The use of minimal residual disease (MRD) measurement as a surrogate marker of molecular response to treatment can potentially improve the evaluation of treatment response and enable estimates of the residual leukemic cell burden during clinical remission, thereby improving the selection of therapeutic strategies and, possibly, long-term clinical outcome. The most specific and sensitive methods for MRD monitoring currently available are polymerase chain reaction amplification of rearranged immunoglobulin and antigen-receptor genes, and flow cytometric detection of aberrant immunophenotypes. Several retrospective studies have demonstrated the strong association between MRD and risk of relapse in childhood acute lymphoid leukemia (ALL), irrespective of the methodology used. The promising results on the predictivity of MRD evaluation at the end of induction treatment has challenged the need for a new definition of remission. There is now urgent need to incorporate MRD data into clinical studies, properly designed to address treatment questions, in order to explore whether a better tailored treatment would result in further improvement in cure rates for children with ALL. However, several critical issues must be resolved before MRD determinations can be routinely considered in clinical decision making.

Key words: childhood acute lymphoblastic leukemia, minimal residual disease, Ig and TcR gene rearrangements.

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Table 1. Applicability of RQ-PCR techniques for MRD detection in childhood acute leukemia.

<table>
<thead>
<tr>
<th>Method</th>
<th>Leukemia subtype</th>
<th>Sensitivity</th>
<th>Main advantages</th>
<th>Main disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR amplification of fusion transcripts</td>
<td>40-50%</td>
<td>10-20%</td>
<td>High sensitivity; rapid; low costly</td>
<td>RNA degradation (false neg.); difficult quantification</td>
</tr>
<tr>
<td></td>
<td>T-ALL</td>
<td>10^-4-10^-6</td>
<td></td>
<td>(unknown number of transcripts per cell); false pos. results for cross-contamination</td>
</tr>
<tr>
<td>PCR amplification of Ig/TcR gene junctional regions</td>
<td>95%</td>
<td>95%</td>
<td>High sensitivity; accurate quantification (fixed number of targets per cell)</td>
<td>Laborious and costly (if patient tailored); clonal evolution (false neg.)</td>
</tr>
</tbody>
</table>

The frequencies and patterns of TcR gene rearrangements in childhood and adult ALL were examined by several Southern blot- and PCR-based studies.\(^5,7,18\) Virtually all B-lineage ALL patients have rearranged immunoglobulin heavy chain (IGH) genes. Most IGH rearrangements represent complete VH-DH-JH recombinations and in 20% of patients incomplete DH-JH rearrangements could be identified.\(^17\) Incomplete IGH gene rearrangements are particularly frequent in infant ALL.\(^19\) In addition, rearrangements of the Igκ deleting element (Kde) occur at a high frequency (approximately 60%).\(^19\) Most T-ALL patients have rearranged TcRβ (TCRB), TcRγ (TCRG) and/or TcRβ (TCRD) genes, and cross-lineage TcR rearrangements and/or deletions are found in more than 90% of patients with B-lineage ALL.\(^20\)

Currently, PCR-based methodologies are more easily and frequently applied to the detection of clonal Ig/TcR gene rearrangements. After PCR identification of Ig/TcR targets at initial diagnosis, clonality must be assessed by homo-heteroduplex analysis or by gene scanning, to confirm their origin from the malignant cells and not from contaminating normal cells with similar Ig or TcR gene rearrangements.\(^21\) The sequence information allows the design of junctional region-spe-
cific oligonucleotides, which can be used as patient-specific junctional region probes in semi-quantitative hybridization experiments (dot blot) or as a primer to quantitatively amplify the rearrangements of the malignant clone (Figure 1). Sensitivities of 1 × 10^{-4} to 1 × 10^{-6} are achievable with both strategies. However, as discussed below, several advantages of RQ-PCR should be considered.

So far, most studies have used the technology hydrolysis probes.14,15,21 This is based on the 5'-3' nuclease activity of Taq DNA polymerase and an internal dual-labeled fluorogenic probe with a 5'-reporter dye and a 3'-quencher dye (Figure 1). During PCR, the 5'-3' nuclease activity of Taq DNA polymerase cleaves the hybridized probe, thereby separating the reporter dye from the quencher dye, resulting in emission of a fluorescent signal that increases during each subsequent PCR cycle. The real-time detection of fluorescence intensity generates quantitative data based on the early cycles of PCR, when the fidelity of amplification is highest. This quantification can be performed over a large dynamic range of four to five orders of magnitude. Hydrolysis probes confer a high degree of specificity to the method, without the need to analyze PCR products, a time-consuming step with a high risk of inter-assay contamination. RQ-PCR is already known for its very efficient reproducibility. The actual amount of DNA can be corrected by quantitative amplification of a control gene.

Instead of positioning the fluorescent probe on the junctional region, a more useful approach consists in the use of a fluorescent probe complementary to germ-line IgH and TcR gene segments, in combination with an ASO primer complementary to the junctional region (Figure 1). This ASO primer approach theoretically results in more sensitive MRD detection compared with the use of germ-line primers, because no competition can occur with the amplification of similar rearrangements in normal cells.

ABI 7700 and 7900 or TaqMan™ (Applied Biosystem, Foster City, CA, USA) are the reference machines, due to the robustness of their performance and their high-throughput; in fact, the 96-well reaction plate provides a convenient tool for simultaneous testing of standard and patients’ samples.22,23 Other machines are now available for RQ-PCR. Of particular interest the
LightCycler (LC) (Roche, Mannheim, Germany) has demonstrated its potential to quantify MRD.\(^{24,25}\) The most compelling feature of LC technology lies in its combination of rapid thermocycling conditions (due to the very thin glass capillaries employed for the PCR assay) with on-line real-time fluorescence detection of PCR product amplification. We have recently evaluated target sensitivity and MRD detection of a large panel of IgH and TcR clonal gene rearrangements by using both the ABI 7700 and the Light-Cycler in parallel.\(^{26}\) Both real-time PCR systems provided specific results for MRD quantification in all the tested follow-up samples, with a sensitivity of at least \(10^{-4}\) in more than 90% of the clonal gene rearrangements used. TaqMan and Light-Cycler real-time PCR technologies produce similar MRD quantification results and the quantification assays can be easily transferred from one detection system to the other. While TaqMan technology offers the possibility of reliably analyzing large sample numbers, PCR in the Light-Cycler is performed extremely quickly. Using the same detection format, both techniques can be applied in combination in multicenter MRD studies.

Independently of the equipment, several criteria should be taken into account for a correct interpretation of RQ-PCR data:\(^{27}\)

(i) the standard curve obtained with the dilutions should have an acceptable slope and correlation coefficient, and the shape of the amplification curves must reflect specific amplification;

(ii) the RQ-PCR analysis should be reproducible. The variation between replicates is higher if the mean Ct value of the replicates is high, which is the case at the highest sensitivity. This implies that one could define two sensitivities: a reproducible sensitivity, indicating the level up to which the data can be precisely quantified, and a maximal sensitivity, indicating the level that can still be detected, although not reproducibly. This is very important when very low MRD levels must be detected;

(iii) the specific amplification should be sufficiently separated from any non-specific (background) amplification from polyclonal cells (peripheral blood DNA from a pool of healthy donors is generally used as a negative control).

The DNA-intercalating SYBR Green dye was also used to monitor nucleic acid amplification of Ig and TcR gene rearrangements.\(^{28}\) However, SYBR Green detects all dsDNA, including primer dimers and other undesired products, and does not allow any verification of product identity. Therefore, the specificity of detection depends only on the specificity of amplification.

### How to incorporate MRD into clinical studies

#### Standardization of the techniques

The standardization of the technique represents a preliminary step to be considered in order to incorporate MRD determinations into a prospective clinical study. The work of an European Study Group on MRD detection in ALL (ESG-MRD-ALL; coordinators: JJM van Dongen and VHJ van der Velden) is in progress, with the aim of standardizing molecular methodologies for clonality assessment and MRD detection by RQ-PCR. This could be a starting point for a common international agreements on guidelines that should be made in the future. Table 2 summarizes the steps of the procedure that must be standardized.

#### How to select Ig/TcR MRD targets

During cell differentiation, Ig and TcR gene rearrangements in B- and T-lineage ALL are prone to subclone formation.\(^{27,28}\) The presence of subclones must be carefully analyzed, in order to avoid monitoring minor clones in patients’ follow-up. In addition, the emergence of subclones that were not detected at diagnosis may occur, and can be responsible for relapses associated with false-negative results during MRD monitoring.\(^{27}\) The analysis of 94 patients with B-lineage ALL, studied at diagnosis and relapse by combining Southern blot and PCR methods, showed that 71% of the potential Ig and TcR targets for MRD analysis identified at diagnosis were preserved at relapse.\(^{29}\) The most stable were IGK-Kde rearrangements (90%) while the least stable were incomplete TCRD rearrangements (63%). Monoclonal rearrangements were significantly more stable than oligoclonal rearrangements.

More recently, PCR-GeneScan and sequencing analyses of Ig/TcR gene rearrangements at diagnosis and subsequent relapse were performed in BM samples from 53 childhood precursor-B-ALL patients.\(^{30}\) At least one stable clonal Ig/TcR target was found in 94% of patients. At relapse, 71% of diagnostic clonal PCR targets were conserved. No significant difference in the stability of different clonal PCR targets was observed (TCRG, 75%; IGK, 71%; IGH, 70%; TCRD, 67%), so it can be concluded that there is no preferential clone-specific target for MRD monitoring. Although it is not clear, one of the reasons for the apparent discrepancy between the two cited reports might be the use of two different methods. Although Southern blot analysis can add detailed information on oligoclonality as well as on the presence of minor clones,\(^{29}\) virtually all running clinical MRD studies are based on a fully PCR-based approach.\(^{26}\)

TCR gene rearrangements are significantly more stable in T-ALL than in B-lineage ALL. In a recent report analyzing 150 Ig/TcR gene rearrangements in 28 chil-
dren and 9 adults with relapsed T-ALL, 88% of clonal rearrangements identified at diagnosis in truly relapsed T-ALL were preserved at relapse. Thus, from a biological point of view, the immunogenotype of T-ALL is more stable than that of precursor-B-ALL. Moreover, clonal stability of Ig/TcR targets between 1st and 2nd relapse has a relevance in the clinical application of MRD monitoring. A recent PCR-study on 48 children with precursor B-ALL at first and second relapse demonstrated that in 52% of the patients, all PCR targets identified at first relapse were preserved at second relapse; in 92% of the patients at least one target and in 73% at least two targets remained stable. Highest stability was found for the IGH and TCRG gene rearrangements.

Overall, the clonal evolution and/or clonal selection events affecting the stability of PCR targets during the course of the disease can potentially generate false-negative results. This should be taken into account in the design of PCR strategies to detect MRD in ALL. Current guidelines suggest that at least two PCR targets must be monitored, preferentially representing two different gene loci. With the current possibilities for IGH, IGK, TCRB, TCRG and TCRD analyses, at least two Ig/TcR targets can be identified in approximately 90% of children with either B-lineage or T-lineage ALL. In addition, as discussed below, the selection criteria for MRD monitoring must also consider stability, sensitivity and specificity of the targets selected.

**The type of technology depends on the clinical question: a method with high sensitivity or low sensitivity?**

Once the clonal rearrangements have been recognized at diagnosis, several methods can be applied to specifically detect the leukemia-derived PCR products during the follow-up of patients who have undergone therapy. The major variable lies in the sensitivity of the test, which can significantly influence the interpretation of the assay’s results. A typical low-sensitivity assay consists of a modified fingerprint analysis, in which the patient- and clone-specific peak corresponding to PCR amplification from residual leukemic cells can be discriminated from the normal background. Polyclonal background levels vary, but usually limit the sensitivity of this approach to the detection of one leukemic cell among 105 to 107 normal cells. This low-sensitivity approach can be considered when the aim of the clinical protocol is to identify patients with high residual tumor load, likely to be at very high risk of relapse. By contrast, in the most sensitive assay available so far, clonal PCR products from homo-heteroduplex analysis are directly sequenced. V, D and J gene segments are then identified, and randomly inserted nucleotides are recognized by comparison with germline sequences in databases. After designing specific primers/probe, highly-sensitive RQ-PCR is then applied (Figure 1). This approach allows identification of the subgroup of patients with very good response to early therapy. The highly-sensitive RQ-PCR approach also allows precise quantification of intermediate MRD levels. Whether patients in these risk groups could profit from treatment reduction/intensification is still unknown.

**Early or late assessment? The impact on clinical decisions**

The clinical impact of MRD strongly depends on the therapeutic time point at which it is assessed. MRD-based stratification can only be introduced into a certain clinical protocol after the actual MRD measurements in that context. Moreover, the earlier the time point is prognostically significant, the higher the possibility is for the MRD monitoring to be clinically applicable, in terms of appropriate modification of the therapy. Several studies have demonstrated the prognostic impact of MRD detection at the end of induction treatment in childhood leukemia. The multicenter study performed by the International Berlin-Frankfurt-Münster Study Group (I-BFM-SG) in 240 children with ALL showed that the combined MRD information (determined at the end of protocol Ia of induction, i.e. 5 weeks from diagnosis -TP1-, and before consolidation treatment, i.e. 3 months from diagnosis -TP2-), identi-
MRD monitoring of childhood ALL by RQ-PCR of Ig/TcR rea rrangements

Requirements for the clinical application of MRD detection by RQ-PCR of Ig/TcR rearrangements

There are prerequisites for the clinical application of MRD detection. Firstly, the predictive clinical value of MRD found in retrospective studies must be reproduced in a prospective study. Furthermore, the application of MRD analysis in large multicenter studies into daily practice requires additional investment in terms of costs, people and structural organization of the work.

The success of MRD monitoring for a single patient requires several steps:

(i) sufficient DNA must be available at diagnosis and at all follow-up time points according to the clinical protocol. This may also require strict monitoring of the BM aspirates in order to repeat the puncture when needed and justified.

(ii) at least two Ig/TcR PCR targets must be available; this requires wide screening of all potential Ig/TcR targets (25 or 21 different PCR for B-cell precursor ALL and T-ALL, respectively).4,12

(iii) the selected targets must be detectable with sufficient sensitivity: at least 10^-4. This implies that several targets for a single patient must be sequenced, and more than one clone-specific primer must be designed and tested for sensitivity against a background of normal cells.

(iv) as indicated above, a reliable method for quantifying and interpreting MRD levels is needed.22

(v) the collection of MRD results must be rapid for them to be clinically useful.

The ongoing cooperative AIEOP-BFM ALL2000 clinical protocol for childhood ALL (in Italy, Germany, Austria and Switzerland) is mainly based on MRD assessment at day +33 and day +78 by two Ig/TcR targets with a sensitivity of at least 10^-4. Concerning the preliminary series of about 2500 patients enrolled into the study, MRD analysis was performed in more than 95% of cases. Considering failures due to target availability and sensitivity, availability of follow-up DNA, and shifts to other treatment protocols, altogether it was possible to stratify almost 75-80% of eligible patients according to MRD levels (unpublished data).

Can we further increase the percentage of cases successfully stratified by MRD?

After applying the complex procedure for high-sensitivity MRD detection indicated above in a diagnostic context, there is not much room for a further increase in the percentage of cases successfully stratified by MRD. The only possibility lies in the incorporation of newly identified molecular targets, which would increase the chance of having at least two sensitive markers per patient.4,44,45

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fies three different risk groups according to MRD level.11 Low-risk MRD (MRD negative at both TP1 and TP2, with a sensitivity of at least 1×10^-4) comprises 43% of the patients, whose 3-year relapse rate was only 2%; by contrast the 15% of cases with a high degree of MRD (>1×10^-4 at TP1 and TP2) had a relapse rate of 75%. The remaining patients (45%) were in an intermediate-risk group, with a 3-year relapse rate of 23%.

Some reports have addressed the question of whether the assessment of MRD at an earlier time point than the end of induction treatment could be equally satisfactory or even better for identifying ALL patients with different treatment outcomes. Two different studies40,41 suggest that there are ALL patients with very high MRD levels may be eligible for early transplantation or experimental treatment. Some studies tried to assess the prognostic value of MRD re-emergence of residual disease, even at the level of 1×10^-4, predicts clinical relapse.10,11 Although the clinical usefulness of late MRD determination is limited, patients with very high MRD levels may be eligible for early transplantation or experimental treatment. Some studies tried to assess the prognostic value of MRD when needed and justified.

It should be considered that the frequency of MRD-positive patients and the MRD levels are higher in T-ALL than in precursor-B-ALL, reflecting the more frequent occurrence of resistant disease in T-ALL.44 If the same MRD-based criteria for risk stratification are applied, fewer T-ALL were classified in the low-risk group (25% versus 46% of patients with precursor B-ALL), and more patients with T-ALL were classified in the MRD-based high risk group (28% compared to only 11% of patients with precursor B-ALL). The relapse-free survival (RFS) rates were also different for T-ALL and precursor B-ALL in the same MRD-based high risk group. Moreover, the prognostic value of MRD levels at TP1 and TP2 was higher in T-ALL (larger RFS gradient), and consistently higher RFS rates were found for MRD-negative T-ALL patients in the first months of therapy.

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MRD monitoring of childhood ALL by RQ-PCR of Ig/TcR
Recently, the monoclonal Vβ4-Jα rearrangements in precursor-B-ALL (with preferential usage of the Jα29 gene segment) were indicated as patient-specific targets for MRD detection, because they show high sensitivity (10^4 or less in most cases) and good stability (88% of rearrangements preserved at relapse).4 More relevant is the identification of TcR β (TCRB) clonal rearrangements in 92% of childhood T-ALL (Vβ-Dβ-Jβ rearrangements in 80%, Dβ-Jβ rearrangements in 53%).4 A TCRB Q-PCR assay with 13 germline Jβ primer/probe combinations and allele-specific oligonucleotides was developed,46 allowing the detection of one leukemic cell within at least 10^6 polyclonal cells in 93% of cases. This means that TCRB monitoring will be of great value for MRD studies in T-ALL, in which the repertoire of Ig/TcR rearrangements is limited and less sensitive.

**Perspectives**

Identification of different MRD subgroups: how to define such differences further?

As striking differences in therapeutic response and outcome may still be observed in ALL patients with the same cytogenetic profile (ie. the t(9;22) and the t(12;21) positive patients) or within the same risk classification group,47 it is likely that other molecular genetic abnormalities and functional activation or inactivation of critical cellular pathways (cell signaling, cell cycle regulation, adhesion, DNA repair, apoptosis, drug resistance) in leukemic cells also affect disease biology and therapeutic response. The use of large scale genomic technologies that measure global patterns of gene expression in leukemic cells, as well as mutational analyses of genes involved in resistance, may identify the molecular basis of therapeutic response or resistance in individual patients.48 In the ongoing MRD-based clinical protocols, the Intermediate risk group still includes the majority of patients; moreover, most of the relapsing cases are in this heterogeneous group. Statistical tools can be used to relate outcome to different combinations of values of MRD at the first two time-points. The availability of MRD also at subsequent time-points might allow the prognosis to be adjusted and updated and monitoring the disease course might identify the subgroup of patients with a higher risk of relapse. This asks for novel statistical approaches that integrate methods for the analysis of survival data with methods for the analysis of longitudinal data, in which the profile of the MRD values in time and not the single values need to be modeled.

How to integrate MRD in the next generation of clinical studies?

Several ongoing clinical studies in childhood ALL have now incorporated the use of MRD at early time points of front-line treatment, to stratify patients to different therapeutic regimens according to MRD levels, and evaluate whether this results in a better outcome. Although the high prognostic value of MRD data obtained at the end of induction treatment has been confirmed in every published study, the reported MRD studies show remarkable differences in the meaning of MRD level information at the end of induction.49 In addition, the MRD-based risk groups are defined differently, resulting in different distributions of patients over the risk groups and different relapse rates. The major differences in risk group definition and corresponding relapse rates might be related to the type of treatment protocol, the timing at which the follow-up samples are taken, or the MRD detection technique used. Consequently, it is impossible to extrapolate data from one clinical treatment protocol to another. This means that for each treatment protocol the MRD information (sampling time-points and required sensitivity) must be defined precisely. In practice, this means that MRD-based treatment interventions should always be designed according to earlier-obtained MRD results from the same treatment protocol. When MRD information from existing treatment protocols is translated into new clinical treatment protocols, several MRD-related aspects will influence the implementation of the new protocol: i) MRD-based stratification can only be introduced in the protocol after the actual MRD measurements (6 to 13 weeks after starting treatment); ii) The treatment blocks before the MRD sampling time-points cannot be changed, because this would directly change the prognostic value of the MRD results; iii) preferably at least two early MRD sampling time-points should be used, because this results in a more accurate definition of MRD-based risk groups. The MRD information at later time-points can potentially be used for treatment modification in MRD-positive intermediate-risk patients.

Information about molecular response to treatment can be used to predict long-term outcome even in relapsed childhood ALL.50-53 In the BFM study, children with MRD levels less than 10^-4 at day 36 of therapy post-relapse had a probability of event-free survival of 86%, compared with zero for children with higher levels of MRD.50 In the current trials for relapsed ALL in BFM countries, the level of MRD at day 36 is decisive for the indication for stem cell transplantation (SCT) in intermediate risk patients. Reported data showed that MRD burden prior to conditioning therapy is the strongest independent predictive factor for relapse post-SCT.50 Moreover, the detection of MRD after transplantation is predictive of an unfavorable outcome.50 These results suggest that clinical studies should be designed that incorporate MRD monitoring pre- and post-SCT in order to direct post-transplant...
interventions and measure their effects. MRD monitoring can be used as a surrogate marker to monitor in vivo response to new drugs in childhood ALL. For example, a randomized phase II/III-study (EsPhALL) has been recently opened to compare the safety and efficacy of imatinib with chemotherapy in pediatric patients with Ph+ ALL. To assess the antileukemic potential of imatinib given to patients with good-risk Ph+ ALL, the pattern of the molecular response will be analyzed by randomized arms, on the basis of 5 MRD measurements at different time-points, by RQ-PCR of both Ig/TcR rearrangements and BCR/ABL fusion gene transcript expression.

**Concluding remarks**

MRD studies are becoming an integral part of the modern management of patients with leukemia. Now that the cure rates of childhood ALL are approaching 80%, the challenge will be how to incorporate MRD information into new studies that pose a therapeutic question. Several critical issues must be resolved before MRD determinations can be routinely considered in clinical decision-making. The selection of the methods and their relative sensitivity depends on the clinical question but also on expertise and facilities available. Highly sensitive PCR techniques (detection limit <1×10^-4) allow the identification of a significant proportion of ALL cases with excellent clinical outcomes in the presence of negative MRD findings at early time-points in treatment. However, MRD negativity does not mean disease eradication and the possibility exists that reduction in treatment intensity will result in an increased rate of relapse, even in patients who readily achieve MRD-negative status. By contrast, patients with 10^2 or more leukemia cells during any phase of remission induction have a very high risk of relapse and are eligible for early transplantation or experimental treatment. How to use intermediate range positive MRD findings (>1×10^-8 but <1×10^-6) is still unclear. Such patients might benefit from further intensification, but that possibility needs to be substantiated by randomized clinical studies. Thus, the German-Austrian BFM and Italian AIEOP study groups have adopted a MRD-based risk group classification for treatment stratification in their ongoing clinical studies. It is hoped that a more sensitive and specific evaluation of remission and early response to treatment could speed further improvement in cure rates for children with ALL. Moreover, in the future, only the combination of simplification and reliability of MRD methods will allow the potential benefits of MRD monitoring to be extended to all children with leukemia.

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