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

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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

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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].

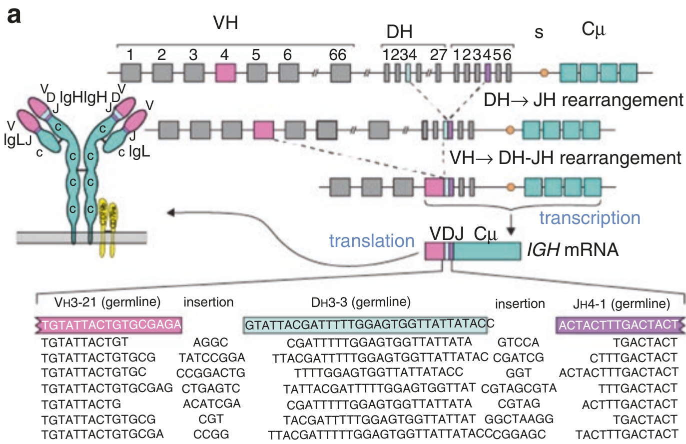
The first large scale PCR-based MRD studies were performed in childhood ALL, using IGH (VH-JH), TRG and TRD gene rearrangements as PCR targets, mainly because of the limited number of primers needed to detect these rearrangements [1, 2]. Soon it appeared that multiple IGH and TRD gene rearrangements 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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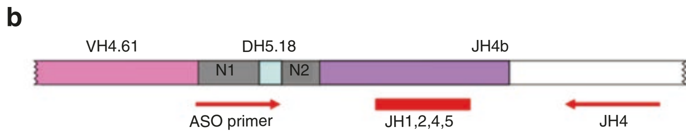
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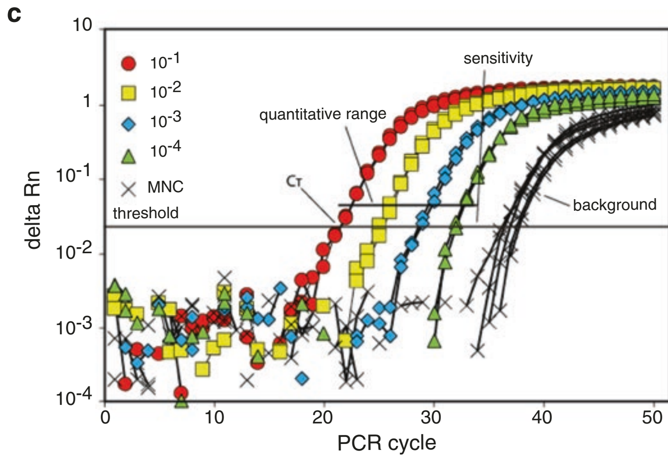
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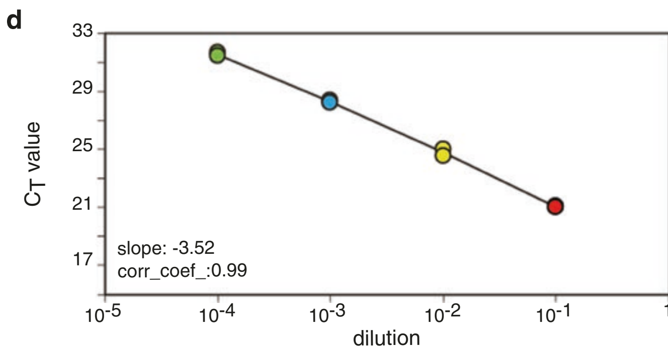
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between treatment protocols and are highly operator dependent procedures, causing substantial inter-laboratory variation. This is a major concern for all clinical studies that wish to exploit MRD measurements.

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

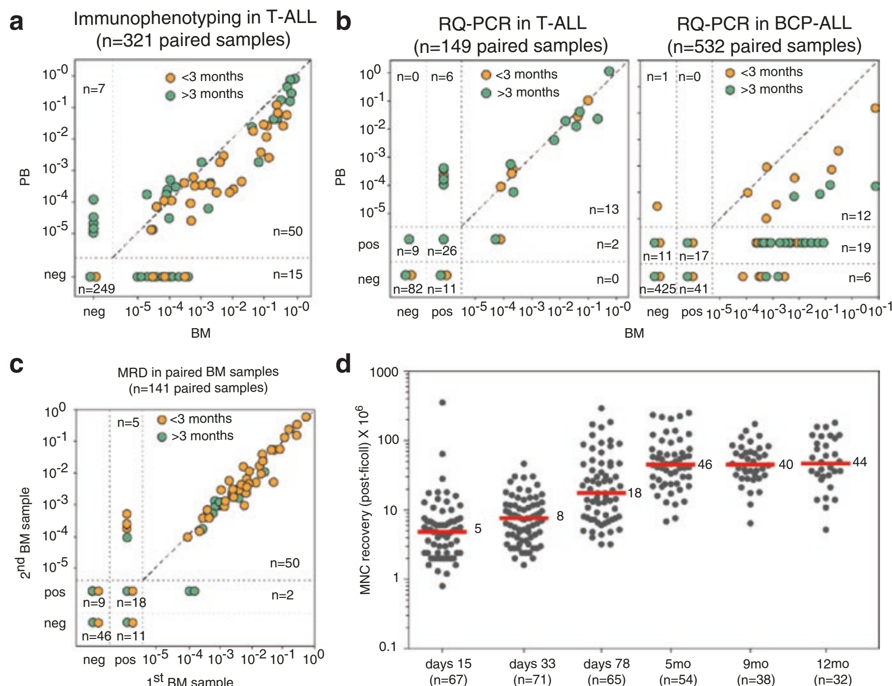


Fig. 6.2 ALL cell frequencies in blood and BM samples during follow-up. (**a**) Frequencies of T-ALL cells, as detected by immunofluorescence microscopy with staining for a T-cell marker and terminal deoxynucleotidyl transferase (TdT) in 321 paired blood and BM samples, obtained from 26 patients [56, 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).

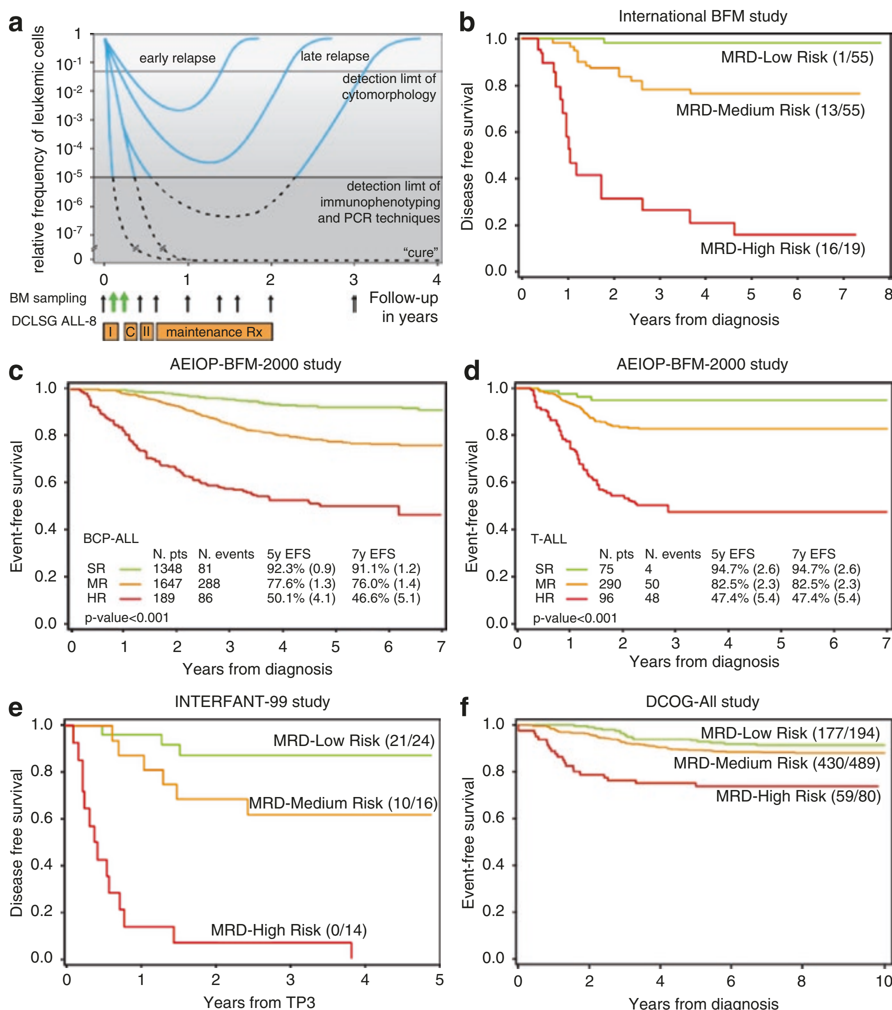
Of note, the overall cell recovery directly relates to the treatment time point, with low cell yields at day 15 and day 33 after starting therapy, but higher cell yields at day 79 and later time points (Fig. 6.2d). The lower cell yields at day 15 are generally not a problem, because at that time most 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 ($\geq 5\times10^{-4}$) at the 3 month time point; MRD-based medium-risk patients had moderate to low MRD levels ($<5\times10^{-4}$) at month 3 after starting therapy (Fig. 6.3b) [1]. Note that the $\geq 5\times10^{-4}$ 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].

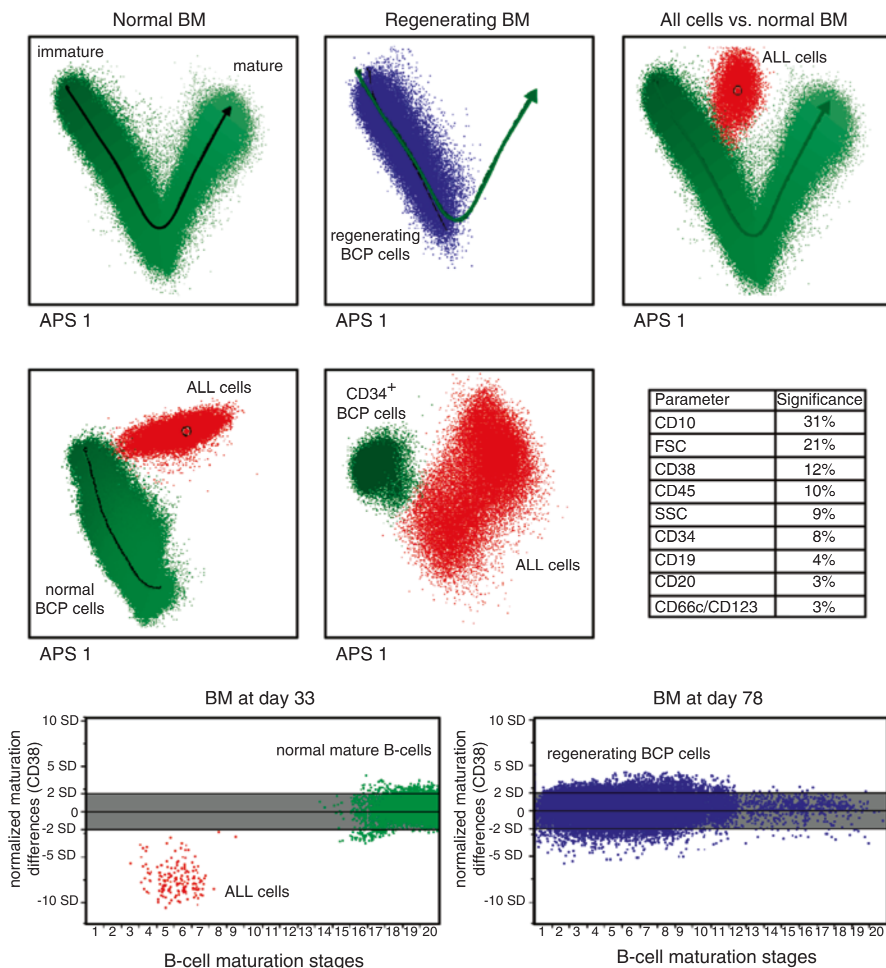
Importantly, EuroFlow-based NGF-MRD strategies provides a full visualisation of the composition of both normal cells and aberrant cells, such as:

- 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

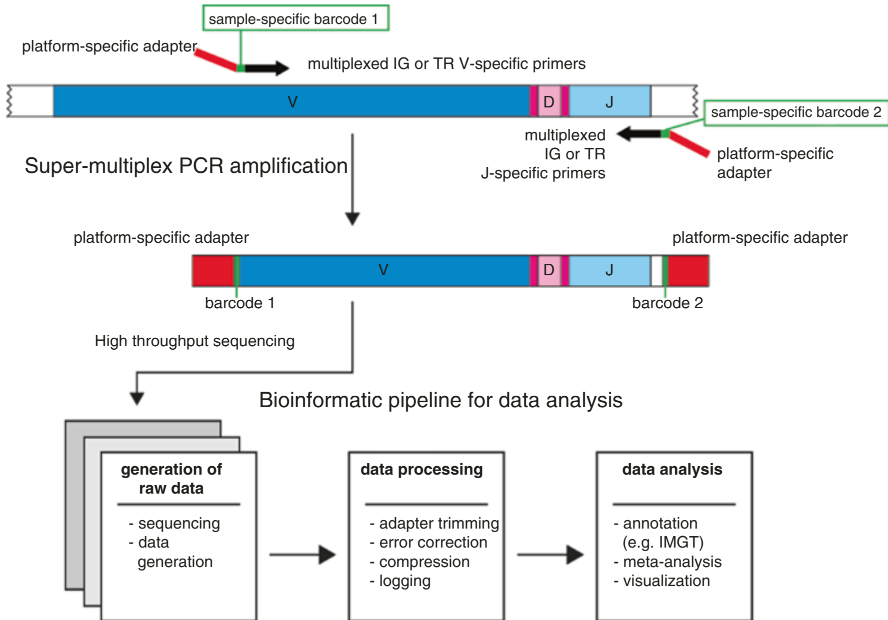


Fig. 6.5 Schematic diagram showing the various steps in HTS of IG and TR for MRD detection. *Top panel*: The IG or TR gene rearrangements are amplified in a single step using a super-multiplex PCR with many different primers, which match with one or more individual V and J genes of the IG and TR genes. 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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