Chapter 3
Minimal Residual Disease in Acute Lymphoblastic Leukemia: Techniques and Application

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Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of diseases with different clinical, phenotypic, and genetic features and variable response to therapy. ALL predominantly occurs in children but affects adults as well. The estimated annual incidence of ALL is 1–4.75 cases per 100,000 people [1, 2]. In newly diagnosed pediatric ALL, 80–85% of cases have a precursor B-cell phenotype (B-ALL), and 12–15% have a precursor T-cell phenotype (T-ALL) [1, 3].

With contemporary chemotherapy protocols, the survival rates among children and adolescence with ALL have improved substantially over the past several decades. Pediatric ALL has been considered a highly curable disease, with 5-year event-free survival (EFS) above 85% [4, 5]. Outcomes for T-ALL, historically inferior to B-ALL, have also significantly improved with recent advances in therapy, with 5-year EFS over 85% [6–8].

A significant reason for the improvement in outcome for ALL is the implementation of risk-stratified therapy based on patient characteristics and types of leukemia as well as response to therapy [4, 9, 10]. Minimal or measurable residual disease (MRD), measured by sensitive methods at various time points post-induction

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therapy, represents the integration of biological features of leukemia, patient characteristics, and chemotherapy regimens, which together determine treatment efficacy. In both pediatric and adult ALL, MRD status is the most powerful prognostic factor and highly predictive of relapse, superseding other historically relevant factors including age, white blood cell count, and cytogenetics [11–25]. MRD has proven utility in risk group assignment and informing tailored management of patients, including intensification or reduction of chemotherapy, hematopoietic stem cell transplantation (HSCT), and novel therapies.

Given the unequivocal prognostic impact of MRD by numerous studies, it is essential to develop sensitive, accurate, and standardized methods for MRD detection and monitoring. The assessment of MRD has evolved substantially over the past decade with improvements in technology. Currently, MRD in ALL is most commonly evaluated by multiparametric flow cytometry and real-time quantitative polymerase chain reaction (RQ-PCR)-based methods. Most recently, new molecular methods, such as high-throughput sequencing (HTS), have evolved into routine laboratory tools to improve the sensitivity and specificity of MRD detection and to enhance prognostication.

Concept of MRD

Current multiagent regimens allow the majority of patients with ALL to achieve durable remission. Traditionally, treatment response is determined by morphology-based methods and clinical criteria [26]. However, many patients achieving morphologic remission ultimately relapse, indicating that morphology-based methods are neither sensitive nor specific enough to detect low levels of leukemic blasts. Highly sensitive methods are required to better assess the reduction in disease burden and to recognize impending relapse.

The first report of detection of morphologically non-evident residual disease in leukemia was published nearly four decades ago, which identified residual leukemic cells in the bone marrow of patients with T-ALL using fluorochrome-conjugated antisera by fluorescence microscopy [27]. That led to the introduction of the fundamental concept of MRD which is used to describe the presence of leukemic blasts after therapy at a level below the limit of conventional cytomorphologic detection (<5% of blasts). It is estimated that MRD is present in 33–47% of adult patients with B-ALL following induction therapy [28]. On the basis of the independent and high prognostic value of MRD for outcome seen in numerous studies, there is a strong rationale to incorporate MRD status into the criteria to define treatment response. The Consensus Development Workshop on MRD from major European study groups established a standardized description of MRD-based response, including “complete MRD response,” “MRD persistence,” and “MRD reappearance,” which allows a standardized assessment of response to treatment and for comparison of MRD results between different treatment protocols [29]. The definition of remission has gradually evolved; recent studies have proposed to use both morphology and MRD when assessing

remission [30, 31]. The current risk stratification strategy in ALL combines conventional risk factors and MRD into a decision algorithm.

Techniques for MRD Detection

To be clinically informative, optimal MRD assays should reliably discriminate leukemic blasts from normal lymphoid cells with high sensitivity consistently during the course of therapy, facilitate a timely report of results, and allow wide implementation with interlaboratory standardization. Multiparametric flow cytometry to identify leukemic blasts by immunophenotypic aberrancy and RQ-PCR-based methods to detect leukemia-specific rearranged immunoglobulin (IG) and T-cell receptor genes (TCR) are the most commonly used MRD assays in clinical practice [32]. Reverse transcriptase quantitative PCR (RT-qPCR) amplification of oncogenic fusion transcripts from balanced chromosome translocation is less commonly used because the identifiable fusion transcripts are only present in a subset of ALL. With recent advances in HTS, much effort has been devoted to the development of HTS-based MRD assays and their implementation in clinical practice. Currently, multiparametric flow cytometry and RQ-PCR analysis of IG/TCR gene rearrangements are informative in >95% of patients with Ph-negative B-ALL and T-ALL. While RT-qPCR-based testing of BCR/ABL1 fusion is a commonly used method for MRD monitoring in Ph-positive B-ALL [33, 34], a persistent signal may not correlate with outcome due to the presence of the translocation in preleukemic stem cells or mature forms derived from those stem cells, so supplementation by another method is now increasingly common.

Multiparametric Flow Cytometry

Methodological Principles of MRD Detection by Flow Cytometry

Discriminating leukemic blasts from normal lymphoid progenitors relies on the immunophenotypic principle that the antigen expression patterns on the normal lymphoid progenitors through all stages of differentiation are highly reproducible and differ from those seen on leukemic blasts, which have altered patterns of antigen expression resulting from underlying genetic mutations [35]. At present, this fundamental principle is applied in two related methodological approaches for MRD detection by flow cytometry.

The first approach is based on identification of a combination of antigens with aberrant expression patterns on the leukemic blasts, designated “leukemia-associated immunophenotypes” (LAIPs) that are not observed in normal lymphoid progenitors [36]. The main types of LAIPs include asynchronous antigen expression, cross-lineage antigen expression, antigen overexpression/underexpression, and

ectopic phenotypes [19, 37]. LAIPs are first identified at diagnosis, using an antibody panel to define regions in multiparametric space that are occupied by leukemic blasts but not normal lymphoid cells. Following treatment, the informative antibody panel identified at diagnosis is used on post-therapy samples, and any leukemic blasts present in the predefined LAIP regions are considered as MRD. Leukemic blasts may have multiple LAIPs recognized in the diagnostic sample, all of which should be carefully followed in the subsequent samples to improve sensitivity and specificity of MRD detection. The increased number of fluorochromes and the ability to analyze more antigens simultaneously would in principle improve the confidence of identification of a leukemic blast population with specific LAIPs.

Although it has been successfully applied in some studies, this strategy has some limitations. First, LAIPs of leukemic clones are not always stable throughout the therapy [38–41], likely due to leukemic blast heterogeneity and subclone selection. One study observed a change of expression of at least one antigen in 69% of the cases with B-ALL between diagnostic and relapsed samples [38]. Such immunophenotypic shifts may lead to false-negative results, if rigid gating strategy with defined regions is used to identify MRD. Second, the immunophenotype of the background normal lymphoid progenitors and leukemic blasts may be altered under the influence of therapeutic drugs [38, 42–44]. It has been shown that steroid treatment in patients with B-ALL can induce immunophenotypic modulation of leukemic blasts including downregulation of immature antigens CD10 and CD34 and upregulation of mature antigens CD20 and CD45. In T-ALL, immaturity-associated antigens, such as TdT, CD99, and CD34, were dramatically reduced during therapy, while lineage-associated markers remained relatively stable [45]. Similarly, the immunophenotype of normal lymphoid progenitors may also change, causing it to appear in the regions predefined for abnormal blasts which results in a false-positive result. In addition, noise from nonspecific binding of reagents can be present in some samples and be counted as part of the LAIP, resulting in a false-positive result. Lastly, this method is entirely dependent on the LAIPs identified at diagnosis. Without the prerequisite knowledge of diagnostic LAIPs, an individualized antibody panel cannot be constructed to define regions for precise MRD detection. This requirement will have significant impact on its application in reference laboratories and tertiary care centers, where only post-therapy samples are available.

An alternative approach, known as “difference from normal,” relies on the theory that the immunophenotype of the leukemic blasts differs from the spectrum of antigenic expression patterns on normal lymphoid cells of similar lineage and maturational stage [46, 47]. At initial diagnosis, this method establishes the specific immunophenotype of leukemic blasts, similar to the identification of LAIPs. As such, this method is a superset of the LAIP approach. In the post-therapy samples, all progenitor populations at varying maturation stages are evaluated to look for discrete populations having immunophenotypic aberrancies that deviate from the antigenic patterns of normal progenitors (Fig. 3.1). The immunophenotype identified in the diagnostic sample can be used as a starting point for post-therapy assessment, but diagnostic LAIPs to define regions for MRD are not required by the “difference from normal” approach. A standard antibody panel emphasizing normal

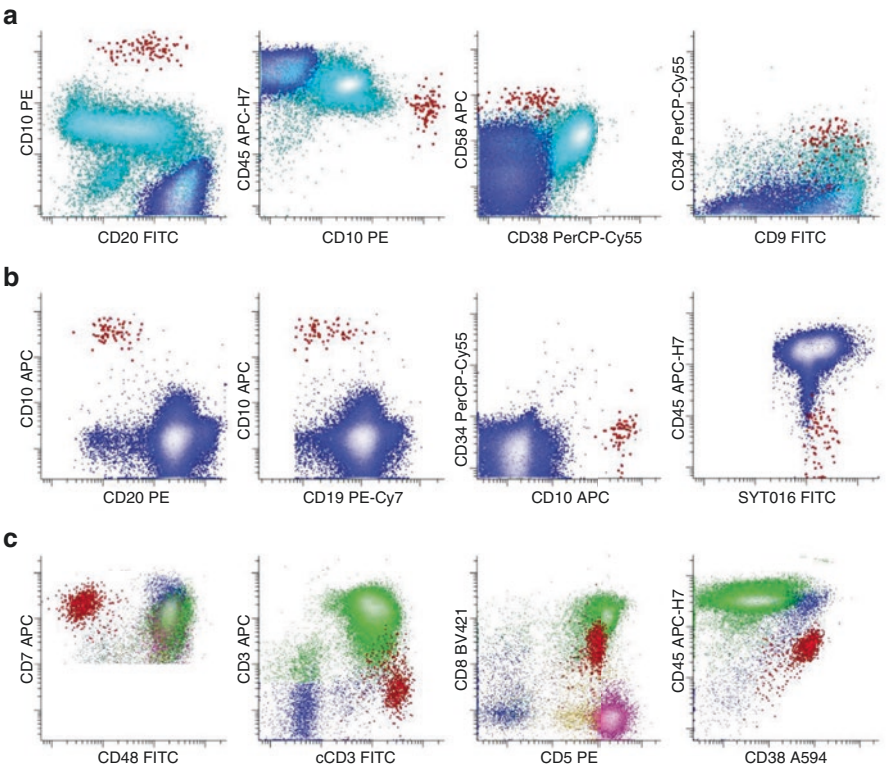

Fig. 3.1 The detection of MRD for acute lymphoblastic leukemia (ALL) following induction therapy by flow cytometry. Bone marrow (**a**, **c**) or peripheral blood (**b**) post-induction therapy was analyzed with an informative antibody panel. The antibody combination allows the identification of residual leukemic blasts by deviation from normal lymphoid progenitors based on lineage and maturational stage

(**a**) B-ALL MRD. The leukemic blasts (red) that represent MRD are characterized by abnormal expression of CD9 (uniform), CD10 (increased), CD34 (slightly increased), CD38 (decreased), CD45 (slightly decreased), and CD58 (increased) relative to immature normal B-cell precursors (cyan) of similar maturational stage. The mature B cells are colored in blue. The population is enumerated at 0.011% of total nucleated cells and 0.023% of nucleated mononuclear cells

(**b**) B-ALL MRD (day 8 post-induction therapy). The leukemic blasts (red) that represent MRD are characterized by abnormal expression of CD10 (increased), CD19 (decreased), CD34 (slightly increased), and CD45 (decreased) comparing to normal immature B-cell precursors, which should not be present in the peripheral blood at this time point. The mature B cells are colored in blue. The population is enumerated at 0.02% of total nucleated cells

(**c**) T-ALL MRD. The leukemic blasts (red) that represent MRD are characterized by abnormal expression of CD3 (absent on the surface, present in the cytoplasm), CD5 (slightly decreased), CD7 (slightly increased), CD8, CD38 (increased), and CD48 (absent) relative to mature T cells (green and pink). The population is enumerated at 0.33% of white cells and 1.1% of nucleated mononuclear cells

patterns of lymphoid maturation and including aberrant antigens commonly identified in LAIPs, rather than individualized antibody panels for LAIPs, can be implemented, which are important factors to consider especially for reference laboratory testing. Immunophenotypic shifts on leukemic blasts during therapy, which may have an impact on MRD detection by LAIP evaluation, do not affect MRD recognition by the "difference from normal" approach in a majority of cases. Despite all these advantages over the LAIP method, "difference from normal" does require extensive expert knowledge of antigenic expression patterns on lymphoid progenitors during normal differentiation and regeneration, making interlaboratory standardization and implementation of data interpretation challenging.

In clinical practice, an integrated strategy using components of both methods simultaneously is commonly applied to improve diagnostic accuracy. Flow cytometry assay sensitivity is largely dependent on the number of events acquired, the antibody panels used, and the degree of immunophenotypic deviation of the leukemic blasts from normal progenitors. Despite the fact that the recommended number of events for acquisition and the number of events to define a clonal leukemic blast population vary significantly among laboratories, a sensitivity of 0.01% can be achieved in a large majority of B-ALL and T-ALL. Assay sensitivity can also vary at different time points post-therapy as some abnormal immunophenotypes may be challenging to differentiate from immunophenotypic aberrancies associated with active marrow regeneration. In a sample with a large number of normal lymphoid progenitors, assay sensitivity may be significantly reduced unless the leukemic population shows prominent abnormalities.

Flow Cytometry Antibody Panels

Desirable MRD assays should have minimal antibody interaction and provide high fluorescence intensities on leukemic blasts with low background. Although there is no consensus on antibody panel selection, different combinations have been tested in B-ALL MRD detection [17, 48–52]. The optimal antibody panels for B-ALL MRD would facilitate identification of leukemic blasts with aberrant, immature immunophenotypes that deviate from normal lymphoid cells. The selection of antibodies for T-ALL MRD emphasizes the ability to identify immunophenotypic features of immature T cells distinct from mature T and NK cells, as the presence of immature T cells in peripheral blood or bone marrow strongly suggests MRD (Table 3.1) [45, 47]. The reagent panels for T-ALL MRD are less well defined and validated because of the low frequency of the disease.

The Children's Oncology Group (COG) assay uses two 6-color reagent combinations for identification of leukemic blasts, and a third reagent combination containing a DNA/RNA binding dye (Syto16) provides a nucleated cell denominator for enumeration (Table 3.1) [12]. Residual leukemic blasts are enumerated as the percentage of total nucleated mononuclear cells, excluding maturing granulocytic cells and including nucleated erythroid cells. MRD measured by the COG assay is highly prognostic in pediatric B-ALL and useful in risk stratification and

Table 3.1 Antibody panels for the detection of MRD in acute lymphoblastic leukemia (ALL)

A. Antibody panels recommended by Euro-Flow Consortium for B-ALL MRD									
	PB	PO	FITC	PE	PerCP5.5	PE-Cy7	APC	APC C750	
1	CD20	CD45	CD81	CD66c/ CD123	CD34	CD19	CD10	CD38	
2	CD20	CD45	CD81	CD73/ CD304	CD34	CD19	CD10	CD38	
B. COG antibody panels for B-ALL MRD									
	FITC	PE	PerCP5.5	PE-Cy7	APC	APC-H7			
1	CD20	CD10	CD38	CD19	CD58	CD45			
2	CD9	CD13 + 33	CD34	CD19	CD10	CD45			
3	Syto16		CD3	CD19	CD71	CD45			
C. COG antibody panels for B-ALL MRD at day 8 post induction (peripheral blood only)									
	FITC	PE	PerCP5.5	PE-Cy7	APC	APC-H7			
	Syto16	CD20	CD34	CD19	CD10	CD45			
D. Antibody panels for B-ALL MRD in patients post anti-CD19 therapy									
	BV421	BV510	FITC	PE	PerCP5.5	PE-Cy7	APC	APC-H7	
	CD10	CD38	CD66b	CD22	CD34	CD20	CD24	CD45	
E. COG antibody panels for T-ALL MRD									
	V450/ BV421	FITC	PE	PE-CF594	PE-Cy5	PE-Cy7	A594	APC	APC-H7
1	CD16	cCD3	CD7		CD56	CD5	CD38	sCD3	CD45
2	CD8	CD48	CD5	CD34	CD56 + 16	CD3	CD4	CD7	CD45
3	Syto16		CD7		CD56	CD3		CD71	CD45

A594 Alexa Fluor 594, *APC* allophycocyanin, *FITC* fluorescein isothiocyanate, *PE* phycoerythrin, *PE-Cy5* PE-cyanine-5, *PE-Cy7* PE-cyanine-7, *PerCP5.5* PerCP-Cy5.5, *PO* pacific orange

risk-directed therapy [12] and has been implemented in a standardized manner in a network of more than 20 laboratories internationally.

Recently, the EuroFlow Consortium described a fully standardized 2-tube, 8-color antibody panel for B-ALL MRD testing (Table 3.1) after multiple rounds of multicenter optimization, which allowed separation between leukemic blasts and normal lymphoid progenitors in >99% of the patients [53]. In samples with sufficient cells (>4 million) analyzed, flow cytometric MRD assay reached a sensitivity of ≤0.001% (10^{-5}), similar to RQ-PCR-based method. The concordance between flow cytometric and RQ-PCR-based MRD data was 98% (97% for samples with MRD < 0.01%).

Over the past several years, immunotherapy has been introduced to patients with relapsed/refractory B-ALL and demonstrated encouraging results. Both chimeric antigen receptor-expressing T cells (CAR-T cells) and bi-specific T-cell engager (BiTE) directed against B-cell marker CD19 deplete both normal B cells and leukemic blasts expressing CD19 [54–58]. As a principal reagent to identify B cells,

CD19 alone is insufficient to enrich for B cells after anti-CD19-targeted therapy. In this context, other B-cell markers must be incorporated to allow for B-cell identification and MRD detection. A novel flow cytometric assay using both CD22 and CD24 as alternative gating reagents for B cells has been described and validated in the setting of immunotherapy targeting CD19 or CD22 (Table 3.1) [59]. The proposed combination showed a good correlation with the standard flow cytometric assay for B-ALL MRD detection and successfully identifies both CD19-positive and CD19-negative leukemic blasts.

Real-Time Quantitative PCR

IG and TCR genes undergo rearrangements during early stages of B and T lymphocyte maturation. Leukemic blasts in ALL originate from one single lymphoid progenitor and therefore carry the same clonally rearranged IG and TCR genes. RQ-PCR-based MRD testing relies on identification of clonally rearranged IG and TCR genes which represent unique sequences in individual leukemic blasts among normal lymphoid cells expressing rearranged genes with different sequences [60]. While IG rearrangements are more frequently detected in B-ALL and TCR rearrangements more commonly found in T-ALL, both B-ALL and T-ALL leukemic blasts can display cross-lineage rearrangement [61, 62]. Clonal IG heavy chain (IGH) and TCR gamma (TCRG) gene rearrangements can be detected in >90% of B-ALL and T-ALL [63, 64]. Approximately 70% of clonal IGH rearrangements are preserved in relapsed B-ALL [65, 66], whereas ~90% of rearrangements are preserved in relapsed T-ALL [67]. Therefore, it is recommended that at least two independent clonal IG/TCR markers are used for MRD detection to reduce false-negative results [68]. With additional PCR targets [such as IG kappa, incomplete IGH, TCR beta (TCRB), TCR delta (TCRD), etc.], 90–95% of ALL patients can be monitored by at least two sensitive MRD targets [60, 64]. This assay generally reaches a sensitivity of 0.01–0.001% (10^{-4}–10^{-5}).

RQ-PCR-based MRD testing is a complex, multistep process. The specific IG or TCR gene rearrangements are sequenced at diagnosis for target identification, allele-specific oligonucleotide (ASO) primers designed complimentary to the unique patient or leukemic-specific junctional region sequences are synthesized, and RQ-PCR conditions are optimized for each target. The ASO primers are then applied to post-therapy samples to identify patient-specific IG/TCR gene rearrangements, and quantification of MRD is achieved by comparing the amplified product to a standard curve established from amplification of serial dilution of a control gene [69]. As a result, the RQ-PCR-based method is time-consuming and laborious and requires extensive knowledge and expertise; therefore, standardization and quality control of the assay are critical for correct interpretation of data and to allow interlaboratory comparison of MRD results. This method has been thoroughly standardized via international collaboration through the efforts of the Euro-MRD

groups, with established guidelines for determination of quantitative range, sensitivity, specificity, and reproducibility for each assay [29, 60, 70].

In addition to IG and TCR gene rearrangements, leukemic-specific gene fusion transcripts, such as *BCR/ABL1* and *MLL* rearrangements, are found in one third of ALL and can also be used as targets for quantitative measurement of residual leukemic cells at mRNA level by RT-qPCR [29]. This assay uses the same primer/probe combination for all patients and is more sensitive (up to 10^{-6}) than DNA-based assay as many copies of mRNA can be present in a leukemic blast.

High-Throughput Next-Generation Sequencing (HTS)

With recent advances in sequencing technology, HTS has become an emerging tool for MRD detection with improved sensitivity compared to flow cytometry and RQ-PCR and has demonstrated a potential as a diagnostic platform. This technique has high-level multiplexing capacity that allows for simultaneous amplification of all possible combinations of the rearranged IG or TCR loci using consensus primers. At diagnosis, HTS detects patient-specific index clonal IG/TCR gene rearrangements using universal primers. The same procedure is applied to the posttreatment samples to identify the index sequences and quantify MRD, eliminating the requirement for patient-specific ASO primers [14]. In addition, HTS can detect small neoplastic subclones present after therapy not identified by flow cytometry or RQ-PCR and monitor clonal evolution that is the source of false-negative results seen with RQ-PCR-based methods. Leukemic clones detected by HTS at relapse can be genetically identical to, evolved from, or completely distinct from diagnostic clones [71]. In theory, HTS-based techniques can reach a sensitivity below 10^{-5} for MRD detection. Some studies using commercial assays have claimed an even lower limit of MRD detection of 10^{-6}–10^{-7} in B-ALL [72, 73].

HTS offers higher sensitivity and precision than other MRD techniques and has been applied to MRD monitoring in ALL. In one cohort of T-ALL, HTS identified at least one clonal TCRB or TCRG rearrangements in 81% of pretreatment samples. At day 29, HTS showed greater sensitivity and specificity than flow cytometry in MRD assessment by detecting the original clonal TCRB sequences [74]. The absence of clonal TCRB rearrangements was associated with early thymic precursor (ETP) or near-ETP subtypes, where rearrangement of TCR had not yet occurred. Similarly in B-ALL, HTS of IGH genes detected clonal IGH rearrangements in 95% of diagnostic samples and successfully identified MRD in day 29 posttreatment samples with a tenfold increase in the lower limit of detection as compared to flow cytometry [75]. These findings suggest the potential clinical utility of HTS in MRD monitoring and risk stratification. Prospective studies will be needed to compare the predictive values of MRD by HTS and standard methods. As HTS becomes more applicable and affordable, consensus guidelines for data interpretation and its clinical use are expected before implementation in ALL MRD surveillance.

Comparison of Methods for MRD Detection

The advantages and logistical challenges associated with different MRD techniques are outlined in Table 3.2. The choice of MRD techniques is mainly dependent on the clinical trial design and available resources. The major advantages of flow cytometry are general applicability to all ALL, wide availability of the assay, rapid reporting of the results allowing for prompt decision making, and simultaneous assessment

Table 3.2 Advantages and limitations of MRD techniques in acute lymphoblastic leukemia (ALL)

	Multiparametric flow cytometry	Real-time quantitative PCR	Reverse transcriptase quantitative PCR	High-throughput sequencing
Target	Leukemia-associated immunophenotypes or "difference from normal" approach	IG/TCR gene rearrangements	Leukemic fusion transcripts	IG/TCR gene rearrangements
Applicability	Essentially all ALL	>95% of ALL	25–40% of B-ALL, 10–15% of T-ALL	>95% of ALL
Sensitivity	3–4 colors: 0.1–0.01%	0.01–0.001%	0.01–0.001%	0.0001%
	6–10 colors: 0.01–0.001%			
Specimen	Viable cells	DNA	RNA	DNA
Turn-around time	1–2 days	~ 4 weeks	1–3 days	1–2 weeks
Availability	Widely available	Widely available in Europe	Widely available	Largely experimental, limited availability
Cost	Moderate expense	More expensive	Moderate expense	Most expensive
Advantages	Rapid resulting	High sensitivity	Rapid resulting	High sensitivity
	Direct quantification	Thorough standardization	High sensitivity	Readily standardized
	Identifies and monitors therapeutic targets	Clinically validated role in risk stratification and treatment decisions by various clinical trials	Does not require patient-specific assays	Detects subclones and clonal evolution
	Provides information on cellular composition		Targets stable during treatment	Provides information on physiological B/T-cell repertoire
				Does not require patient-specific assays

Table 3.2 (continued)

	Multiparametric flow cytometry	Real-time quantitative PCR	Reverse transcriptase quantitative PCR	High-throughput sequencing
Disadvantages	Inadequate interlaboratory standardization	High cost	Only applicable to ALL harboring detectable fusion transcripts	High cost
	Requires expert knowledge for data interpretation	Requires diagnostic sample to identify patient-specific IG/TCR gene rearrangements	Limited standardization	Requires diagnostic sample to identify patient-specific index IG/TCR gene rearrangements
	False negativity resulting from immunophenotypic shifts or confounding regenerating progenitors	Requires construction of patient-specific primers	Instability of RNA	Requires complex bioinformatics
		Time consuming and labor intensive	Uncertain quantification of leukemic blasts	Limited clinical validation
		False negativity resulting from clonal evolution		

of cellular characteristics required for targeted therapies. Unlike RQ-PCR that requires patient-specific primers, flow cytometry uses standardized antibody panels for essentially all patients. The main challenge in performing MRD detection by flow cytometry is the lack of reproducibility across laboratories due to considerable variability in instrumentation, reagents and procedures, data analysis software, and reporting [76, 77]. Because the data interpretation is inherently subjective, expert knowledge of normal and regenerative antigenic expression pattern of lymphoid progenitors and experience with immunophenotypic shifts post-therapy are required for accurate data interpretation. Therefore, interlaboratory standardization of methodologies is necessary to ensure comparability of MRD results between different laboratories and treatment protocols. As shown for the COG assay, training the laboratories to use a standardized assay, along with systemic education and feedback on MRD data interpretation, can reduce discordance among interpreters [78]. The recent technical innovations including flow cytometers that allow for more colors and automated data analysis [79] could improve sensitivity, specificity, and time effectiveness of MRD detection.

RQ-PCR-based MRD assay is the gold standard method in ALL and has been extensively optimized and standardized in Europe. Although it is labor-intensive, time-consuming, and expensive, RQ-PCR analysis of IG/TCR rearrangement for MRD is 1-log more sensitive (10^{-4}–10^{-5}) than that achieved by standard flow cytometry [52, 80, 81]. This assay requires a laborious initial characterization of IG/TCR gene rearrangements in leukemic blasts and construction of patient-specific ASO primers for posttreatment testing, making it challenging and expensive in a routine clinical setting. Other limitations include false-negative results due to clonal evolution or emergence of a new clone and false-positive results caused by nonspecific amplification of IG/TCR genes in background lymphoid progenitors [82]. Currently, MRD assessment by flow cytometry is the standard of care in ALL in the United States, whereas RQ-PCR-based testing is commonly used in European clinical trials.

Most studies have shown that flow cytometry and RQ-PCR analysis of IG or TCR rearrangements generate concordant MRD measurements, for MRD levels >0.01% [48, 52, 80, 83–85]. The discordant cases were frequently seen with low levels of MRD (<0.01%), mostly flow-negative/RQ-PCR-positive. The discordance can be explained by the higher sensitivity of the RQ-PCR assay, presence of nonviable blasts detected by RQ-PCR but not by flow cytometry, nonspecific amplification of normal DNA resulting in false-positive RQ-PCR results, immunophenotypic changes post-therapy, and the presence of confounding regenerating blasts which may reduce the sensitivity of flow cytometry. In B-ALL, the concordance between flow cytometry and RQ-PCR was time point-dependent; most discordance was found at day 33 post-therapy (70% concordant), as compared with day 15 (86% concordant) and day 78 (87% concordant) [52]. Patients with discordant MRD results at day 33 had an intermediate clinical outcome much closer to concordantly negative cases than to the concordantly positive cases, suggesting that the presence of very low level of discordant MRD at day 33 is not strongly predictive of outcome.

HTS platforms may mitigate some of the limitations of flow cytometry and RQ-PCR. Similar to RQ-PCR, a sample containing a relatively large number (>5%) of leukemic blasts is required for identification of clonally rearranged IG/TCR gene index sequences. Importantly, HTS uses a standardized assay with multiplexed primers for both diagnostic and subsequent samples and is therefore less laborious and time-consuming than standard RQ-PCR assays. HTS has the ability to detect minor subclones and monitor clonal evolution, reducing false-negative results seen by RQ-PCR assays. In addition, HTS also allows the evaluation of the heterogeneity of the normal lymphoid repertoire. Comparing to flow cytometry, HTS is less likely to be affected by immunophenotypic shifts and the presence of regenerating blasts, and data interpretation is less subjective. Nevertheless, flow cytometry provides more rapid reporting than either RQ-PCR or HTS, which is important when rapid clinical decision-making is needed, e.g., at End of Induction (EOI).

Early studies have suggested a higher analytic sensitivity of MRD detection at 10^{-6}–10^{-7} in B-ALL by HTS than that can be achieved by flow cytometry and RQ-PCR [73]. Subsequent studies further demonstrated HTS could detect MRD in posttreatment samples that was not identified by flow cytometry in both B-ALL [75]

and T-ALL [74]. In both studies, the MRD results were highly concordant between HTS and flow cytometry at the limit of detection of 0.01%. HTS additionally detected very low levels of MRD not identified by flow cytometry in a significant subset of patients. Comparing with RQ-PCR, MRD results in B-ALL by both methods were concordant in 85–96% of patients [73, 86]. Using an MRD threshold of 0.01%, HTS was comparable to flow cytometry in predicting outcome and risk stratification [87]. Despite these promising results, clinical relevance of MRD measured by HTS needs to be further defined in randomized trials before implementation of HTS into routine MRD monitoring and risk stratification.

Clinical Application of MRD

Multiple published trials have demonstrated the indispensible prognostic value of MRD in both pediatric and adult ALL regardless of disease subtype, therapeutic regimen, method and timing of MRD assessment, and threshold of MRD cutoff [88]. As a result, MRD status has been incorporated into clinical trials to assess response to initial treatment, for subsequent MRD-based risk stratification, and to direct future therapy. It is important to recognize that the clinical impact of MRD is strictly dependent on the timing of MRD assessment and MRD threshold for decision-making determined by therapeutic protocols. Therefore, MRD data cannot be directly extrapolated from one treatment regimen to another but rather must be evaluated under the same therapeutic conditions.

Prognostic Implication of MRD

MRD in Frontline Chemotherapy

Many studies unanimously support the significant prognostic impact of MRD in ALL, and therefore MRD serves as a critical component for risk stratification. The first large-scale prospective study AIEOP-BFM-ALL2000 in childhood and adolescent B-ALL introduced standardized assessment of MRD by RQ-PCR (sensitive of at least 10^{-4}) at two time points for risk stratification [9]. MRD negativity at day 33 post-induction is the strongest predictor for excellent 5-year EFS, and high levels of MRD ($\geq 10^{-3}$) at day 78 are highly predictive of relapse. Similar conclusions were drawn in pediatric patients with T-ALL enrolled in the same protocol [89]. Other study groups confirmed the independent prognostic impact of MRD, however, using different timing and methods of MRD testing and different cutoff values of MRD [11, 12, 18, 90, 91]. In a Swedish multicenter study, a MRD level ≥0.1% at day 29 quantified by both flow cytometry and RQ-PCR predicted high risk of relapse in children with B-ALL [81]. In T-ALL, MRD detected by RQ-PCR was superior to flow cytometry in predicting relapse.

The independent prognostic effect of MRD is also recognized in adult ALL using RQ-PCR [16, 25, 92] or flow cytometry [15]. Conventional prognostic factors lose their prognostic value when MRD status was included in the analysis [16, 25]. A retrospective study of adults with MRD-positive ALL ($\geq 10^{-4}$) by flow cytometry or RQ-PCR who received standard treatment of care between 2000 and 2014 was recently performed using the European ALL study group database [93]. The data showed relatively short relapse free survival (RFS) and overall survival (OS) in patients with MRD-positive ALL, particularly at higher MRD levels, while lower baseline MRD level was a strong predictor for better RFS. Early complete molecular response during induction therapy was associated with an excellent outcome [94].

Several studies have assessed the prognostic utility of HTS-based monitoring for MRD and have reported that MRD measured by HTS predicts risk of relapse in both pediatric [73, 86, 95] and adult ALL [96]. In the recent COG studies AALL0331 and AALL0232, using a MRD threshold of 0.01%, HTS was equivalent to flow cytometry in its ability for risk stratification in childhood B-ALL at EOI [87]. Reducing the threshold of HTS below 0.01% at EOI did not improve risk stratification in general but allowed identification of a small subset (19.9%) of standard-risk MRD-negative patients who had an outstanding outcome and required no further therapy. Although low-positive MRD ($<10^{-4}$) and high-positive MRD ($\geq 10^{-4}$) were similarly associated with decreased leukemia-free survival [95], HTS provided opportunity to identify additional patients with MRD who would benefit with intensified therapy. Despite enhanced sensitivity of HTS to $<10^{-6}$, the clinically actionable MRD threshold for most patients appears to be unchanged. Comparison of the clinical utility of these methods should be addressed in prospective studies before definite adoption of HTS to replace other methods in MRD quantification for optimal risk stratification.

MRD in Pre- and Post-hematopoietic Cell Transplant

The prognostic impact of MRD status prior to hematopoietic cell transplant (HCT) is well established in children and adults [72, 97–101]. Many studies have also explored the importance of post-HCT MRD status, and all demonstrated that any evidence of MRD is significantly associated with increased risk of relapse [99, 100, 102–105]. Studies have also been performed to evaluate whether more sensitive HTS better predicts relapse than standard methods in the setting of pre- and post-HCT. In a small cohort of adult B-ALL patients, MRD detected by HTS within 30 days prior to HCT predicts post-HCT relapse [100]. After HCT, MRD $\geq 10^{-6}$ detected in blood samples had shown a 100% positive predictive value for relapse. Comparing to flow cytometry, pre- and post-HCT MRD detected by HTS predicts relapse and survival more accurately than 6-color flow cytometry in pediatric patients with B-ALL [72]. Therefore, post-HCT MRD monitoring by HTS is useful in detecting impending relapse for early intervention before overt relapse.

MRD In Ph-Positive ALL

Ph-positive B-ALL accounts for about 25% of adult ALL [1]. While MRD is the most significant prognostic factor in Ph-negative ALL, the utility of MRD assessment in Ph-positive is not well defined. In patients treated with frontline chemotherapy combined with tyrosine kinase inhibitors (TKI), complete molecular response measured by RT-qPCR at 3 months was associated with superior RFS and OS even without HCT compared with those with lesser molecular response [106, 107]. The French GRAAPH-2003 study, however, showed that early MRD evaluation did not significantly influence OS and disease-free survival [108]. The French GRAAPH-2005 study further confirmed that early MRD response was less discriminant than WBC [109]. HCT improved outcome in patients with persistent MRD, but patients who achieved major molecular response did not benefit from HCT. To explore the predictive value of MRD in the setting of HCT, recent studies on Ph-positive ALL patients treated with chemotherapy and TKI support the prognostic relevance of MRD before HCT [110, 111]. Achieving a complete molecular response prior to HCT significantly reduced the risk of relapse after HCT. Future prospective studies using MRD-based stratification may be necessary to clarify remaining issues and shed light on optimal management in Ph-positive ALL.

MRD in Targeted Therapy

In the era of immunotherapy, MRD assessment can recognize patients that may benefit from novel therapeutic agents, in particular, in patients who are not candidates for HCT. Inotuzumab ozogamicin directed against CD22 [112], BiTE blinatumomab [58, 113], and CAR-T cells directed against CD19 [55, 56] have been used in relapsed/refractory ALL and have shown to improve survival, in part mediated through inducing complete MRD response. The FDA has approved the use of blinatumomab in childhood and adult ALL patients with MRD $\geq$ 0.1% in first or second CR based on a phase 2 trial [114]. Of 116 patients evaluated, patients who achieved MRD negativity (78%) had significantly longer RFS and OS than patients with persistent MRD. In a subsequent large phase 3 study randomizing Ph-negative relapsed/refractory ALL patients between blinatumomab and standard of care salvage chemotherapy, patients receiving blinatumomab had significantly higher rates of complete remission (CR) and negative MRD status and longer EFS and OS than with chemotherapy [115]. This is the first time the FDA used MRD endpoint as the basis for approval of a therapeutic agent. Regarding anti-CD19 CAR-T cells, several groups have shown that most of the responding patients became negative and maintain this status for several months or years [56, 116–118]. However, relapse rates are high even in patients achieving MRD negativity, different from frontline chemotherapy. After CAR-T therapy, MRD appears to be insufficient to predict long-term remissions. High-sensitivity MRD assays and early time point testing may be necessary to identify a subset of patients with rapid and deep response and

good prognosis. The prognostic relevance of MRD in the setting of novel therapies needs to be further elucidated in clinical trials.

Therapeutic Implication of MRD

Although there are profound differences in trial design, methods and timing of MRD testing, and threshold for MRD-directed therapy, the major prospective studies have provided strong evidence that risk-directed therapy based on the presence of MRD improves survival and reduces the relapse rate in intermediate- and high-risk pediatric ALL [7, 12, 119–122]. MRD levels at various time points post-therapy have been validated to predict relapse and incorporated into post-remission therapy regimen, including therapy intensification and HCT [123]. Similarly in adult ALL, high-risk patients with unfavorable MRD status can benefit from more intensive therapy, such as HCT, with significantly improved EFS and OS [15, 25, 92, 124].

On the other hand, MRD may be used to identify good responders that may benefit from treatment de-escalation to reduce toxicity [125, 126]. In MRC UKALL 2003 study of pediatric and young adult ALL, there was no significant difference in EFS in low-risk patients defined by MRD status at EOI (undetectable or $<10^{-4}$) who received one or two delayed intensification courses [125], implying that treatment reduction is feasible for low-risk patients. HCT does not appear to be beneficial in patients with low levels of or no detectable MRD and therefore should be avoided [15, 25].

It is very likely that MRD has different clinical and therapeutic meanings dependent on the underlying biology and genotype. Genome sequencing of pre- and post-treatment childhood B-ALL identifies two distinct evolutionary patterns, a highly dynamic pattern and a quasi-inert evolutionary pattern, governing early and late relapse, respectively [127]. If confirmed in other cohorts, these findings have clinical implications and emphasize the need for adapted treatment strategies to prevent therapeutic escape.

Practical Issues

Specimen Types for MRD Testing

MRD is tested and quantified in either peripheral blood or bone marrow, although the sample type has an apparent impact on assay sensitivity. MRD levels show a strong correlation in paired peripheral blood and bone marrow in T-ALL, but poor correlation in B-ALL with lower levels of MRD detected in peripheral blood [128, 129]. Therefore, bone marrow is the preferred sample to achieve optimal sensitivity for MRD detection in B-ALL. Recent studies demonstrate that although paired

peripheral blood and bone marrow samples showed comparable clonal distribution in most of patients with B-ALL, the peripheral blood does not consistently represent the clonal composition in the bone marrow, further implicating the importance of sample selection in MRD detection [130, 131].

Timing and Methods for MRD Assessment

MRD is a time point- and context-dependent variable that has different prognostic implications at different time points following treatment. Although there is a lack of full agreement on optimal timing and methods of MRD assessment, in B-ALL a threshold of 0.01% at EOI is commonly used to identify patients with a greater risk of relapse and is largely technology independent. In T-ALL the data are less robust, but a threshold of 0.01–0.1% at the End of Consolidation therapy appears to identify poor-risk patients, in part a reflection of slower leukemic blast clearance in T-ALL compared with B-ALL. In addition, the MRD threshold to identify poor-risk patients appears dependent on the underlying biology, for example, an MRD threshold of 0.1% at EOI is optimal to identify poor-risk patients within the pediatric standard-risk B-ALL cohort having double trisomies, while an MRD threshold of 0.01% provides similar risk stratification for the remaining pediatric standard-risk patients [132, 133]. Based on the intent of MRD assessment, a general strategy for MRD testing will likely require more than one technology. At early time points (EOI), flow cytometry or RQ-PCR-based methods, if applicable, can provide adequate sensitivity and rapid resulting for prompt decision-making. Further from therapy, especially post-remission surveillance, a high-sensitivity assay, such as HTS, is desirable as it is more likely that any level of MRD will be associated with a higher risk of overt hematologic relapse.

MRD Monitoring Post-remission

There is no consensus regarding the clinically appropriate interval for MRD monitoring in patients who are in remission and no longer receiving therapy. Given the different rates of response to therapy and different relapse kinetics of various leukemia subtypes, the schedule of MRD monitoring may vary significantly. In the German Multicenter ALL (GMALL) trial, the utility of MRD as an indicator of impending relapse was prospectively evaluated in 105 MRD-negative patients by RQ-PCR with a 3-month interval after consolidation [22]. Thirteen of 15 patients (89%) with MRD detected within the quantitative range of RQ-PCR subsequently relapsed after a median interval of 4.1 months, indicating that MRD positivity during early phase of post-consolidation is predictive of subsequent hematologic relapse. Base on these data, molecular relapse defined as conversion to quantifiable

MRD by RQ-PCR led to salvage therapy prior to hematologic relapse in GMALL trial. As to the length of MRD monitoring, a follow-up of 12 months after the end of the first year of therapy with 3-month interval seems adequate to detect most of the patients who later relapsed. A recent study confirmed the previous findings by demonstrating MRD positivity detected by flow cytometry at any time points after achieving CR was associated with a high risk of relapse in a series of 546 MRD-negative ALL patients [134]. This study monitored MRD at the time of CR and at ~3-month interval thereafter. MRD was detected in 55 patients with a median of 14 months, and 44 of 55 patients (80%) subsequently developed hematologic relapse after a median of 3 months. These findings support the concept that MRD detected in CR can predict hematologic relapse, making post-remission MRD monitoring necessary if pre-emptive intervention is planned.

MRD as a Surrogate Endpoint for Outcomes

There is a clinical need to identify novel endpoints that facilitate the assessment of drug efficacy at early stages than those allowed by conventional endpoints. To be considered, alteration in surrogate endpoints must reflect changes in outcomes. Although MRD is strongly correlated with outcomes in ALL for drugs in current use [88] and available at an early time point, it cannot reflect the long-term therapeutic or toxic effects of drugs. Several studies have indicated MRD response does not reflect the effect of drugs on outcomes [135, 136]. For the first time, a meta-analysis of individual data from two large phase 3 trials for childhood ALL [8, 135] was performed to formally evaluate whether MRD status at EOI frontline treatment is an accurate surrogate endpoint for EFS [137]. While the analysis confirmed the strong prognostic effect of early MRD response on EFS regardless of treatment, MRD at EOI was found to be a poor surrogate for treatment effect on EFS at the trial level. As MRD is commonly used for risk stratification in ALL, subsequent treatment is modulated based on MRD itself and may attenuate potential surrogacy. The data suggests the limitation of a powerful prognostic factor in being a surrogate endpoint in the setting of frontline ALL treatment. Using MRD as a primary endpoint for accelerated approval of a novel drug would require demonstration of a direct linkage between a change in therapy based on MRD and traditional clinical endpoints, such as EFS and OS.

Summary

MRD has emerged as a strong independent predictor of outcomes in pediatric and adult ALL. MRD monitoring provides an assessment of response to therapy that is more informative than that provided by morphologic evaluation. Novel techniques, such as HTS, have the potential to overcome the limitations of standard flow

cytometry and RQ-PCR-based methods, although reporting is not yet rapid enough for early clinical decision-making. MRD status post-therapy is crucial for risk assessment and for determining those patients that may benefit from therapeutic reduction or intensification to improve clinical outcome. However, the prognostic significance of MRD is dependent on the clinical scenario, leukemic biology, and timing of MRD testing. Disease and treatment-specific protocols should dictate the schedule of MRD monitoring, the optimal methods for MRD detection, and the cutoff value for MRD status, to ensure optimal risk stratification and personalized therapy. Integration of complex genetic information and MRD is likely to increasingly drive personalized clinical protocols. Future prospective studies are ultimately needed to prove the efficacy of MRD-adapted treatment in randomized trials.

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