# Molecular monitoring of childhood acute lymphoblastic leukemia using antigen receptor gene rearrangements and quantitative polymerase chain reaction technology

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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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Over the past three decades, remarkable advances have been made in the treatment of acute lymphoblastic leukemia (ALL) in children.[1,2] Yet significant challenges remain. Although the use of modern combination chemotherapy and post-induction therapeutic intensification now yield long-term remissions in nearly 75% of children affected by ALL, 25% ultimately relapse with disease that is highly refractory to current therapy. Conversely, another 25% of children who now receive dose intensification are likely *over-treated* and probably could have been cured using less intensive regimens resulting in fewer toxicities and long-term side effects.[2]

Current risk classification schemes use clinical and laboratory parameters such as patient's age, initial white blood cell count, and the presence of specific ALL-associated cytogenetic abnormalities to stratify patients into *low*, *intermediate*, *high*, and *very high* risk categories.

The rate of clearance of leukemic cells from the bone marrow during the early phase of therapy is an independent prognostic factor in ALL.[1,2] Patients who respond slowly have a high risk of relapse, and patients who fail to achieve a complete remission within 4 to 6 weeks of the induction treatment, or who have a poor response to the first week of corticosteroid treatment have a particularly dismal prognosis. Complete remission is commonly defined as restoration of normal hematopoiesis, with less than 5% of blast cells by morphological examination of the bone marrow (BM). However, patients with nearly 5% leukemic blast cells in their BM can harbor as many as $10^{10}$ leukemic cells, and these patients may receive the same treatment as patients with a greater reduction in the leukemic cell burden. Conversely, normal hematopoietic progenitors, which may represent 5% or more of the cells in regenerating marrow, can be erroneously interpreted as residual leukemic cells. Although morphological examination can be readily applied at any center, it has low precision, because about 20% of patients with a good initial response eventually relapse, and a third of patients with a poor response may survive long term when treated with intensive chemotherapy alone.

Sequential monitoring of minimal residual disease (MRD) with specific and sensitive methods (capable of recognizing one leukemic cell among $10^4$ or more normal BM cells, at least 100-fold more sensitive than morphologic examination), recently compelled the redefinition of complete remission in patients with ALL,[3] and further

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

| *Method* | *Leukemia subtype* | | *Sensitivity* | *Main advantages* | *Main disadvantages* |
|---|---|---|---|---|---|
| | *precursor-B ALL* | *T-ALL* | | | |
| PCR amplification of fusion transcripts | 40-50% | 10-20% | $10^{-4}$-$10^{-6}$ | High sensitivity; rapid; low costly | RNA degradation (false neg.); difficult quantification (unknown number of transcripts per cell); false pos. results for cross-contamination |
| PCR amplification of Ig/TcR gene junctional regions | 95% | 95% | $10^{-4}$-$10^{-5}$ | High sensitivity; accurate quantification (fixed number of targets per cell) | Laborious and costly (if patient tailored); clonal evolution (false neg.) |

improved the clinical utility of risk assessment. Several techniques have been developed over the past 10 to 15 years to complement morphology in assessing response to treatment, including immunologic and molecular methods fluorescent *in situ* hybridization (FISH), *in vitro* drug response and colony assays.[4-7] This improvement drastically changed the definition of remission, which now depends on the sensitivity of the detecting methodology.[3] In ALL, the most reliable methods for MRD detection include flow cytometric profiling of aberrant immunophenotypes, polymerase chain reaction (PCR) amplification of fusion transcripts and chromosomal breakpoints, antigen-receptor genes, aberrant genes, or aberrantly expressed genes. These approaches are widely used for MRD monitoring because they are sufficiently specific, sensitive ($10^{-4}$ to $10^{-6}$), quantitative, and relatively easily applicable.[4-7] Quantitative MRD data can be obtained by real time quantitative PCR (RQ-PCR) technology. RQ-PCR permits accurate quantification of PCR products by the detection of fluorescent signals generated by the PCR-dependent degradation of a target-specific fluorescent probe, at the beginning of the exponential phase of the PCR amplification process. RQ-PCR analysis can be performed with SYBR Green I or hydrolysis probes as a detection system, in several RQ-PCR instruments.

Table 1 summarizes the applicability of the RQ-PCR methods for MRD detection in childhood ALL. A prerequisite for applying MRD measurements in clinical studies is that the data should be available for all patients. RT-PCR amplification of fusion genes can only be applied to a limited subgroup of patients, and its prognostic value is still not fully understood. By contrast, PCR-based MRD detection of Ig and TcR gene rearrangements can be applied in 95% of childhood ALL cases. Accordingly, most of the clinical studies of MRD in childhood ALL have used one of the different PCR approaches for the detection of antigen-receptor gene rearrangements.[4-7] MRD analysis by molecular[8-13] or highly sensitive ($10^{-4}$) immunologic methods[14,15] can predict outcome on the basis of the reduction of the leukemia cell burden during the first months of therapy. The present review will highlight some technical aspects of the detection of MRD by quantitative PCR, mainly focusing on Ig and TcR genes, and discuss the issues to be considered when this method is incorporated in clinical studies of childhood ALL treatment.

### MRD detection by RQ-PCR of Ig/TcR rearrangements (technical considerations)

Ig and TcR genes are the most widely applicable genes and therefore can be considered a *universal* target for MRD detection in childhood ALL (Table 1). As a result of somatic rearrangements of Ig and TcR gene loci, a unique clonotypic marker is made from the joining of the germline variable (V), diversity (D) and joining (J) gene segments. The uniqueness of each rearrangement further depends on random insertion and deletion of nucleotides at the junction sites of the V, (D), and J gene segments, making the junctional regions of Ig and TcR genes *fingerprint-like* sequences.[5-7]

The frequencies and patterns of TcR gene rearrangements in childhood and adult ALL were examined by several Southern blot- and PCR-based studies.[5-7,16] 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.[18] 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 TcRδ (*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.[5-7] The sequence information allows the design of junctional region-spe-

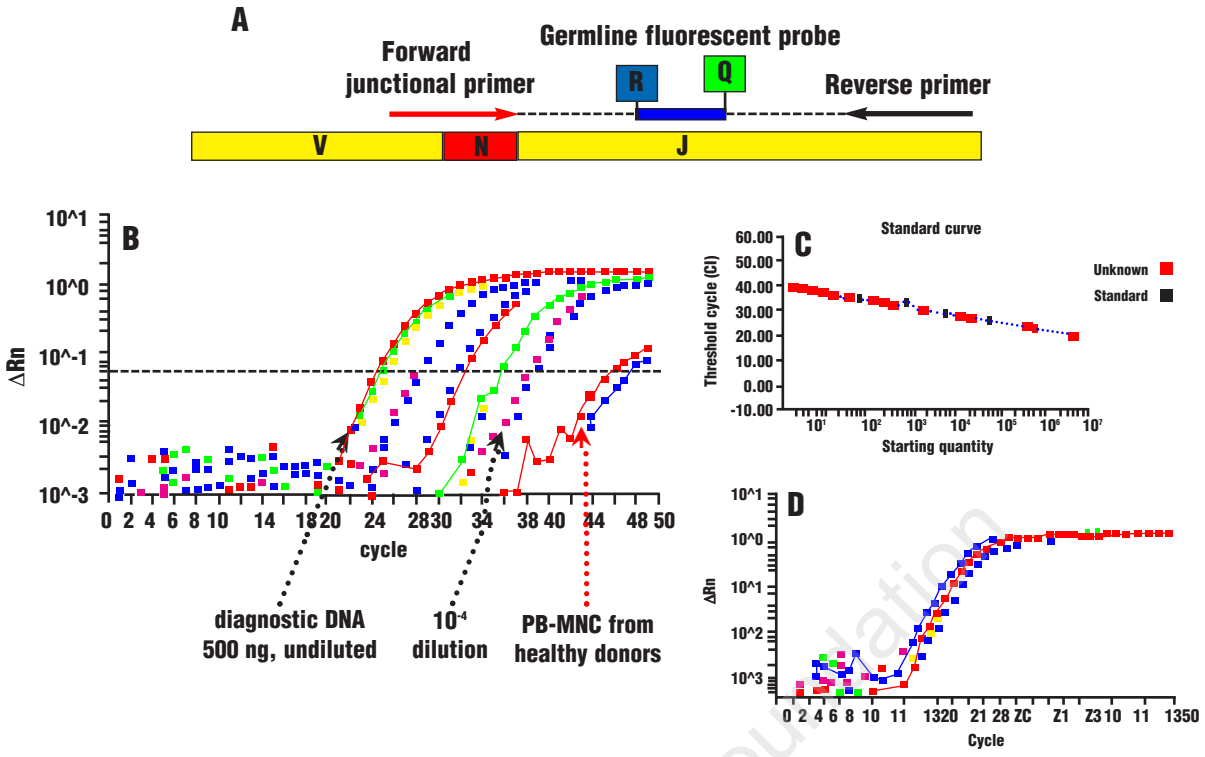


**Figure 1.** Quantitative PCR for monitoring Ig/TcR rearrangements. *Panel A*: clonal PCR products are sequenced, junctional regions are identified and specific oligonucleotides can be designed. Junction-specific primers can be used to detect malignant cells among normal lymphoid cells during follow-up of patients, in combination with fluorescent probes and reverse primers on the germline region of each rearrangement. Serial dilutions of the diagnostic DNA can be performed to verify the sensitivity and specificity of each PCR assay (*Panel B*), and to obtain a regression curve (*Panel C*) for the precise quantification of fluorescent levels at the single time points. An independent gene (ie. albumin) must be amplified at diagnosis and each time point in order to assess the actual amount and quality (amplificability) of the DNA in each reaction (*Panel D*).



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 $1\times10^{-4}$ to $1\times10^{-6}$ 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 germline 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 Biosytem, 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.[25] 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:[22]

(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.[26] 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.[30]

*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-

**Table 2.** Standardization of PCR analysis of IG/TCR genes.

| *To be standardized* | *Meaning* |
|---|---|
| Cell sampling | Timing and logistics of cell sampling and processing of the BM aspirate |
| DNA extraction and storage | Methods of DNA extraction; solution and temperatures of DNA storage |
| Control | Choice of the appropriate control gene (stability over different conditions) |
| Detection of PCR targets at diagnosis | Multiple screening in unique conditions to uncover all targets and facilitate the routine work |
| Selection of PCR targets | Definition of the priority list based on sensitivity and stability of different targets |
| Required sensitivity | Sensitivity tests (varying primers and annealing temperatures) to get more efficient PCR conditions |
| Quantification of MRD levels and sensitivity | Performance and requisites of the actual MRD test |
| Guidelines for interpretation of RQ-PCR results | Definition of reproducible ranges and detection limits, definition of false positive and false negative tests |

dren and 9 adults with relapsed T-ALL, 88% of clonal rearrangements identified at diagnosis in truly relapsed T-ALL were preserved at relapse.[28] 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.[31] 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 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.[32-36] 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 $10^2$ to $10^3$ normal cells.[37,38] 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[5,7,22,32] (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.[8-13,15,39] 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-

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 $1\times10^{-4}$) 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 ($\geq1\times10^{-3}$ at TP1 and TP2) had a relapse rate of 75%. The remaining patients (43%) 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 studies[40,41] suggest that there are ALL patients with very early (day 15-day 19) and profound cytoreduction, who are therefore candidates for future studies designed to test less intense and hence less toxic regimens of chemotherapy. However only data from larger series of patients would confirm these promising preliminary observations. By contrast, the persistence of residual blasts beyond 4 to 6 months of therapy or the re-emergence of residual disease, even at the level of $1\times10^{-4}$, predicts clinical relapse.[11,12] 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 testing in BM samples from children at the end of therapy or later.[13,42] In a recent report, MRD was detected in 28% of patients at 24 months after diagnosis, and was highly predictive of relapse. However, it is still not clear whether possible patients' selection or treatment modification might have influenced the study results.[13]

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.[43] If the same MRD-based criteria for risk stratification are applied, fewer T-ALL were classified in the low-risk group (23% 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.

### 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 RQ-PCR monitoring of Ig/TcR rearrangements needs considerable technical expertise in molecular biology, sample collection and handling, DNA extraction, PCR screening, sequencing and primer design, RQ-PCR application and interpretation. All these steps require standardization and quality control rounds to guarantee reproducibility between laboratories (as previously described).[5,7,22]

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).[5,7,32]

(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.[22,32]

(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.[34,44,45]

Recently, the monoclonal Vδ2-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).[45] 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%).[44] A TCRB RQ-PCR assay with 13 germline Jβ primer/probe combinations and allele-specific oligonucleotides was developed,[34] allowing the detection of one leukemic cell within at least $10^4$ 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,[46,47] 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.[50,51] In the BFM study, children with MRD levels less than $10^{-3}$ at day 36 of therapy post-relapse had a probability of event-free survival of 0.86, 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.[52] Moreover, the detection of MRD after transplantation is predictive of an unfavorable outcome.[53,54] 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.[55]

## 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 $<1\times10^{-4}$) 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 ($>1\times10^{-4}$ but $<1\times10^{-2}$) 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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