatment outcome for childhood ALL. (GLOBOCAN 2012 and World Bank 2015 data)

can be administered by trained general practitioners while middle income countries can cure ~60% through more complicated blocks by trained paediatricians. One way to stratify care is to use the World Bank [2] definition of low (n = 31) and middle-income (n = 104) income countries which have gross national income per capita of <US $1000 and US$1000 to US$12,000 respectively. However, disparity in income and medical resources may differ between provinces and rural versus urban areas in large countries.

13.3 Diagnosis

Most children with ALL present typically from symptoms of pancytopaenia – recurrent fevers from neutropaenia, increased tiredness from anemia, petechiae and mucosal bleed from thrombocytopaenia. Uncontrolled growth of lymphoblasts in bone marrow, lymph nodes, liver and spleen cause bone pain, lymphadenopathy and hepatosplenomegaly respectively. Unfortunately some of these signs and symptoms are similar to dengue fever, malaria and tuberculosis. So diagnosis can be delayed by weeks, placing the child in a worse clinical state. Malnutrition and worm infestations are common, decreasing tolerance to and increasing morbidity of therapy.

cALL is rare. Every child with suspected ALL should be referred to a hospital experienced in diagnosing and managing of cALL. Most provincial hospitals only will see few cases per year and will lack experience and supportive care to best manage them. Diagnosis of ALL [3] requires examination of a bone marrow aspirate by an experienced haematologist with specialised equipment (Table 13.1). Sterile, disposable bone marrow needles should be used to aspirate a diagnostic sample from the posterior superior iliac spine and placed into an EDTA tube, usually an adult FBC tube, to prevent clotting. The bone marrow can then be smeared onto a clean microscopic slide. Bone marrow stains using May-Grunwald Giemsa or Wright-Giemsa stains are recommended as they have good nuclear staining, allowing appreciation of open chromatin and nucleoli and cytoplasmic inclusions.

13.4 Where Should cALL Be Treated in LMICs?

Unlike in HIC where cALL is managed by paediatric oncologists in paediatric cancer centres, in LMIC, cALL can be managed either in a paediatric unit in a general hospital or in a children's hospital or adult cancer or haematology hospital. They can be managed by paediatricians with interest in paediatric hematology-oncology or by adult haematologist focusing on paediatric oncology. Each care model has its own merits and limitations (Table 13.2). In some countries, like India and Philippines, pediatric oncologists in private hospital manage a sizeable number of cALL successfully. These private hospitals have a charity foundation or receive special land rights in return for treating a certain number of poor patients.

Table 13.1 Recommended tests for diagnosis and risk stratification in LMICs

Resource available	Diagnosis	Risk stratification criteria
Basic	Morphology and cytochemistry Chest X-ray for mediastinal mass	NCI criteria NCI Standard Risk: Age 1-10 and WBC <50 Mediastinal mass -> T-ALL Day 8 peripheral blood response ABC <1 BM at Day 15, end induction
Limited	Morphology and cytochemistry Immunophenotyping (restricted panel) DNA index RT-PCR of *BCR-ABL1*, *MLL-AFF1*, and *ETV6-RUNX1*	NCI criteria Immunophenotype (T cell vs B cell) Molecular subgroup Favorable: DNA index >1.16 or *ETV6-RUNX1* Unfavorable: *BCR-ABL1*, *MLL-AFF1. DNA index <1* Day 8 peripheral blood response ABC <1 BM at Day 15, end induction
Enhanced	Morphology Immunophenotyping DNA index RT-PCR of *BCR-ABL1*, *MLL-AFF1*, *ETV6-RUNX1*, and *TCF3-PBX1* Cytogenetics for hyperdiploidy >50 or hypodiploidy <44 Fluorescence in-situ hybridisation of chromosomes 4, 10, and 17, and *BCR-ABL1* *Pharmacogenetics for Mercaptopurine metabolism: TPMT and NUDT15 variants*	NCI criteria Molecular subgroups Favorable: DNA index >1.16 or Hyperdiploidy > 50 chr, or triple trisomy 4, 10,17 and *ETV6-RUNX1* Unfavorable: *BCR-ABL1*, *MLL-AFF1, Hypodiploidy < 44 chr* Minimal residual disease measurements by IgH or T-cell receptor rearrangements, flow cytometry, or deep sequencing Pharmacogenetics

In LMICs, working cooperatively on the strengths of each hospital is the way forward. Communication and collaboration as a national group using a common protocol allow easy understanding and transition of care. This may involve centralising cytogenetics and flow cytometry in haematology hospitals. Transferring to children's hospital for paediatric surgical and ICU care for children who developed severe complications may be life-saving.

Rural patients can be followed up in provincial hospitals during maintenance therapy. University Malaya doctors using the Malaysia -Singapore (Ma-Spore) protocol make weekly telephone calls with provincial hospitals to get updated FBC results and progress of children referred out. If parents are Internet savvy or can use smart phone apps like Whatsapp, FBC charts and maintenance doses of mercaptopurine and methotrexate can be communicated easily by them. Good summary of medical records need to be maintained both by patients and hospital. These can be done using chemocards which summarise WBC, ANC, drug doses and schedules. Parents should maintain a large A4 hard cover exercise books where records can be entered and important blood results and protocols pasted. Having a family

Table 13.2 Comparing paediatric versus adult cancer or blood hospitals in LMIC

Type of hospital	Paediatric in General Hospital or Children's Hospital	Adult Cancer Hospital	Adult Blood Hospital
Doctors	Paediatrician focusing on paediatric oncology	Medical Oncologist	Hematologist focusing on paediatric oncology
Funding	Least funded. Charity to raise funds for childhood cancer	Best government funding	Good funding
Diagnosis	Limited BM morphology and cytogenetics	Limited BM morphology, cytogenetics	Good BM morphology, flow cytometry, cytogenetics, molecular
Imaging[a]	US, CT, MRI	US, CT, MRI, PET-CT	Limited US, CT
Blood support	Limited. Need to get blood from blood centres	Good	Excellent. Random platelet units
Supportive care	Paediatric ICU Paediatric dialysis	Medical ICU Adult dialysis	Medical ICU No dialysis
Surgery	Paediatric Surgery	Excellent oncologic surgery	Limited. Transfer to pediatric hospital
No of patients	Overcrowded with mainly general paediatrics including thalassemia or sickle cell disease.	Severely overcrowded	Least crowded

[a]*US* ultrasonography, *CT* Computerised tomography, *MRI* Magnetic resonance imaging, *PET-CT* positive emission tomography with computerised tomography

coordinator employed by foundations who tracks families and compliance and trained to answer common queries, frees the doctor and nurses from non-health care work. The coordinator help apply for financial subsidy, family financial support and housing.

13.5 Improving Outcome Through Reducing Toxic Deaths, Abandonment and Relapse

The overall results for cALL from LMICs are poor because of high incidence of toxic deaths, abandonment of therapy and relapse. Jogjakarta [4] reported a 5-year EFS of 31% with toxic deaths in 16%, abandonment in 15% and relapse in 38% of patients treated on the Indonesian WK-ALL 2000 protocol. Interestingly, relapse rates in LMICs are not more than twice those from HIC. Reducing intensity of therapy will reduce toxicity and abandonment will improve overall outcome.

LMICs have limited supportive care. Less intensive induction chemotherapy using 2–3 non-myelosuppressive drugs is recommended (Table 13.3). Steroid like prednisolone or dexamethasone and vincristine with IT methotrexate can achieve complete morphological remission in 90% of children with ALL. Adding L-asparaginase significantly increase costs and toxicity but may be manageable in centres with good supportive care. In fact, COG, UKALL and Ma-Spore groups

Table 13.3 Proposed protocol for LMICs based on resource

Basic/Low income country	Limited/Middle income country
Induction (two-drug), for 4 weeks Oral Pred 20 mg/m^2/day in three divided doses for Day 1, then 60 mg/m^2/day × 27 days IV VCR 1.5 mg/m^2/dose[a] weekly day 1/8/15/22 IT MTX[b] day 8/15/22	**Induction (3-drug SR; 4 drug HR) for 5 weeks**
	Oral Pred 20 mg/m^2/day in three divided doses for Day 1, then 60 mg/m^2/day × 6 days
	IV VCR 1.5 mg/m^2/dose[a] weekly D1/8/15/22/29
	Oral Dexa 6 mg/m^2/day for 21 days from D8 to 29
	IM L-Asp 6000 U/m^2/dose twice a week Day 8/11/15/18/22/25
	IV DNR 25 mg/m^2/dose on Day 8 for HR patients only
	IT MTX[b] Day 8/15/29
	IT MTX[b] Day 8/11/15/22/29 for CNS 2/3 BMA at Day day 29 to check morphological complete remission
Interim maintenance #1 for 8 weeks Oral MP 37.5–50 mg/m^2/day for 8 weeks (before bedtime) Oral MTX 20 mg/m^2/week for 8 weeks IT MTX[b], weeks 1/3/5/7 BMA at week 1 to check morphological complete remission	**Consolidation (4 weeks)**
	IV VCR 1.5 mg/m^2/dose[a] weekly on Day 1 for SR
	IV VCR 1.5 mg/m^2/dose[a] weekly on Day 1/8/15 for HR
	Oral MP 50 mg/m^2/day for 28 days (before bedtime)
	IT MTX[b] Day 1/8/15
	IT MTX[b] Day 1/8/15/22 for CNS 2/3
Delayed intensification #1 for 4 weeks IV VCR 1.5 mg/m^2/dose[a], day 1/ 8/15/22 Dexa 6 mg/m^2 per day, for 28 days IT MTX[b], days 1 and 15	**Interim maintenance (7 weeks)**
	IV VCR 1.5 mg/m^2/dose[a] every 10 days
	IV MTX 100 mg/m^2 escalating by 25 mg/m^2 every 10 days
	IT MTX[b] Day 31 for CNS 1
	IT MTX[b] Day 1/11/21/31/41 for CNS 2/3
Interim maintenance #2 for 8 weeks Same as interim maintenance part 1	
Delayed intensification #2 for 4 weeks Same as delayed intensification part 1	**Delayed intensification (SR/HR for 7 weeks)**
	IV VCR 1.5 mg/m^2/dose[a] weekly D1/8/15/22
	Oral Dexa 6 mg/m^2/day for 21 days
	IM L-Asp 6000 U/m^2/dose every 3 days for six doses
	IT MTX[b] Day 1/29
	IT MTX[b] Day 1/8/29 for CNS 2/3
	IV CPM 500 mg/m^2/dose on day 29 with IV mesna 500 mg/m^2
	S/C or IV AraC 75 mg/m^2/dose on D29 to 32 and D36 to 39
	Oral MP 50 mg/m^2/day (before bedtime) for 10 days

(continued)

Table 13.3 (continued)

Basic/Low income country	Limited/Middle income country
Maintenance, 4-week block, repeated until 2 years of maintenance Oral MP 37.5–50 mg/m^2/day for 28 days Oral MTX 15 mg/m^2/week, for 4 weeks Oral Dexa 4 mg/m^2/day for 5 days during week 4 IV VCR 1.5 mg/m^2/dose[a] week 4 IT MTX[b] week 4 for first year	**Maintenance (SR/HR), 12-week block, repeated until 2 years of maintenance** Oral MP 50 mg/m^2/day for 28 days Oral MTX 20 mg/m^2/week, for 4 weeks Oral Dexa 6 mg/m^2/day for 5 days during week 1 for SR Oral Dexa 6 mg/m^2/day for 5 days during week 1/5/9 for HR IV VCR 1.5 mg/m^2/dose[a] week 1 for SR IV VCR 1.5 mg/m^2/dose[a] week 1/5/9 for HR IT MTX[b] week 1 for first year

BMA bone marrow aspirate, *Dexa* dexamethasone, *DNR* daunorubicin, *IM* intramuscular, *IV* intravenous, *IT* intrathecal, *MP* mercaptopurine, *MTX* methotrexate, *L-asp* E coli L-asparaginase, *Pred* prednisolone, *VCR* vincristine. DNR can be replaced with IV doxorubicin at the same dose

[a]Maximum dose of vincristine capped at 2 mg

[b]IT MTX dose 6 mg in 3 ml for < 1 year old; IT MTX 8 mg in 4 ml for 1–2 years old; IT MTX 10 mg in 5 ml for 2–3 years old; IT MTX 12 mg in 6 ml > 3 years old

start majority of their patients with only 3 drug induction chemotherapy. Despite this sadly, many LMICs use 4-drug induction with anthracyclines with high induction deaths due to toxicity.

Starting with less intensive therapy has significant advantages. Firstly, tumour lysis syndrome can be managed without risk of acute kidney injury. Adding three additional doses of L-asparaginase in the Indonesian WK-ALL 2000 protocol [5] increased toxicity without improving EFS. Adding IV anthracycline during induction chemotherapy causes severe prolonged myelosuppression, mucositis and need for prolonged antibiotics and blood product support. These cause increased morbidity, deplete family resources and increase abandonment. CCG-105 study showed that intensive 4-drug induction is not better than 3-drug induction if delayed intensification [6] is given.

13.6 Definition of Abandonment

Refusal to start induction chemotherapy or failure to complete ALL chemotherapy will likely cause relapse and death from disease. Abandonment is defined as stopping chemotherapy for > 6 weeks during any phase of ALL protocol treatment. Loss to follow up after completing maintenance therapy is not abandonment and can be censored. LMICs consistently show abandonment > 6% and this is correlated to low national income, high economic hardship and higher out-of-pocket medical payment [7]. For large countries, there may be even differences in abandonment rates between provinces with lower abandonment among city dwellers. Cultural beliefs that cancer is incurable or chemotherapy drugs are poisons reinforce abandonment behaviours.

13.7 Painful Procedures

A main reason for abandonment is perceived suffering. Children are naturally fearful of hospitals and painful procedures. Seeing a sick child screaming from multiple needle sticks and painful bone marrow aspiration and intra-thecal chemotherapy, often leads to abandonment of treatment by the family. Improving family understanding and provision of social and financial support from dedicated social workers are critical in countering such behaviour.

13.8 IV Lines and Central Lines

EMLA cream for needle punctures, IV conscious sedation for painful bone marrow and IT initially are useful. LMICs may only have butterfly needles. Plastic Jelco needles are ideal. Reusing needles is not acceptable as the risks of blood transmission of Hepatitis C and HIV are too high.

Port-a-cath or Hickman lines are expensive and difficult to maintain. Unless staff are trained and has acceptable rates of infections with central lines, these are best avoided in LMICs. Alternative is to place a temporary peripheral IV catheters in the brachiocephalic vein in the elbow. We use a closed system where all the fluids bags are all spiked up at the time of access of the central line (Fig. 13.2). All chemotherapy

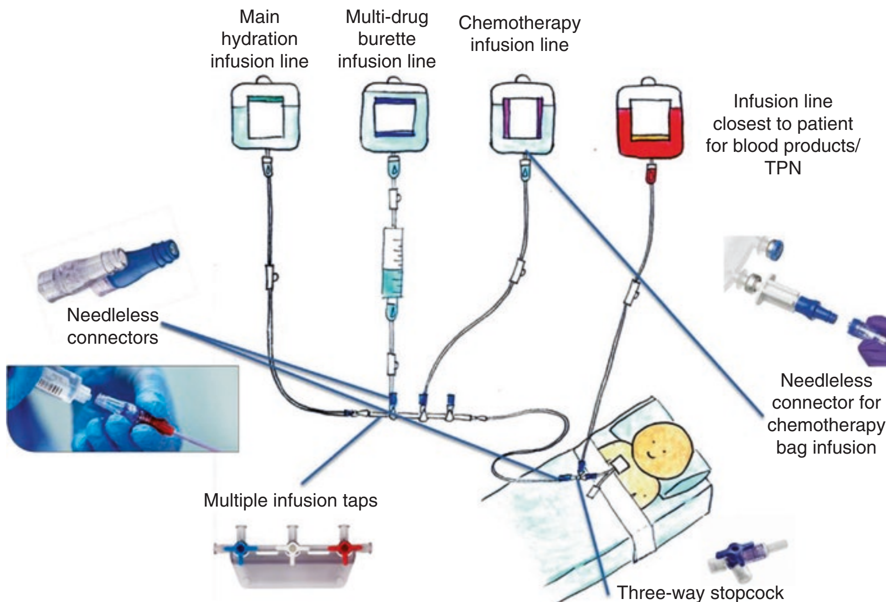

Fig. 13.2 Closed system access to central line using a system of three way stopcock, needless connectors and burettes. The system is set up from the start, minimal opening of the line is needed. Fluids are spiked up in large bags and replaced infrequently

and IV medications are mainly administered in a closed system via a burette and a self-sealing clave that can be cleaned thoroughly using >5 alcohol swabs. This significantly reduces line infections.

IV conscious sedation using IV midazolam 0.2 mg/kg/dose; IV atropine 0.02 mg/kg/dose and IV ketamine 2 mg/kg/dose can be administered safely. Patients should fast for about 4–6 h before IV sedation and pulsed oximetry monitoring should be done during procedure. Parents can be taught to monitor breathing and heart rate post procedure for 1 h. After initial success, some patients can have intrathecal chemotherapy with help of just EMLA cream as local anaesthetic.

13.9 Outpatient Therapy

Inpatient beds are expensive to maintain as it requires supportive infrastructure, doctors and dedicated nurses to run. Outpatient day therapy allows each bed or chemotherapy chair to be recycled a couple of times a day, thus is more cost effective. A dedicated team of 2–3 doctors, 5–6 nurses and a pharmacist to prepare the chemotherapy drugs is necessary. Laboratory results must be available quickly or consultations can be done the day prior to procedure. Chemotherapy drugs are pre-ordered and prepared. Each bed or chair can then be cleaned quickly and recycled 2–5 times each day. Even IT chemotherapy that requires patient to lie flat for 4 h can be done. Governments and insurance should cover outpatient care.

13.10 Halfway Homes

cALL treatment requires around 6–9 months of intensive therapy. Many LMICs are large and sparsely populated with limited transport infra-structure, making travel difficult. Parents sleep in the same bed as the child, cook and eat in the ward and patients are often not discharged because they don't have a place to stay local to hospital. Inpatients wards are overcrowded, with an increased risk of hospital acquired infections. Providing a halfway home is cost-effective and parents can be encouraged and trained to help clean, cook and maintain it with the help of a coordinator.

The non-profit St Jude India Child Care Centre [8] have 18 child care centres. Each centre is run locally with a local team of volunteers who work to a standard operating procedure, which includes instructions on cleaning and maintenance. The key is for local volunteers to be led by a team leader who will raise funds, look for a suitable place to rent and partner local hospitals who care for childhood cancer. Each centre has multiple partitioned rooms with a bed for the mother and child. Each family has a dedicated gas cooker and locker and is given specific rations to cook fresh food for their child. The family stays for about 6 months during the intensive phase of ALL therapy. Parents and patients are taught how to maintain cleanliness, drink boiled water and eat freshly cooked food. This is important as many

children die during maintenance therapy from infections when they return home. Use of mosquito nets and covering food with nets to reduce flies are simple yet effective.

13.11 Infections: Varicella, Measles, TB

Varicella zoster virus is highly contagious and can be severe and even rapidly fatal in children with ALL. Especially in tropical LMICs, many children and even their parents are not immune to varicella. For example, in Guatemala, the incidence of varicella infection in the National Unit of Pediatric Oncology is 23.4 per 1000 person years with a median age of 5.2 years with 14% requiring critical care and a 3.4% mortality [9]. In Singapore, despite varicella vaccination, 32% cALL are seronegative with another 13% losing their varicella immunity after treatment. Patel et al. reported similar frequencies [10]. It is common to have large varicella outbreaks in the LMIC hospitals. Post exposure prophylaxis with oral aciclovir 200 mg/dose (<2 years old) or 800 mg/dose (>2 years old) qds can be used in LMIC centres instead of varicella hyperimmune globulin [11]. As many parents lack immunity to varicella, active varicella vaccination of mothers can be effective with little risk of spread.

In many LMIC, dengue fever and even recently Zika virus are common. Many patients are mis-diagnosed with recurrent dengue hemorrhagic fever when they present with fever and severe thrombocytopaenia causing treatment delay. After dengue or varicella infections patients have prolonged myelosuppression, requiring treatment interruptions and lower doses of chemotherapy. Worms like Ascaris can cause bowel obstruction. Routine deworming is encouraged in LMIC especially when patients are from rural areas.

Although measles vaccination is mandatory and provided free of charge in LMICs, not all children seroconvert. Due to misconceived fears of autism, contamination of porcine source in Muslim countries, fake vaccines and improper vaccine storage, outbreaks of measles infections are common in LMICs. As immunocompromised patients may present with fatal measles pneumonitis without even skin rash, a high index of suspicion is needed. All centres should maintain an up-to-date register of whether their patients have been vaccinated or are immune to varicella and measles.

13.12 Drugs Reliability of Supply: Generic Drugs, Drug Registration

Despite most drugs being included in the WHO List of Drugs, shortage of chemotherapy drugs often plagues LMICs. In 2016, Indonesian government misguided by banned mercaptopurine, which was apparented used for therapeutic abortion, limiting access to one of the most important drug in ALL. Hospitals often lack a good pharmacy to track the availability of drugs and their budget may run out by end of

financial year. Families often have to buy their own drugs from private pharmacies outside the hospital. Pharmaceutical companies lack motivation to register and import chemotherapy drugs used in cALL in LMICs as these are cheap generic drugs. Health ministries are often slow to approve import of drugs. Fortunately common drugs like vincristine, prednisolone/dexamethasone are commonly available and can be sourced from many generic making companies. L-asparaginase, mercaptopurine and oral and preservative free intra-thecal methotrexate are more limited. Foundations should try to coordinate stockpiles and transport of drugs from different countries. In such emergency shortages, standard regulatory rules should be relaxed. The Max Foundation has successfully made available imatinib from Novartis for LMICs.

13.13 Training Doctors and Nurses

Training families on management of fever, side-effects of treatment, administration of medications like steroids with food and mercaptopurine is best on an empty stomach 2 h after dinner and not with milk, is essential for the optimal delivery of treatment. A standardized schedule for the whole hospital e.g., cotrimoxazole is given BD on Monday and Tuesdays while oral MTX is given on Fridays. Similar to asthma action plan, there should be an ALL action plan. Protocol copies should be printed, completed and filed in each patient's case notes. A hard cover book should be provided to families with follow up notes and results in them.

Treatment protocol should be simple. For example, the Indonesian ALL protocol has only Standard risk and High risk protocols, each protocol condensed into two pages which the family can carry to consultations. Treatment is protocolized, to ensure a minimum standard and allow identification of gaps when reviewed systematically. A team of leaders with local experience are involved in designing a practical protocol. Random, unsubstantiated changes, which confound analysis and future improvements, should be avoided. As most of LMICs doctors lack in depth training in delivering chemotherapy, the most effective protocols are probably use limited number of drugs that are not myelosuppressive and in fixed repeated combinations. This allows easy understanding and management of side-effects.

A simple concept is to do the possible first. LMICs can start with treating children with NCI standard risk, especially those from the city. This reduces the problems of transport infrastructure and treatment abandonment. Children from rural areas are at increased risk of infections due to lack of clean water, long distance to hospital, higher risk for fungal infections from atap roofs and infections like malaria and dengue.

It is important to collect baseline and survival data. Data provide ways to identify the critical gaps that then can be tackled systematically and reassessed if the intervention is successful. For example, if abandonment due to lack of money, transport and housing can be addressed by improved financial support of even paying families such as by providing coupons for public transport and half-way houses.

13.14 Staff

Majority of the doctors and nurses from LMICs are not trained in pediatric oncology. Doctors lack mentors who have proper training to manage ALL and its complications. Similarly nurses may not appreciate the importance of aseptic technique and how to administer chemotherapy and antibiotics. Training the trainer who are senior members of the team is critical. There is a need to develop a standardized curriculum from which adaptation and translation can be done. In fact, St Jude Global [1] is planning to set up a school for paediatric oncology training where doctors, nurses and health care professionals can be trained and certified.

Doctors and nurses from LMICs in government hospitals are badly paid. Successful programmes start with raising funds to pay the salaries of a leader, dedicated team of senior doctors and nurses (Table 13.4). Experienced staff to manage the sickest children cannot be retained without the adequate remuneration.

Advances in affordable technology – emails, web conferencing, courier, Internet-enabled data repository like RedCap – can overcome current limitations and

Table 13.4 Important elements for setting up an ALL programme in developing countries

Leader – well trained in pediatric oncology, respected, leadership qualities, consensus builder, able to get a team running, mature, visionary – able to project needs to future, communicator, fund raiser
Team – paediatrician, haematologist specialist, paediatric oncology trained nurses. Nurses from developing countries tend to be less trained and do not speak good English
Coordinator – administrator
Facility – inpatient – dedicated paediatric oncology unit including isolation rooms– reduces infections from general paediatric cases like pneumonia, viral infections. Lots of wash basins. Well maintained clean, well ventilated facility
Outpatient facility – allow right siting of care, recycling outpatient beds which are less expensive to maintain
Cancer pharmacy – reconstitution of drugs, checking drug doses and route of administration. Keep track of drugs to avoid shortages
ICU including ventilator support, haemodialysis and continuous monitoring
Blood bank – preferably 24/7 support, safe screened blood components including platelets, fresh frozen plasma and RBC concentrates
Laboratory support – FBC, Chemistries, Blood culture, Fungal culture
Diagnostic imaging – Ultrasonography, CT scan, MRI, PET-CT scan, Echocardiography
Support – community support like children's cancer foundation – providing psychosocial care for family, family mentors by survivors – able to overcome cultural and non medical issues, fund raising, covering costs of chemotherapy US$15,000 with family support for transport US$6000. Public and health professional education
Governmental support – provision of hospital infrastructure, drug approval, funding
Transport and communications infrastructure. – Prepaid bus tickets
Building half way houses for out of town families to stay during intensive phase of therapy
Funding – initial start up then with time local foundations take over
Twinning with aspirant centres – St Jude Global Program
Mentorship with leaders – provide guidance, consultations training, weekly web-based conferencing. Adaptation of protocol to local context

significantly change care. Unstained bone marrow slides can be shipped by courier or mail to reference centres where they can be properly stained, scanned and uploaded for review by volunteer expert haematologists. Flow cytometry plots can be reviewed on line. Molecular tests can be carried out on dried samples on FTA cards or fixed, unmounted, unstained glass slides.

What we need is a non-intensive, well-tolerated backbone protocol that can be easily used in resource-limited countries. Unfortunately, even the IBFM ALL-Intercontinental (ALL-IC) protocol requires intensive resources outside the reach of most LMICs. The developing world needs to learn to draw up a cost-effective, resource-suitable protocol with the help of the leaders of ALL. The free exchange of experience through the International BFM meetings, Ponte de Legno meetings and publications in English literature have done a lot to rapidly learn how to treat ALL.

13.15 Ethics, IRBs and Databases: Avoiding Unnecessary Excessive Regulations

Currently even data collection study requires approval by institutional review boards (IRB). This is unfortunate as hospitals in LMICs often lack IRBs. Doctors who are already overwhelmed with clinical work do have time to submit unnecessarily complex forms and applications. Applying HIC ethical standards to LMICs which lack the infrastructure and manpower is unhelpful. Champions for each national cooperative groups can design a common universal simple consent form that can be used for all hospitals.

Imposing excessive regulation like personal data protection and complicated ethics and health regulatory requirements will hamper early effective implementation of ALL treatment in developing countries. An example is the now defunct Pediatric Oncology Database (POND) which successfully supported multiple paediatric cancer centres to collect simple data and survival outcomes. But concerns about personal data security have unfortunately undermined an otherwise simple to use and intuitive online database. New databases like ReDCap will help but they require more specialized training to use.

In summary, cALL is a curable disease even in developing countries. Resource-stratified treatment protocols, identifying an inspiring leader, training a dedicated team of doctors and nurses and provision of socio-economic support for families of children with ALL are some of the critical components of a successful programme. Copying resource-intensive protocols and ethics requirements from HIC are inappropriate.

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