Prognostic value of low-level MRD in adult acute lymphoblastic leukemia detected by low- and high-throughput methods

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Key Points

- Adult patients with acute lymphoblastic leukemia with low MRD positivity at week 16 form an intermediate-risk group.
- NGS improves the risk assignment of patients with MolNE MRD.

Persistence of minimal residual disease (MRD) after induction/consolidation therapy in acute lymphoblastic leukemia is the leading cause of relapse. The GMALL 07/2003 study used MRD detection by real-time quantitative polymerase chain reaction of clonal immune gene rearrangements with \(1 \times 10^{-4}\) as discriminating cutoff: levels \(\geq 1 \times 10^{-4}\) define molecular failure and MRD-negativity with an assay sensitivity of at least \(1 \times 10^{-4}\) defining complete molecular response. The clinical relevance of MRD results not fitting into these categories is unclear and termed “molecular not evaluable” (MolNE) toward MRD-based treatment decisions. Within the GMALL 07/03 study, 1019 consecutive bone marrow samples after first consolidation were evaluated for MRD. Patients with complete molecular response had significantly better outcome (5-year overall survival [OS] = 85% ± 2%, n = 603; 5-year disease-free survival [DFS] = 73% ± 2%, n = 599) compared with patients with molecular failure (5-year OS = 40% ± 3%, n = 238; 5-year DFS = 29% ± 3%, n = 208), with patients with MolNE in between (5-year OS = 66% ± 4%; 5-year DFS = 52% ± 4%, n = 178). Of MolNE samples reanalyzed using next-generation sequencing (NGS), patients with undetectable NGS-MRD (n = 44; 5-year OS = 88% ± 5%, 5-year DFS = 70% ± 7%) had significantly better outcome than those with positive NGS-MRD (n = 42; 5-year OS = 37% ± 8%; 5-year DFS = 33% ± 8%). MolNE MRD results not just are borderline values with questionable relevance but also form an intermediate-risk group, assignment of which can be further improved by NGS.

Introduction

Minimal residual disease (MRD) detection is standard practice in the treatment of adult patients with acute lymphoblastic leukemia (ALL) across Europe, with 73% of patients being tested for MRD in first complete remission.1 With standard chemotherapy regimens, ~90% of patients achieve hematological remission, defined as <5% of blasts in bone marrow based on morphologic assessment and resolution...
of extramedullary involvement. However, with the use of modern technologies, such as multiparameter flow cytometry and real-time quantitative polymerase chain reaction (RQ-PCR), 30% to 50% of patients have persistent MRD below the detection level of routine microscopy, which is the main cause of subsequent relapse.1,4

Detectable MRD after induction-consolidation therapy has been associated with poorer disease-free (DFS) and overall survival (OS) across various therapies, disease subtypes, detection methods, and time points.5,6 MRD is therefore widely used to stratify patients into risk groups, to adjust the intensity of chemotherapy, to decide on the addition of targeted therapies, for timely recognition of impending relapse, and as an early measure of disease response in clinical trials. Most studies employ $1 \times 10^{-4}$ as a cutoff for defining MRD persistence/relapse detected by allele-specific RQ-PCR of clonal immunoglobulin and T-cell receptor (TR) gene rearrangements.7 Also, within the German Multicenter ALL trial (GMALL) 07/2003 and the subsequent observational study, RQ-PCR was prospectively used for MRD detection and a cutoff of $1 \times 10^{-4}$ at treatment week 10 (w+10), and w+16 was used to identify patients with molecular failure (MolFail).8 This approach minimizes the risk of obtaining false-positive RQ-PCR results owing to a nonspecific amplification of healthy background lymphoid cells in regenerating bone marrow.8,9 Although MRD positivity < $1 \times 10^{-4}$ is assumed to reflect real low-level disease in most cases, its prognostic relevance has not yet been defined in adult ALL. Even though the EuroMRD group developed precise guidelines for clinical situations where false positivity or false negativity have to be prevented, nonspecific amplification of background immunoglobulin/TR rearrangements might be mixed up with real low-level positivity below quantitative range.10

Here, we analyzed the prognostic relevance of MRD results not fitting to the categories MolFail (quantifiable MRD $\geq 1 \times 10^{-4}$) or complete molecular response (MolCR: MRD negativity with an assay sensitivity of at least $1 \times 10^{-4}$) after consolidation treatment I at week 16 (w+16) of the GMALL 07/2003 protocol, including Ph− ALL patients aged 15 to 55 years. Where possible, we retrospectively analyzed these samples with next-generation sequencing (NGS)-based immunoglobulin/TR assays to evaluate whether the reportedly better specificity of NGS may enhance prognostication.

**Methods**

The entire analyzed cohort contained 1019 high-risk and standard-risk consecutive patients from the GMALL 07/2003 study with Ph− ALL, whose MRD in bone marrow aspirate at w+16 was analyzed at the Unit for Hematological Diagnostics in Kiel by the EuroMRD-based immunoglobulin/TR RQ-PCR. Of the samples, 178 did not fall into the MolCR (MRD negativity with sensitivity at least $10^{-4}$) or the MolFail (MRD $\leq 10^{-4}$) groups because they were either MRD− with an insufficient assay sensitivity, MRD− below quantitative range,10 or MRD− below $1 \times 10^{-4}$. Together, these patients were classified as molecular not evaluable (MolNE), and their clinical characteristics are summarized in Table 1. Risk stratification on the GMALL 07/2003 and its therapeutic consequences are summarized in the supplemental material. The study was approved by the Institutional Review Board of the Christian Albrechts-University in Kiel and performed in accordance with the Declaration of Helsinki.

For all patients, sequences of dominant immunoglobulin/TR rearrangements at the time of diagnosis, obtained by the classical low-throughput analysis employing multiplex BIOMED-2 PCRs11 and Sanger sequencing, were available. In 96 patients with diagnostic DNA available, EuroClonality NGS-based marker screening employing IGH-VJ-FR1 and TRB-VJ primers12 was performed to confirm the results of the routine low-throughput marker screening and as the basis for the NGS-based MRD quantification in w+16 samples. MRD at w+16 was quantified by NGS in 86 patients with available DNA with IGH-VJ-FR1 (60 rearrangements) and TRB-VJ (33 rearrangements) EuroClonality primers and 1-step PCR.12,13 For each patient, 3 replicates each containing 500 ng were analyzed, making the sensitivity of the assay comparable to RQ-PCR ($1 \times 10^{-4}$). For more details on NGS-based marker identification and MRD detection, please see the supplemental material.

**Results and discussion**

Of 1019 patients with Ph− acute lymphoblastic leukemia treated according to the GMALL07/2003 protocol, 603 (59%) were classified as MolCR according to the RQ-PCR MRD level at w+16, 238 (23%) were classified as MolFail, and 178 (17%) patients were classified as MolNE. Among patients with MolNE, 50 (28%) were MRD− with insufficient sensitivity, 4 (2%) had quantifiable MRD $< 1 \times 10^{-4}$, and 124 (70%) had not-quantifiable MRD (with 57 of the respective RQ-PCR assays reaching a quantitative range of at least $1 \times 10^{-4}$). The MolNE group of patients showed an intermediate prognosis (5-year OS = 66% ± 4%, 5-year DFS = 52% ± 4%, 5-year remission duration [RD] = 59% ± 4%) as compared with MolCR (5-year OS = 85% ± 2%, 5-year DFS = 73% ± 2%, 5-year RD = 80% ± 2%) and patients with MolFail (5-year OS = 40% ± 3%, 5-year DFS = 29% ± 3%, 5-year RD = 37% ± 4%; Figure 1A). This is in line with the results from a study on 304 adult patients with Ph− ALL, which showed that DFS of patients with postinduction MRD $< 10^{-4}$ (52%) clustered between patients in

**Table 1. Characteristics of the MolNE patient group**

| Characteristics                  | Patients (N = 178) |
|----------------------------------|--------------------|
| Age, y                           | No. %              |
| Median (range)                   | 29 (15-64)         |
| ≥35 y                            | 112 63             |
| >5 y                             | 66 37              |
| Risk stratification              |                    |
| Standard risk                    | 131 74             |
| High risk                        | 47 26              |
| Immunophenotype                  |                    |
| C-/pre-B-ALL                     | 111 62             |
| Pro-B-ALL                        | 11 6               |
| Early-T-ALL                      | 7 4                |
| Mature T-ALL                     | 7 4                |
| Thymic-T-ALL                     | 42 24              |

**White blood cell count, $\mu$L**

| Median (range) | 11400 (400-463900) |

B-ALL, B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia.

For all patients, sequences of dominant immunoglobulin/TR rearrangements at the time of diagnosis, obtained by the classical low-throughput analysis employing multiplex BIOMED-2 PCRs and Sanger sequencing, were available. In 96 patients with diagnostic DNA available, EuroClonality NGS-based marker screening employing IGH-VJ-FR1 and TRB-VJ primers was performed to confirm the results of the routine low-throughput marker screening and as the basis for the NGS-based MRD quantification in w+16 samples. MRD at w+16 was quantified by NGS in 86 patients with available DNA with IGH-VJ-FR1 (60 rearrangements) and TRB-VJ (33 rearrangements) EuroClonality primers and 1-step PCR. For each patient, 3 replicates each containing 500 ng were analyzed, making the sensitivity of the assay comparable to RQ-PCR ($1 \times 10^{-4}$). For more details on NGS-based marker identification and MRD detection, please see the supplemental material.

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complete molecular remission (63%), patients with MRD ranging from $10^{-4}$ to $<10^{-3}$ (47%), and patients with MRD $\geq 10^{-3}$ (15%).$^{14}$ Furthermore, a pediatric study on 455 children with B-ALL showed that patients with MRD levels between $1 \times 10^{-4}$ and $1 \times 10^{-5}$ at the end of remission induction therapy had a significantly higher risk of relapse than patients with lower or undetectable MRD.$^{15}$

NGS-based MRD detection was performed to check RQ-PCR-based MRD results in w+16 samples of 86 patients of the MolNE group (67 patients with RQ-PCR$^+$ MRD and 19 with RQ-PCR$^-$ MRD with insufficient sensitivity). The NGS assay detected MRD in 42/86 samples (49%), including 41/67 (61%) RQ-PCR$^+$ and 1/19 (5%) RQ-PCR$^-$ with insufficient assay sensitivity. Of note, 26/87 (39%) of RQ-PCR$^+$ MolNE samples were NGS-MRD$^-$, suggesting a high rate of false positive MRD detection because of nonspecific amplification in this group. These data suggest that RQ-PCR negativity is generally confirmed by NGS and prognostically favorable even if the assay sensitivity does not formally reach the level of $1 \times 10^{-4}$ according to EuroMRD criteria.$^{10}$

RQ-PCR is the only method formally checking the sensitivity of each individual assay, whereas multiparameter flow cytometry and NGS only assume certain sensitivities based on input sample amount.

The 5-year OS and DFS of patients with NGS-MRD$^+$ (n = 44; 5-year OS = 88% ± 5%, 5-year DFS = 70% ± 7%, 5-year RD = 75% ± 7%) was significantly higher than for patients with NGS-MRD$^-$ (n = 42; 5-year OS = 37% ± 8%, P < .0001; 5-year DFS = 33% ± 8%, P = .0035; 5-year RD = 41% ± 9%, P = .0035; Figure 1B). Outcome in patients with RQ-PCR$^+$ MolNE was excellent. The single NGS-MRD$^+$/RQ-PCR$^-$ patient in this group died in complete remission after allogeneic stem cell transplantation. When patients with MolNE with negative RQ-PCR and insufficient assay sensitivity were excluded from the analysis, the difference between the OS of the 2 groups remained highly significant (NGS-MRD$^-$ patients: n = 26, 5-year OS = 84% ± 9%; NGS-MRD$^+$ patients: n = 41, 5-year OS = 37% ± 8%, P = .0024; supplemental Figure 2). For more information on NGS-based marker screening and MRD detection results, please see the supplemental material.

![Figure 1. Prognostic impact of MRD levels at end of consolidation (w+16), as shown by Kaplan-Meier estimates of OS, DFS, and RD.](image-url)
In summary, we show that NGS improved the specificity of the MRD analysis and enabled a more precise risk prediction for patients with low-level nonquantifiable RQ-PCR MRD positivity. Although exact MRD quantification using immunoglobulin/TR NGS is still a challenge,\textsuperscript{13} distinction of MRD positivity and MRD negativity is possible and reliable, thanks to a more specific, nucleotide sequence-based readout avoiding false positive signals resulting from nonspecific amplification of background cells, as often observed in RQ-PCR.\textsuperscript{8,9,16} This limited specificity of RQ-PCR MRD assessment in this group of samples has been reported previously and represents a challenge for a reliable relapse risk prediction.\textsuperscript{8,9,16}

Our results might seem to be in contrast with previous publications,\textsuperscript{17,18} mainly reporting false negative RQ-PCR MRD results. This illusive discrepancy is attributable to sample selection: whereas the other studies are performed on unselected samples, we focused on samples with low-level RQ-PCR MRD with a therefore higher probability of false positive RQ-PCR results. An NGS-MRD assessment using higher DNA amounts could potentially improve the sensitivity of the analysis and therefore the prognostication in the MoIIR group, as suggested by others.\textsuperscript{19}

Overall, NGS-based MRD analysis in ALL seems to be especially helpful in patients with RQ-PCR MolNE and may help to discriminate true MRD from false positivity in this considerable and clinically important group. The better specificity of NGS could potentially also improve the predictive value of MRD in the MoIIR and MolFail groups; however, this is beyond the scope of this report. Still, further prospective studies are necessary to prove that NGS-MRD-based stratification can improve the outcome of patients with MolNE. Moreover, such studies could also help refine the definition of and assignment to the MolNE group and its 3 distinct subgroups (MRD$^+$ with insufficient sensitivity, quantifiable MRD $<1 \times 10^{-4}$, and not-quantifiable MRD), and eventually consolidate its significance and use beyond the GMALL study group.

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Authorship
Contribution: J.K., N.G., and M.B. designed the study; M.K., J.K., H.T., N.D., C.D.B., B.S., J.B., N.A., K.W., N.G., J.B., K.N., S.R., A.V., and M.B. contributed and interpreted data; J.K. and H.T. performed and analyzed experiments; M.K., J.K., N.G., and M.B. designed and performed statistical analyses; C.D.B., N.G., and M.B. supervised the project; M.K. and M.B. drafted the first version of the manuscript; and all authors revised and approved the final version of the manuscript.

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References
1. Pigneur A, Montesinos P, Cong Z, et al. Testing for minimal residual disease in adults with acute lymphoblastic leukemia in Europe: a clinician survey. BMC Cancer. 2018;18(1):1100.
2. Abou Dalle I, Jabbour E, Short NJ. Evaluation and management of measurable residual disease in acute lymphoblastic leukemia. Ther Adv Hematol. 2020;11:2040620720910023.
3. Bassan R, Spinelli O, Oldani E, et al. Improved risk classification for risk-specific therapy based on the molecular study of minimal residual disease (MRD) in adult acute lymphoblastic leukemia (ALL). Blood. 2009;113(18):4153-4162.
4. Gööbüget N, Kneba M, Raff T, et al; German Multicenter Study Group for Adult Acute Lymphoblastic Leukemia. Adult patients with acute lymphoblastic leukemia and molecular failure display a poor prognosis and are candidates for stem cell transplantation and targeted therapies. Blood. 2012;120(9):1868-1876.
5. Bassan R, Brüggemann M, Radcliffe HS, Hartfield E, Kreuzbauer G, Wetten S. A systematic literature review and meta-analysis of minimal residual disease as a prognostic indicator in adult B-cell acute lymphoblastic leukemia. Haematologica. 2019;104(10):2028-2039.
6. Berry DA, Zhou S, Higley H, et al. Association of minimal residual disease with clinical outcome in pediatric and adult acute lymphoblastic leukemia: A meta-analysis. *JAMA Oncol.* 2017;3(7):e170580.

7. Hoelzer D, Bassan R, Dombret H, Fielding A, Ribera JM, Buske C; ESMO Guidelines Committee. Acute lymphoblastic leukaemia in adult patients: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* 2016;27(suppl 5):v69-v82.

8. van der Velden VHJ, Wijkhuijs JM, van Dongen JJM. Non-specific amplification of patient-specific Ig/TCR gene rearrangements depends on the time point during therapy: implications for minimal residual disease monitoring. *Leukemia.* 2008;22(3):641-644.

9. Fronkova E, Muzikova K, Mejstrikova E, et al. B-cell reconstitution after allogeneic SCT impairs minimal residual disease monitoring in children with ALL. *Bone Marrow Transplant.* 2008;42(3):187-196.

10. van der Velden VHJ, Cazzaniga G, Schrauder A, et al; European Study Group on MRD detection in ALL (ESG-MRD-ALL). Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. *Leukemia.* 2007;21(4):604-611.

11. van Dongen JJM, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia.* 2003;17(12):2257-2317.

12. Brüggemann M, Kotrová M, Knecht H, et al; EuroClonality-NGS working group. Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker identification in acute lymphoblastic leukaemia; a EuroClonality-NGS validation study. *Leukemia.* 2019;33(9):2241-2253.

13. Knecht H, Reigl T, Kotrová M, et al; EuroClonality-NGS Working Group. Quality control and quantification in IG/TR next-generation sequencing marker identification: protocols and bioinformatic functionalities by EuroClonality-NGS. *Leukemia.* 2019;33(9):2254-2265.

14. Bassan R, Spinelli O, Oldani E, et al. Different molecular levels of post-induction minimal residual disease may predict hematopoietic stem cell transplantation outcome in adult Philadelphia-negative acute lymphoblastic leukemia. *Blood Cancer J.* 2014;4(7):e225.

15. Stow P, Key L, Chen X, et al. Clinical significance of low levels of minimal residual disease at the end of remission induction therapy in childhood acute lymphoblastic leukemia. *Blood.* 2010;115(23):4657-4663.

16. Kotrova M, van der Velden VHJ, van Dongen JJM, et al. Next-generation sequencing indicates false-positive MRD results and better predicts prognosis after SCT in patients with childhood ALL. *Bone Marrow Transplant.* 2017;52(7):962-968.

17. Starza Della I, Novi De LA, Santoro A, et al. Digital droplet PCR and next-generation sequencing refine minimal residual disease monitoring in acute lymphoblastic leukemia. *Leuk Lymphoma.* 2019;60(11):2838-2840.

18. Kotrova M, Muzikova K, Mejstrikova E, et al. The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL. *Blood.* 2015;126(8):1045-1047.

19. Ladetto M, Brüggemann M, Montillo L, et al. Next-generation sequencing and real-time quantitative PCR for minimal residual disease detection in B-cell disorders. *Leukemia.* 2014;28(8):1299-1307.