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Running head: Bone marrow morphology in pediatric ALL

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Minimal residual disease monitoring cannot fully replace bone marrow morphology in assessing disease status in pediatric acute lymphoblastic leukemia

Minimal residual disease (MRD) monitoring has a strong prognostic value in childhood lymphoblastic leukemia (ALL) and is currently utilized in all major pediatric ALL protocols. MRD monitoring is done by multiparameter flow cytometry, IG/TCR quantitative PCR or reverse transcriptase quantitative PCR of leukemic fusion transcripts providing a reliable measurement of treatment response.

However, occasionally bone marrow (BM) aspirates may not yield representative material or be misinterpreted due to treatment-induced changes in MRD marker profile, undetected subclones at diagnosis, contamination with peripheral blood or cell adhesion and stroma cell interactions posing a risk for underestimating MRD levels and misclassifying resistant disease that may be detected by traditional BM morphology methods, immunohistochemistry, karyotyping and FISH. We present four cases with high MRD levels where MRD monitoring failed to provide the correct stratification information. Through these cases we discuss the continued need to consider all available information including BM smears, touch imprints and trephine biopsy preparations not only at diagnosis but throughout remission monitoring in pediatric ALL.

**Key words:** Minimal residual disease; acute lymphoblastic leukemia; pediatric; bone marrow morphology
Introduction

Minimal residual disease (MRD) monitoring during therapy has become the most important independent prognostic factor in childhood acute lymphoblastic leukemia (ALL) (1, 2). In childhood ALL MRD monitoring is done by multiparameter flow cytometry, quantitative polymerase chain reaction (PCR) of clonal rearrangements in immunoglobulin and/or T-cell receptor genes (IG/TCR), or reverse transcriptase quantitative PCR (qRT-PCR) of leukemic fusion transcripts (1, 3, 4). New methods such as high-throughput next-generation sequencing (NGS) are emerging with the potential to further improve the sensitivity and specificity of MRD detection (1, 4, 5). Currently flow cytometric methods can identify a distinctive leukemia-associated immunophenotype in most patients with ALL and can detect MRD levels at 0.01%-0.001% or lower depending on the amount of cells analyzed. Whereas molecular methods using clonal IG/TCR rearrangements and patient-specific PCR primers or PCR-amplified DNA fragments of these genes to deep-sequencing analysis, may detect MRD at levels of 0.001% or lower (6).

Nevertheless, as leukemia associated markers can be unstable during therapy and bone marrow aspirate may be contaminated with peripheral blood relying solely on these highly sensitive methods when evaluating MRD status may pose a risk of missing or misclassifying resistant ALL disease readily detected by traditional bone marrow (BM) morphology methods. We present four cases where MRD monitoring may have failed to provide the correct stratification information highlighting the continued need to consider all available information including BM smears, touch imprints and trephine biopsy preparations not only at diagnosis but throughout treatment duration including post induction and post remission monitoring.

Case reports

Case I

A 7 year old boy diagnosed with precursor B ALL. BM aspirate and biopsy yielded marrow smears of high cellularity with 64% lymphoblasts. Flow cytometry identified 36% lymphoblasts in the BM. By flow cytometry the aberrant clone was
immunophenotypically characterized as CD19+, cytCD79a+, CD22+, CD20dim, CD10+, CD34+, TdT+, CD45dim, CD123+, CD66c+, MPO-, cytCD3-, CD13+, CD33+, cytlgM-, CD117-, smlgK-, smlgL-. The immunophenotype was informative (all blast were CD34+/CD123+ in addition to aberrant expression of CD66c (100% of blasts), CD13 (40%), CD33 (30%) and CD4 (50%) and suitable for MRD monitoring by flow cytometry in spite of the presence of several small subpopulations (about 1-2% of all cells) showing negative or weak expression of CD10, CD34 and CD58. Standard antibody combination was used for MRD monitoring (CD66c-CD123-CD34-CD19-CD22-CD45 and CD10-CD20-CD34-CD19-CD33-CD45). Due to the occurrence of immunophenotypically heterogeneous subpopulations PCR MRD monitoring was performed in parallel.

Karyotyping showed a hyperdiploid clone (50,XY,+X,+1,+5,del(7)(p12),del(12)(p11p12),+21[13]/46,XY[12]), whereas FISH showed trisomy 1, 19, 21 and monosomy 12. He received induction treatment according to the Nordic Society of Paediatric Haematology and Oncology (NOPHO) ALL 2008 protocol non-high risk arm for B-cell precursor ALL including prednisolone, vincristine and doxorubicin as described earlier (7). Multiplex PCR (HemaVision®-28N, Dna Diagnostic, Risskov, Denmark) was negative for all 28 investigated translocations (8).

At day 15 flow cytometry indicated a MRD level of 1.1%. Quantitative PCR based on clonal IG/TCR rearrangements found MRD at 4%. However, on the trephine biopsy infiltration of blast cells, immunohistochemically showing CD34 and TdT positivity were seen in more than 50% of marrow cells. Induction therapy was continued unchanged according to the pre-B phenotype and protocol.

At day 29 flow cytometric evaluation showed a MRD level of 0.4%. Quantitative PCR based on clonal IG/TCR rearrangements suggested a MRD level of 4%. However, at morphological evaluation 20% blast was seen on BM imprints and approximately 50% blasts in the trephine marrow biopsy. These 50% blasts remained positive for CD34 and for TdT, although for the latter slightly less pronounced than at day 15. These were at first considered to be haematogones (9) mimicking lymphoblasts and he continued to receive consolidation treatment according to the intermediate risk arm. However, FISH investigation of BM smears and biopsy confirmed trisomy 1, 19 and 21 as well as
monosomy 12 as seen at the time of diagnosis. The patient’s treatment was converted to high-risk (HR) block treatment followed by allogeneic hematopoietic stem cell transplantation (hSCT). He is alive in 1st continuous remission (1.CR).

Case II
A 7 year old girl diagnosed with precursor B ALL. BM aspiration and biopsy yielded smears of high cellularity with 90% lymphoblasts (Figure 1). Flow cytometry identified 49% lymphoblasts. The aberrant clone was by flow cytometry characterized as positive for CD10bright, CD19, CD22, cytCD79a, TdT and CD38dim, partly positive for CD34 and with aberrant expression of CD49f, CD123bright and CD135. Blast cells where negative for smIgK, smIgL, cytIgM, CD20, cytCD3, MPO and all other evaluated myeloid and T-cell specific markers. The immunophenotype was informative and suitable for MRD monitoring by flow cytometry. NOPHO ALL 2008 standard antibody combinations were used for MRD monitoring in addition to a patient specific marker combination (CD66c-CD123-CD34-CD19-CD22-CD45 and CD10-CD49f-CD34-CD19-CD38-CD45).

Cytogenetic analysis revealed three spots for 1q23, chromosome 9 centromere, 9p and 9q34 as well as four spots for 21q22 and one spot for 12p13 in 85% of the cells. However these findings were not reproduced by karyotyping (46,XX[20]). She received induction treatment according to the NOPHO-ALL 2008 protocol non-HR arm (7).

At day 15 flow cytometry suggested a sample dominated by blast cells characterized by ongoing apoptosis/cell death (high side-scatter (SSC) and low forward scatter (FSC)) (10). This sample contained 73% or 24% blast cells depending on whether or not the apoptotic cells were considered. Morphological examination revealed BM smears containing approximate 44% blast cells and a trephine biopsy containing no less than 50% blast cells. Induction therapy was continued unchanged according to the pre-B phenotype and protocol.

At day 29 flow cytometric evaluation showed a MRD level of 1%. She consequently continued to receive intermediate risk therapy. However morphological evaluation of imprints revealed pronounced dominance of lymphoid cells, some of which had blast morphology. The trephine biopsy revealed a hypo-cellular BM dominated by scattered
mononuclear cells with expression of CD79a, TdT, CD34 and PAX5 with the proportion of lymphoblasts estimated to be at least 25% of marrow cells. Subsequently FISH analysis on touch imprints confirmed four spots for 21q22 and 1 spot for 12p13 in 20% of nuclei as seen at the time of diagnosis (Figure 1). The patient’s treatment was converted to HR block treatment followed by allogeneic hSCT. She is alive in 1.CR.

**Case III**

A 2 year old boy diagnosed with precursor B ALL. BM aspiration and biopsy yielded smears dominated by lymphoblasts. The trephine biopsy was infiltrated by blast cells positive for CD34, PAX5 and CD79a with an infiltration rate of >90%. Flow cytometry identified 79% lymphoblasts all characterized as positive for CD19, cytCD79a, CD22, CD10, CD123 and negative for MPO, cytCD3, cytIgM, smIgK, smIgL as well as all other myeloid and T-cell specific markers. Blasts were divided into two subpopulations, 66% of which were positive for CD34 and TdT, negative for CD20 and with CD38dim expression, while the other was negative for CD34 and TdT, positive for CD20 and with normal expression of CD38. The immunophenotype were informative for both subpopulations and suitable for MRD monitoring by flow cytometry. Two standard NOPHO ALL 2008 and one patient specific antibody combination were used for MRD monitoring (CD10-CD20-CD34-CD19-CD33-CD45; CD66c-CD123-CD34-CD19-CD22-CD45 and CD10-CD49f-CD34-CD19-CD20-CD45) with corresponding result. Karyotyping revealed a hyperdiploid clone with trisomy 21 (51,X,-Y,+X,+4,+10,+17,+18,+21[5]/46,XY[20]), which was confirmed by FISH. He received induction treatment according to the NOPHO-ALL 2008 protocol non-HR arm. Multiplex PCR was negative for all 28 investigated translocations (8). At day 15 flow cytometry suggested residual disease of 25%. The touch imprint revealed approximately 25% blasts. The trephine biopsy showed infiltration of blast cells, immunohistochemically PAX5 positive constituting more than 50% of marrow cells. Induction therapy was continued unchanged according to the pre-B phenotype and protocol.

At day 29 flow cytometric evaluation showed an MRD level of 0.3%. However, by morphological evaluation 10% blasts were seen on touch imprints and approximately 45% blasts were seen in the trephine marrow biopsy. These 45% blasts remained
positive for CD10 and CD20. FISH analysis on touch imprints confirmed the presence from diagnosis of trisomy 21 in 15%. Accordingly the patient was re-stratified to HR block treatment with subsequent allogeneic hCST. He is alive in 1.CR.

Case IV
A 9 year old boy diagnosed with precursor T ALL. At diagnosis BM smears, touch imprints and trephine biopsy were dominated by blasts cells. Flow cytometry identified 78% lymphoblasts in the BM. By flow cytometry the aberrant clone was immunophenotypically characterized as CD45dim, cytCD3+, CD5+, TDTdim, CD8dim, CD2dim, CD7+, CD123dim, CD45RA+ and CD38+, CD48-, smCD3-, CD4-, CD10-, CD99-, CD1a-, CD117-, CD34-, TCRa/b-, TCRg/d-, cytTCRB-, CD33-, CD56-, CD13-, HLA-DR-, CD44- and CD22-. The immunophenotype was informative and suitable for MRD monitoring by flow cytometry. The antibody combination prioritized for flow cytometry MRD monitoring was CD48-CD7-cytCD3-CD4-CD8-CD56-CD16-CD45. PCR MRD monitoring was performed in parallel from day 29 according to the pre-T phenotype and protocol. Cytogenetic analysis revealed a clone with t(5;14) as the only aberration (46,XY,t(5;14)(q15;q32)[13]/46,XY[2]) confirmed by FISH to not involve TLX3. Multiplex PCR was negative for all 28 investigated translocations (8). He received induction treatment according to the NOPHO-ALL-2008 protocol high risk arm for ALL including dexamethasone, vincristine and doxorubicin as described earlier (11).

At day 15 flow cytometry suggested a MRD level of 3.5%. Morphological evaluation of the trephine biopsy however revealed incomplete remission of precursor T-ALL with 25-30% lymphoblasts. By immunohistochemistry they were CD3+ in the cytoplasm, CD2w/- and CD5w/-.

As soon as these results were evident he was converted to HR block treatment according to the T-ALL phenotype at presentation and treatment protocol. Before switching therapy a new BM examination was performed.

This examination revealed a very hypocellular BM. By immunohistochemistry, 35-40% T cells were detected and compared with flow cytometry, which showed that two-thirds of the T cells were lymphoblasts corresponding to approximately 25% T lymphoblasts.
similar to the BM examination done at day 15. This was confirmed by quantitative PCR based on clonal IG/TCR rearrangements showing 30% MRD. Following HR block treatment, he subsequently received allogeneic hSCT and is alive in 1.CR.

All patients were diagnosed and treated at H. C. Andersen Children’s Hospital, Odense University Hospital, Odense, Denmark according to the NOPHO ALL 2008 protocol from September 2008 to October 2018. During this period 90 patients were treated at our center and thus this issue affected 4/90 patients (4.4%).

In all four cases standard techniques was applied for obtaining the BM aspirate samples and the trephine biopsy from the posterior superior iliac spine using standard disposable needles (12, 13). Samples for MRD monitoring were always taken from the first puncture of the iliac crest after visually inspection of bone marrow smears drawing volumes of 2 x 2.5-5 mL of marrow. During this time period a uniform sampling protocol using three separate puncture sites were implemented still with MRD samples taken from the first puncture. Samples for morphological evaluation and cytogenetics were drawn after repuncture in another area of the medulla and finally trephine biopsies were taken after the third repuncture of the iliac crest. MRD was measured at the Department of Clinical Immunology (NOPHO National Danish MRD laboratory), Rigshospitalet, Copenhagen, Denmark, either by RQ-PCR based techniques using patient-specific clonal Ig/TCR gene rearrangements according to the BIOMED-2 guidelines (14, 15) and/or flow cytometry using protocol-defined six- to eight color MRD panels for identification and monitoring of leukemia associated immunophenotypes according to the NOPHO ALL 2008 guidelines (16, 17). According to the protocol BCP-ALL should primary be stratified using MRD-flow and T-ALL primary using PCR-MRD if informative markers are available.

A minimum of 100,000 cells were analyzed at diagnosis to identify blast-heterogeneity. If any subpopulations were detected at this sensitivity level these were taken into account at MRD follow-up. At follow up, patients were analyzed using the protocol-defined and patient-specific MRD antibody-combinations if alternative markers were available. In all MRD samples quality was evaluated in terms of total cell count in the bone marrow and presence of CD3+ normal T-cells or mature neutrophils in unexpected large fractions depending on the MRD time point.
Informed consent was obtained from the legal guardians of all patients prior to publication.

Figure 1 exemplifies bone marrow morphology at diagnosis and at treatment day 29 as described in case II.

Table 1 shows blast percentages by morphology, immune histochemistry, flowcytometry and PCR at diagnosis, day 15 and day 29.

**DISCUSSION**

Following induction therapy the majority of patients with ALL achieve complete remission traditionally defined by morphologic criteria requiring the presence of <5% blasts in the BM by morphology and peripheral count recovery (4). MRD monitoring has redefined the concept of remission in pediatric ALL providing a reliable measurement of the drug sensitivity of leukemic lymphoblasts guiding better risk-directed therapy. Several studies have demonstrated a strong association between MRD levels and treatment outcome in childhood ALL (1, 2, 4). Accordingly MRD techniques are currently utilized during and after induction therapy and for post remission monitoring to assess the response to therapy and to provide important information on the depth of post induction remission in essentially all major pediatric ALL protocols (6).

However, in few instances like in the present four cases, BM aspirates may not yield representative material or be misinterpreted posing a risk that the highly sensitive MRD monitoring methods may miss or misclassify resistant disease readily detected by morphology, immunohistochemistry, karyotyping and FISH.

BM aspiration is subject to sampling error resulting in cells of interest being absent or underrepresented in the aspirate. Although sampling technique may be crucial and should preferably be standardized with regard to sampling sequence, number of repunctures and bone marrow volumes drawn it is unlikely that a uniform sampling protocol will totally eliminate this issue. Accordingly, there may also be treatment-induced changes in MRD marker profile or undetected subclones at diagnosis and thus failure to detect or underestimation of residual disease. Furthermore, contribution of bone microenvironment, cell adhesion and cell stroma interactions may also influence sample quality (18-20). To a certain extent this may be overcome by always combining aspiration with trephine biopsy and touch imprint preparations (18, 21). BM touch imprints and
trephine biopsies play a crucial role in the diagnostic workup of acute leukemia, particularly when there is severe pancytopenia and a dry tap or a bloody tap on BM aspiration. Touch imprint provides important morphologic information to arrive at an initial diagnosis on the day of examination while the trephine biopsy sections are awaited. Although the value of touch preparation is well reported in the literature, it may be overlooked in practice (21). Notably, in dry tap patients diagnostic flow cytometry and genetic analysis including molecular PCR-MRD and flow cytometry MRD and can all be performed from cells or DNA extracted from trephine samples.

In the modern age MRD monitoring provides a quick and reliable assessment of treatment response and drug sensitivity in pediatric ALL. This has allowed for better risk assessment and refined stratification strategies ultimately resulting in better treatment outcomes (6). However, as illustrated by these four cases, thorough evaluation of BM morphology to guide the need for MRD assessment on trephine samples in these rare cases is essential in assessing disease status in pediatric acute lymphoblastic leukemia. Consequently a full work-up including BM smears, trephine biopsy and touch imprint should always be carried out in ALL not only at diagnosis but through-out disease monitoring in addition to MRD monitoring in order to improve ALL risk assessment and treatment outcomes. Since it is of paramount importance in MRD guided treatment stratification that MRD measurements can be trusted further studies comparing MRD measured in aspirates and cell extracts from biopsies may be warranted.
REFERENCES


FIGURES & TABLES

Figure 1: Bone marrow morphology at diagnosis and at treatment day 29 as described in case II
A: Giemsa stain of bone marrow trephine biopsy at diagnosis, B: Terminal deoxynucleotidyl transferase stain of bone marrow trephine biopsy at diagnosis, C: FISH on bone marrow aspirate at diagnosis with dual color dual fusion probe for 12p13.2 and 21q22.3 (Vysis) showing one green spot for 12p13.2 and 4 red spots for 21q22.3, D: Giemsa stain of bone marrow trephine biopsy at treatment day 29, E: Terminal deoxynucleotidyl transferase stain of bone marrow trephine biopsy at treatment day 29, E: FISH on touch imprint with dual color dual fusion probe for 12p13.2 and 21q22.3
(Vysis) showing one green spot for 12p13.2 and 4 red spots for 21q22.3 on treatment day 29.

**Tabel 1.** Blast percentage by morphology, immune histochemistry, flow cytometry and PCR at diagnosis, day 15 and day 29 in childhood ALL (n=4)

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<th>III</th>
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<td>Pre-B ALL</td>
<td>Pre-B ALL</td>
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**Blood & bone marrow findings at diagnosis**

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<th>Case III</th>
<th>Case IV</th>
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</tr>
<tr>
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<td>&gt;90 %</td>
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</tr>
<tr>
<td>Imprint</td>
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</tr>
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**Blood & bone marrow findings at day 15**

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**Blood & bone marrow findings at day 29**

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*Sample dominated by blast cells characterized by ongoing apoptosis/cell death, see text.
**For case IV day 27 before converting to high risk block therapy. BM; bone marrow, NA; not available, NR; not reported; PCR; polymerase chain reaction.