Minimal residual disease quantification by PCR in childhood acute lymphoblastic leukaemia

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In Belgium approximately 70 children are diagnosed with acute lymphoblastic leukaemia annually. For these children, the monitoring of minimal residual disease has an important prognostic value. The level of minimal residual disease during the first three months of therapy is used to recognise subgroups that differ substantially in outcome. Two techniques are used for minimal residual disease monitoring: the Genescan method and the allele specific oligonucleotide polymerase chain reaction. The Genescan method is a less sensitive method (10^{-3}) but is fast and less expensive. The allele specific oligonucleotide polymerase chain reaction requires more time and budget but has a sensitivity of 10^{-4}-10^{-5}. Both techniques have proven their value in minimal residual disease monitoring in childhood acute lymphoblastic leukaemia.

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Introduction

Acute lymphoblastic leukaemia (ALL) is the most common childhood cancer. Each year, approximately 70 children are diagnosed with ALL in Belgium. At diagnosis, leukemic cells in the bone marrow can easily be detected by light microscopy. Starting intensive chemotherapy will lead to a fast decrease of the cancer cells. The remaining cancer cells will no longer be detected by light microscopy and the patient is declared to be in complete remission. However, the remaining leukemic cells can be detected by molecular techniques such as the polymerase chain reaction (PCR). The quantification of minimal residual disease (MRD) is based on the detection of the immunoglobulin (Ig) or T-cell receptor (TCR) genes.

The antigen specificity of the B- and T-cells relies on the so-called V(D)J recombination (*Figure 1*). During early B- and T-cell differentiation the genes coding for the variable (V), diverse (D) and joining (J) gene segments of the Ig and the TCR genes rearrange. This rearrangement is a random process: different V, (D) and J segments are coupled randomly with the deletion of a part of the germline sequence and the insertion of
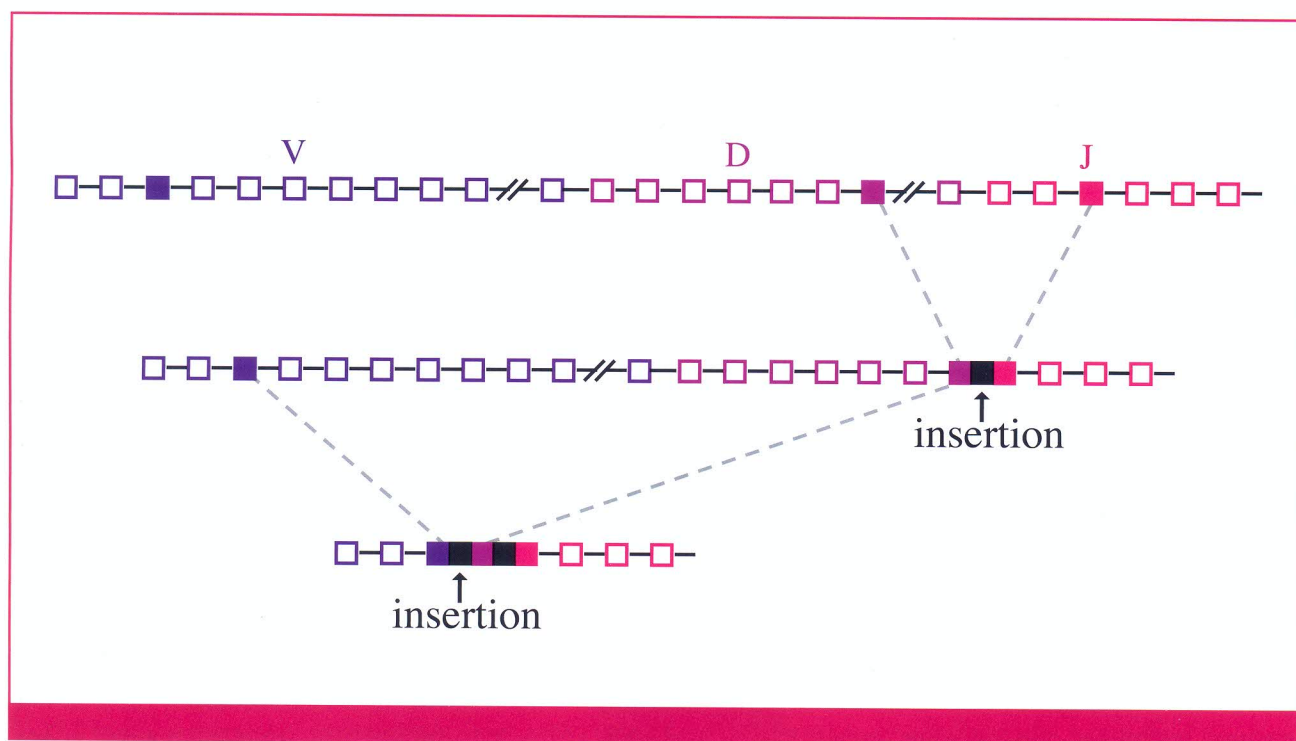
Figure 1. V(D)J recombination during early B- and T-cell differentiation. A diverse (D) gene segment is coupled to a joining (J) gene segment with the deletion of the intervening gene segments and the insertion of random nucleotides. In a second step a variable (V) gene segment is coupled to the rearranged DJ gene segment.

random nucleotides, leading to unique junctional regions and a huge diversity of the Ig and TCR repertoire.

In ALL patients a clonal expansion of one malignant B/T progenitor cell has taken place. All the leukemic cells of one clone carry the same identical and unique DNA sequence. This DNA sequence can be used as a patient specific marker for the detection of MRD. Monitoring MRD has an important prognostic value and is used to stratify patients into different risk groups. Based on the level of MRD, therapy can be adapted to overcome undertreatment or overtreatment. This article will focus on the detection of MRD in Belgian children with ALL using Ig and TCR rearrangements as targets for PCR.

MRD – EORTC-CLG guidelines

For the risk stratification and the protocols of the Belgian children with ALL, the paediatric oncologists follow the European Organisation for Research and Treatment of Cancer – Children's Leukaemia Group (EORTC-CLG) guidelines, a Berlin-Frankfurt-Münster (BFM) based protocol (Riehm 1973), adapted according to the results of previous studies (EORTC 58881 and 58951 protocols and the International Berlin-Frankfurt-Münster Study Group (I-BFM-SG)).[1-4] These studies have clearly shown that there are two very

important time points to measure MRD: time point 1A after induction therapy (day 35) and time point 1B after consolidation therapy (day 90).

Children with MRD $\geq 10^{-2}$ at time point 1A have a significantly higher risk of relapse (70-100%) compared to the children with MRD $< 10^{-2}$ (2-10%).[1,2,5,6] The MRD status at this time point is the most important prognostic factor, independent of other risk factors such as age, blast count, prednisone response, immunophenotype or chromosomal aberrations. Children at low or intermediate risk but with MRD $\geq 10^{-2}$ at time point 1A are switched to a high risk treatment protocol (*Table 1*). MRD results are needed as soon as possible after induction treatment in order to adapt therapy quickly. Therefore, MRD is quantified by the Genescan method: a fast and less expensive method but also a technique with a limited sensitivity for quantification. Monitoring MRD at the second time point (1B) provides better insights into the kinetics of the decrease in MRD and the relapse risk.[1-3] Patients with MRD $\geq 10^{-3}$ at time point 1B are at high risk and will be prepared for stem cell transplantation (SCT). Patients with MRD $\geq 10^{-2}$ after induction therapy (and hence in the very high risk group) but with MRD $< 10^{-3}$ after consolidation therapy are not considered for SCT. Because the sensitivity of the Genescan method is between 10^{-2}

Table 1. Overview of the EORTC-CLG guidelines.

Risk group	Time point 1A: Genescan analysis	Time point 1B: ASO-PCR
Low	MRD $\geq 10^{-2}$ → switch to high risk	MRD $\geq 10^{-3}$ → switch to the high risk, SCT indicated
Intermediate	MRD $\geq 10^{-2}$ → switch to high risk	MRD $\geq 10^{-3}$ → switch to high risk, SCT indicated
High	SCT indicated in case of poor pre-phase response and MRD $\geq 10^{-2}$	MRD $\geq 10^{-3}$ → SCT indicated

Based on different parameters the patients are stratified into different risk groups. Dependent on the MRD results during therapy the children can be switched to another risk group or they can be considered for stem cell transplantation (SCT).

and 10^{-3} - dependent on the clonal marker - the MRD quantification at time point 1B is measured by a more sensitive technique: the real-time quantitative allele specific oligonucleotide (ASO) PCR. With this technique a sensitivity of 10^{-4}-10^{-5} can be reached. Due to the fact that ASO-PCR is technically very demanding and requires a lot of expertise, the technique is centralised in one centre in Belgium, the Molecular Haematology lab in UZ Brussel. This lab receives the diagnosis and follow-up bone marrow samples of all Belgian children diagnosed with ALL. Since 2009 the lab has fulfilled the ISO norm 15198.

Target identification: detection of the Ig and TCR rearrangements

The different Ig and TCR rearrangements at diagnosis are detected by PCR followed by Genescan analysis. All the leukemic cells of one clone carry the same rearranged junctional region which has a unique sequence with a specific size. This unique sequence will be amplified by PCR using universal primer sets.[7,8] After amplification of the clone specific sequence, the size of the amplicon is detected by capillary gel electrophoreses on a sequencer (Genescan analysis). In ALL a single monoclonal peak will be detected and the size of this peak is highly clone-specific. Healthy persons will have heterogeneous amplicons due to the huge variety in possible rearrangements in all normal lymphocytes leading to a polyclonal Gaussian curve (*Figure 2*).

The tested Ig and TCR genes are: Ig heavy chain (complete and incomplete) (IGH), Ig kappa (IGK), TCR gamma (TCRG), TCR delta (TCRD) and TCR beta (complete and incomplete) (TCRB). An additional marker tested in T-ALL is the SILTAL-1 locus. Deletions involving this locus occur in 12-26% of T-ALL.[9] Due to a 90 kb deletion of chromosome 1, the SIL gene will fuse with the TAL-1 gene leading to an overexpression of TAL-1. In more than 95% of the Belgian patients, one or more markers can be detected in the diagnosis sample; more than 90% have two or more markers. These markers can be used for monitoring MRD in follow-up samples.
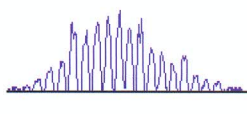
Time point 1A: quantification of MRD by Genescan

The Genescan method is used for the quantification of MRD after induction therapy (time point 1A). At diagnosis, the MRD lab makes an inventory of the clone-specific Ig/TCR markers. The quantification of MRD by Genescan relies on the detection of one or two of these markers in the follow-up sample. The identification of these markers is based on the size of a specific PCR product; the quantification is based on the competitive PCR method as described by Guidal et al. and Cavé et al.[10,11] Therefore, a reference tube and a test tube are made. The reference tube contains 5.10^4 polyclonal mononuclear cells, 5.10^1 leukemic cells of the patient at diagnosis and 5.10^1 cells of an internal standard and mimics MRD of 0.1%. The internal standard is a DNA sample of bone marrow mononuclear cells of an ALL patient that has the same rearrangement as the patient to be tested but with a different size. The test tube consists of 5.10^4 mononuclear cells of the follow-up sample and 5.10^1 cells of the internal standard. If the diagnostic marker is still present in the follow-up sample, MRD is quantified by comparison of the height ratios of the PCR peaks of the patient's leukemic cells and the internal standard with the reference tube (*Figure 3*).
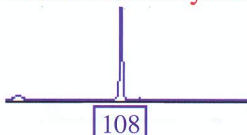
Germline sequences:

IGHV3-33*01	IGHD7-27	IGHJ3*02
...CGAGAGA	CTAACTGGGGA	AATGGTC...

A. Normal

	insertion		insertion		Genescan analysis:
...CGAGAG	T	AACTGGG	AT	GGTC..	
...CGAG	TGGGA	CTAACTGG	TAATA	ATGGTC...	
...CGAGA	TCGG	AACTGGGGA	TGCGT	TC...	
...C	AATCGGT	ACTGGGG		GGTC...	
...CGAG	GGTAT	TAACTGG	CGTAG	GTC...	Polyclonal

B. ALL

	insertion		insertion		Genescan analysis:
...CGAGAG	TGTAC	CTAACTGGGGA	TGGG	GGTC...	
...CGAGAG	TGTAC	CTAACTGGGGA	TGGG	GGTC...	
...CGAGAG	TGTAC	CTAACTGGGGA	TGGG	GGTC...	108
...CGAGAG	TGTAC	CTAACTGGGGA	TGGG	GGTC...	Monoclonal peak of
...CGAGAG	TGTAC	CTAACTGGGGA	TGGG	GGTC...	108 nucleotides

Figure 2. Target identification in ALL; Genescan analysis. **A.** Due to all the different rearrangements in normal B cells a polyclonal Gaussian curve is detected in healthy persons. **B.** The leukemic cells of one clone in ALL carry the same rearranged junctional regions leading to the detection of one monoclonal peak.

With this test, a sensitivity of 0.1% can be reached in most cases. After induction therapy, around 10% of the patients still have detectable cancer cells that can be quantified by Genescan analysis. From 2010 till 2012 the Genescan quantification after induction therapy was performed in 185 Belgian patients of whom 141 were at low or intermediate risk. Four out of these 141 patients (3%) had MRD $\geq 10^{-2}$ at time point 1A. These children were switched to the high risk group to prevent a possible relapse. To date, no relapses have occurred in this group.

Time point 1B: quantification of MRD by ASO-PCR

The real-time quantitative allele specific oligonucleotide polymerase chain reaction (ASO-PCR) is used for the detection of MRD after consolidation therapy (time point 1B).[12] For this technique, the junctional regions of the different clone specific markers - present at diagnosis - are sequenced. After a homology search of this sequence with all the known human germline Ig and TCR gene segments, the clone specific junctional region(s) of the gene can be found. Patient specific primers are designed at the junctional region and tested for sensitivity and specificity by real-time temperature gradient PCR. After optimisation of the PCR reaction, the MRD level of the follow up sample can be measured. Therefore, a serial dilution of the DNA of the diagnosis sample into the DNA of a germline pool of at least six healthy persons is made. The range of the serial dilution is 10^{-1}-10^{-5}. Out of this serial dilution a standard curve can be made with the real-time PCR software and the MRD level of the follow-up samples can be derived from this standard curve (*Figure 4*).

The analysis of the real-time PCR data is made according to the guidelines of the EuroMRD consortium.[13]
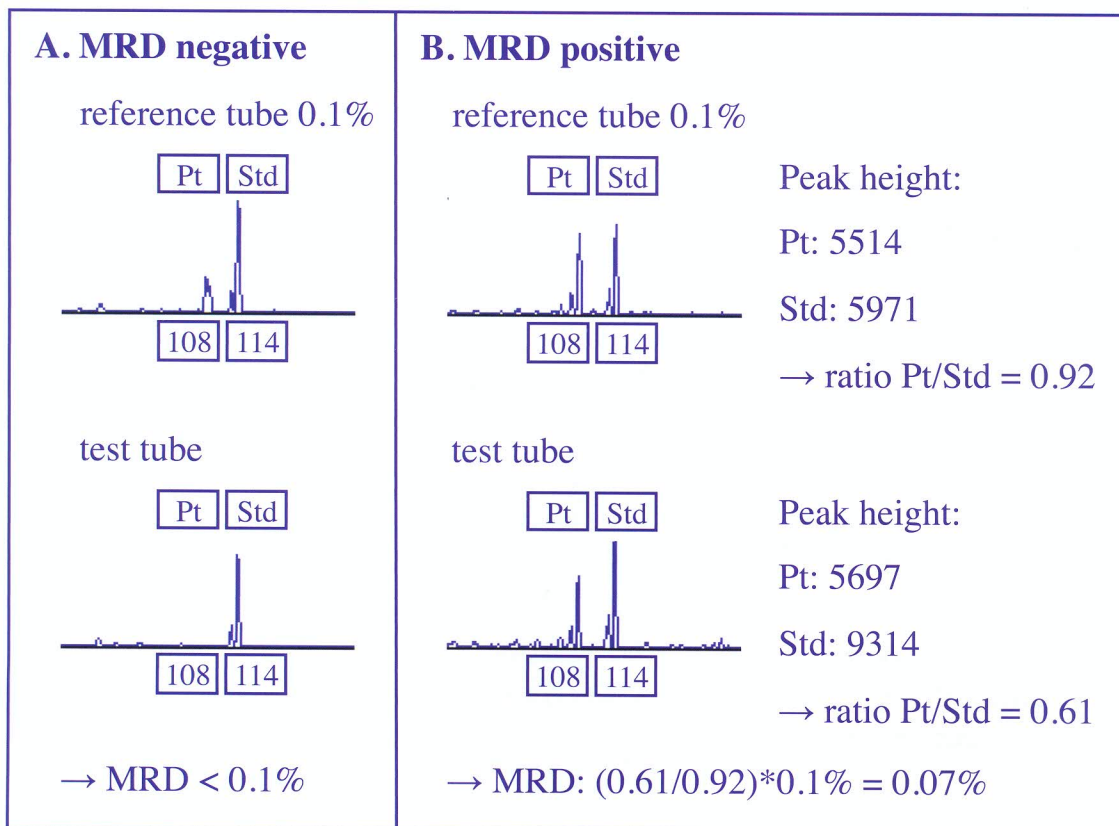
Figure 3. MRD quantification by Genescan analysis at time point 1A. For each quantification a reference tube and a test tube are made. Pt = peak size of the patient at diagnosis (108 bp), Std = peak size of the standard (114 bp) **A.** The diagnostic peak isn't visible anymore in the test tube. The patient is negative for this marker (< 0.1%) **B.** The peak of diagnosis is still visible in the follow up sample (in the test tube). After calculation the patient has an MRD of 0.07%.

The EuroMRD consortium consists of 43 MRD-PCR laboratories (18 countries in Europe, Israel, Singapore, Japan and Australia) and organises quality controls for the ASO-PCR twice a year. Due to these quality controls and the annual EuroMRD meetings, the quantification of MRD by ASO-PCR is a highly standardised technique across Europe. Since 2005 the MRD lab of UZ Brussel is member of the EuroMRD consortium and has to fulfil the different criteria set up by this group concerning lab organisation and experience. Since 2011 the MRD lab of UZ Brussel is also ISO 15198 accredited for the ASO-PCR.

Because some rearrangements can still change during treatment, two different targets are used for follow-up to avoid false negative results. The quantitative range of the first target has to be 10^{-4} and the sensitivity of the second primer 10^{-4}. This is achieved in 72% of the Belgian patients. The result of the ASO-PCR is known within two weeks from the sample arrival in the MRD lab.

From 2010 till 2012 the ASO-PCR after consolidation therapy was performed in 167 Belgian patients of whom 34 (20%) were at high risk. Twenty seven of these high risk patients would have received SCT if MRD data were not available. Now, only thirteen patients received SCT of whom five relapsed. SCT could be avoided in fourteen of the 27 high risk patients (51%). Thirteen of these fourteen patients are in complete remission or continuing therapy, only one patient relapsed. These results show that MRD assessment at time point 1B is important to prevent overtreatment.
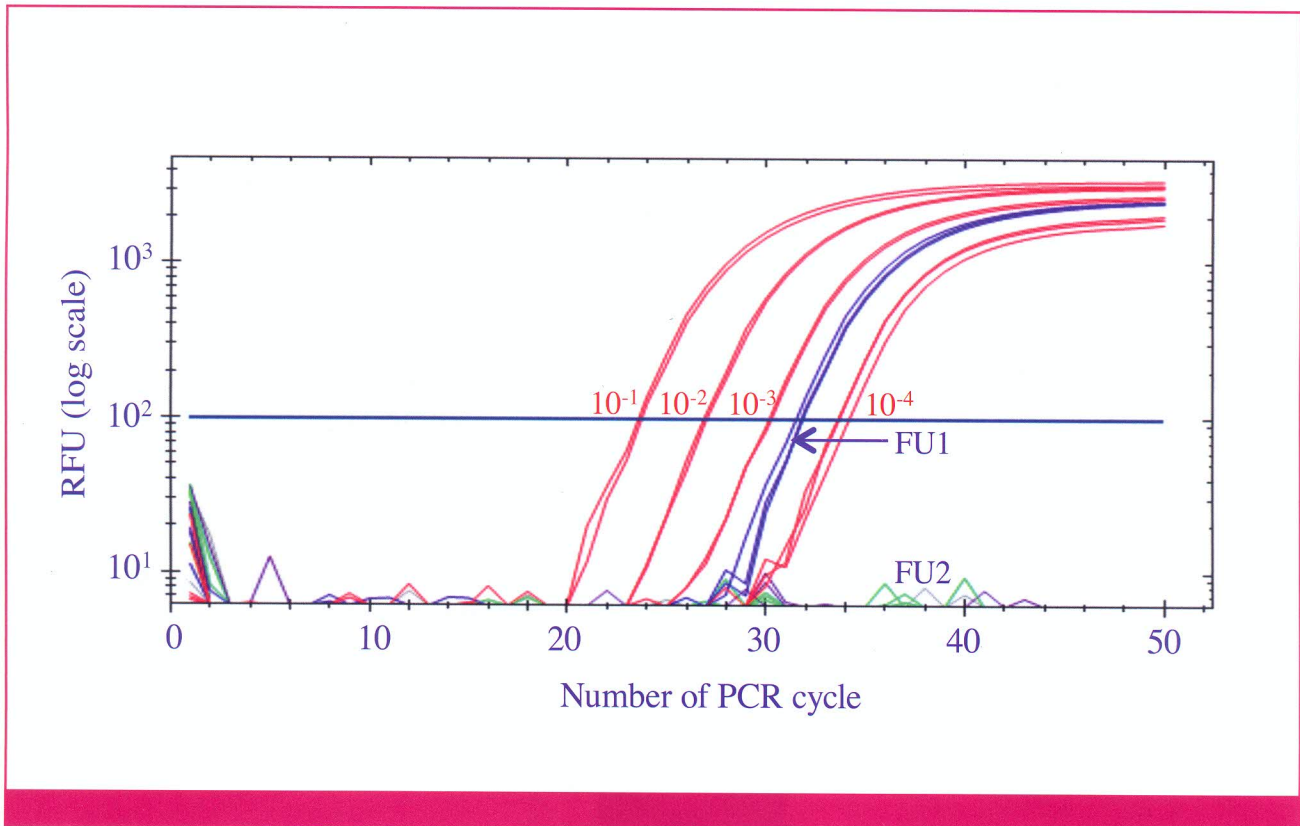
Figure 4. MRD quantification by ASO-PCR. Red: standard curve dilution, range 10^{-1}-10^{-5}. Follow up 1 (FU1): positive, MRD of 5.10^{-4}; Follow up 2 (FU2): negative.

MRD detection in relapsed patients

A retrospective study of the BFM group showed that relapsed ALL patients with a low MRD level at the end of reinduction therapy ($<10^{-3}$) have a significantly better event free survival (EFS) (86%) compared to the patients with a high MRD level (0%).[14]

A recent prospective study in intermediate risk relapsed ALL patients showed that the MRD level after induction therapy is the most important and independent risk factor for long-term outcome.[15] Relapsed children with MRD $<10^{-3}$ after induction therapy had an EFS at ten years of 76% compared to 18% for children with MRD $\geq 10^{-3}$. In order to increase cure, only the children with MRD $\geq 10^{-3}$ were considered for SCT.

Between 2010 and 2012 twenty seven Belgian patients relapsed. Twenty four of these patients were candidates for SCT if no MRD results were available. Based on the MRD results by ASO-PCR, only sixteen patients received SCT, meaning prevention of a high risk toxic treatment in eight patients. Of these eight patients (30%) who didn't receive SCT, seven are in continuous complete remission and one is in complete remission but continuing therapy.

MRD detection before SCT

Different studies have proven the importance of MRD assessment before SCT. Children with no detectable MRD ($<10^{-5}$) just before SCT have a better EFS at five years (75.2 - 78%) compared to the MRD positive children (29.8 - 32%).[16,17] Based on the MRD results before SCT, patients with high MRD levels can be considered for alternative therapies - such as phase I/II trials, new experimental drugs or immunotherapy - before or after SCT to reduce the risk of relapse.

Conclusion

In Belgium, MRD quantification in newly diagnosed childhood ALL is performed after induction therapy (time point 1A) and after consolidation therapy (time point 1B).

The MRD assessment after time point 1A is performed by Genescan analysis. At this time point children at low or intermediate risk with MRD higher than 10^{-2} are switched to a high risk treatment protocol for more intensive therapy.

The MRD quantification at time point 1B is performed by the ASO-PCR. This time point is important to decide whether or not SCT is indicated for a patient

Key messages for clinical practice

1. MRD levels during the first three months of therapy are used to recognise different subgroups in childhood ALL that need different treatment approaches.

2. MRD quantification after induction therapy is performed by Genescan analysis. Children at low or intermediate risk with MRD $\geq 10^{-2}$ are switched to a high risk treatment protocol for more intensive therapy.

3. MRD quantification after consolidation therapy is performed by ASO-PCR. Several children at high risk with MRD $< 10^{-3}$ after consolidation will not benefit from stem cell transplantation.

4. MRD quantification after relapse is the most important and independent risk factor for long-term outcome. If the MRD level after reinduction therapy for relapse is $> 10^{-3}$, SCT is indicated.

5. Before SCT the MRD level should be as low as possible. Children with no detectable MRD just before transplantation have a higher EFS compared to MRD positive children.

at high risk and hence to avoid overtreatment.

The ASO-PCR technique is also used for the MRD quantification after induction therapy in relapsed patients and before SCT and guides decisions on SCT and/or alternative therapies.

In conclusion, Genescan analysis and ASO-PCR are two powerful tools to perform MRD quantification and both are necessary to perform MRD monitoring in childhood ALL.

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