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Prognostic relevance of limit of quantification as low-level cutoff for flow cytometry-based measurable residual disease assessment in acute myeloid leukemia

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TO THE EDITOR

Measurable residual disease (MRD) assessed by multiparameter flow cytometry (MFC) is prognostic in patients with acute myeloid leukemia (AML), with current European LeukemiaNet (ELN) guidelines defining positivity above 0.1% leukemia-associated immunophenotype (LAIP)-positive cells among total white blood cells (WBC) [1]. However, MRD levels below 0.1% are not equivalent to complete disease eradication. Approximately 30% of MRD-negative cases relapse [2], raising the question if lower levels of residual disease may have clinical significance.

A threshold of 50 LAIP-positive events in samples with at least 500,000 WBC has been proposed as a low-level MRD cutoff [3, 4] to reduce potential false negative results. It represents the technical limit of quantification (LOQ), the smallest number of events required to reliably distinguish a cluster of cells.

Since the LOQ is a low-level threshold, its reliability depends on assay background levels defined as the expression of LAIPs in healthy or regenerating bone marrow (BM) after chemotherapy without residual disease. These background levels can vary substantially between different LAIPs and flow cytometry platforms [5–7]. Therefore, we investigated the prognostic value of LOQ as a low-level threshold while considering the impact of technical background levels across different LAIPs measured across different platforms.

We included 939 adult AML patients in complete remission (CR) after two cycles of intensive chemotherapy with detailed MFC-MRD data from HOVON trials, GIMEMA AML-1310, and AMLSG-Brazil (detailed description: Supplementary methods, Table S1, Table S2, and Fig. S1).

We calculated the LOQ as (50/WBC) × 100 and classified patients into three MRD groups: LOQneg (<LOQ, $N = 277$, 29.5%), MRDnegLOQpos (≥LOQ but <0.1% $N = 492$, 52.4%), and MRDpos (≥0.1%, $N = 170$, 18.1%). The median WBC was 944,934 events (range: 500,626–3,968,084; IQR: 764,548–1,009,690), resulting in a median LOQ threshold of 0.0053% (IQR: 0.0050–0.0065%). MRDpos patients were more frequently ELN adverse risk (38%, Table S3) compared to MRDneg patients (LOQposMRDneg: 24%, LOQneg: 20%), and less often achieved early CR (75% after C1 vs. 84% and 85%, respectively). As previously described for the AML-1310 [3] and HO132 trial [8], in the current cohort, the three MRD levels also showed significant overall survival (OS) and relapse-free survival (RFS) differences (Figs. S2A, S3, respectively). In pairwise comparison, LOQneg patients had significantly worse OS and RFS compared to MRDnegLOQpos patients in both univariable (Table S4) and multivariable analysis (Table 1 and Table S5, respectively).

To enable LAIP harmonization and the investigation of potential LAIP-dependency of the prognostic value of LOQ, we first classified patients into categories based on LAIP expression characteristics as described by Grimwade and Freeman [9] (asynchronous, cross-lineage, under-expression, over-expression, and mature LAIPs) and then combined categories based on LAIP background levels as reported by Rossi [5]. The three groups, from lowest to highest background level, were asynchronous/cross-lineage (A/C) LAIPs ($N = 620$, 66.0%, Fig. S2B), under/over-expression (U/O) LAIPs ($N = 259$, 27.6%), and mature LAIPs ($N = 35$, 3.7%). As these subgroups were formed by pooling individual LAIPs, it should be considered that not all LAIPs within a single category have similarly high or low background levels. Twenty-five cases (2.7%) could not be classified due to insufficient information on the leukemia-defining marker. The distribution of LAIP-categories was similar across HOVON trials (Fig. S2C), while the majority of mature LAIPs ($N = 30$, 85.7%) were found within the AML-1310 trial, representing 21.6% of all LAIPs within that study. This discrepancy is attributable to different panel designs and gating strategies between institutions, as a tube specifically devoted to identifying mature LAIPs was utilized exclusively within the AML-1310 trial.

We found a different prognostic value of LOQ for the three LAIP groups. A/C-LAIPs had a median LOQ of 0.0052% and LOQposMRDneg patients had significantly worse outcomes compared to LOQneg patients for OS (Fig. 1A) and RFS (Fig. S3) in both univariable (Table S4) and multivariable analysis (Table 1, Table S5). In contrast, we found no significant survival differences between LOQneg and LOQposMRDneg for U/O-LAIPs (Fig. 1B and Fig. S3, median LOQ 0.0054%) and mature LAIPs (Fig. 1C and Fig. S3, median LOQ 0.0071%). For mature LAIPs, outcomes were favorable overall with a 3-year OS of 84% and RFS of 71%, potentially reflecting lower relapse-initiating capacity of more differentiated cells, although results should be interpreted with caution due to a limited number of patients (Table S4) and need further evaluation in future studies. Cumulative incidence of relapse (CIR) analysis was comparable to RFS analysis (Fig. S4). We found no significant differences in the prevalence of ELN risk groups between LAIP subgroups (Table S6).

While the three LAIP categories demonstrated distinct prognostic significance, intra-category heterogeneity is likely, given that each category was derived from aggregating individual LAIPs. To investigate this, we performed LAIP-specific survival analysis, however, despite our large cohort, only the most prevalent LAIP CD7+ ($N = 251$) had sufficient patients per MRD subgroup. Multivariable analysis showed significantly worse OS (Table 1) and RFS (Table S5) for LOQposMRDneg compared to LOQneg. The

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Table 1. Multivariable analysis for overall survival per LAIP subgroup.

	Entire Cohort ($N = 939$)			A/C-LAIPs ($N = 620$)			U/O-LAIPs ($N = 259$)			CD7-LAIP ($N = 251$)		
Variable	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***	**HR**	**95% CI**	***p***
MRD status												
LOQneg	—	—		—	—		—	—		—	—	
LOQposMRDneg	1.38	1.09, 1.75	**0.008**	1.62	1.16, 2.27	**0.005**	1.03	0.65, 1.62	0.914	1.8	1.07, 3.01	**0.026**
MRDpos	1.82	1.35, 2.45	**<0.001**	1.93	1.30, 2.87	**0.001**	1.73	0.95, 3.15	0.072	2.01	0.99, 4.08	0.052
AML type												
De novo	—	—		—	—		—	—		—	—	
Secondary/TR	0.90	0.59, 1.37	0.608	0.85	0.50, 1.43	0.542	0.98	0.50, 1.92	0.964	0.29	0.07, 1.14	0.077
Peripheral WBC count at diagnosis												
<100 $\times 10^9$/L	—	—		—	—		—	—		—	—	
≥100 $\times 10^9$/L	1.66	1.21, 2.28	**0.002**	1.65	1.15, 2.38	**0.007**	1.84	0.94, 3.61	0.077	1.48	0.85, 2.58	0.166
Age in years												
<60	—	—		—	—		—	—		—	—	
≥60	1.42	1.11, 1.82	**0.005**	1.77	1.33, 2.35	**<0.001**	0.86	0.51, 1.45	0.565	2.95	1.91, 4.54	**<0.001**
Achievement of first CR												
After Cycle 1	—	—		—	—		—	—		—	—	
After Cycle 2	1.76	1.38, 2.26	**<0.001**	1.68	1.26, 2.24	**<0.001**	2.14	1.29, 3.55	**0.003**	1.73	1.10, 2.71	**0.017**
ELN 2017 risk classification												
Favorable	—	—		—	—		—	—		—	—	
Intermediate	1.48	1.13, 1.94	**0.005**	1.45	1.05, 2.02	**0.026**	1.89	1.10, 3.24	**0.020**	1.67	1.03, 2.70	**0.037**
Adverse	2.18	1.68, 2.85	**<0.001**	2.18	1.58, 3.02	**<0.001**	2.6	1.52, 4.45	**0.001**	1.8	1.09, 2.96	**0.021**
Consolidation												
No consolidation	—	—		—	—		—	—		—	—	
Autologous HSCT/ CC	0.62	0.45, 0.84	**0.002**	0.87	0.60, 1.27	0.466	0.27	0.15, 0.50	**<0.001**	1.08	0.62, 1.89	0.786
Allogeneic HSCT	0.71	0.53, 0.95	**0.023**	0.98	0.68, 1.39	0.899	0.33	0.19, 0.57	**<0.001**	1.14	0.62, 2.07	0.680

A/C asynchronous/cross-lineage, *CC* chemotherapy-based consolidation, *CI* confidence interval, *CR* complete remission, *HSCT* hematopoietic stem cell transplantation, *HR* hazard ratio, *LAIP* leukemia-associated immunophenotype, *LOQ* limit of quantification, *MRD* measurable residual disease, *TR* treatment-related, *U/O* under/over-expression, *WBC* white blood cell.

CIR for LOQposMRDneg patients was similar to that of MRDpos (≥ 0.1%) patients (Fig. S5)

We also evaluated the feasibility of a uniform 0.01% low-level cutoff, which, in contrast to LOQ at 50 events, remains constant irrespective of the number of WBC acquired and is recently more often considered as a lower cutoff for MRD-negativity in studies exploring low-level MRD. For all LAIP-subgroups and CD7+, in multivariable analysis, this cutoff did not show significant survival differences between patients below 0.01% compared to those between 0.01% and 0.1%, except for RFS in A/C-LAIPs (Figs. S6–S8; Tables S7, S8). Rank statistics for optimal cutoff detection did not reveal a uniform low-level threshold across subgroups (Fig. S9).

Defining a uniform low-level cutoff across all LAIPs is challenging, due to differences in background levels, as illustrated by four different LAIPs in regenerating BM (Fig. S10). Background levels were highly variable across LAIPs, with some exceeding 50 events, highlighting that although the technical LOQ of the assay has been validated at 50 events for other hematologic diseases [10], it does not apply to all LAIPs in AML. Since phenotypes with higher background require higher thresholds to reliably distinguish true residual leukemia cells from normal progenitors, LAIP-specific thresholds, although cumbersome, are likely unavoidable when assessing low-level MFC-MRD.

The LOQ successfully stratified MRD-negative patients (<0.1%) into two distinct prognostic groups for patients with A/C-LAIPs, particularly CD7+. Although the survival differences between LOQneg and LOQposMRDneg might be sufficient to justify clinical implementation, the LOQ as cutoff would classify 71% of patients as MRD-positive. This is a substantially larger group compared to previous studies (around 20%) in which MRD-guided consolidation was successfully implemented [11, 12]. Furthermore, these studies have shown that for intermediate risk patients with MRD below 0.1%, allogeneic stem cell transplantation can be deferred, with the opportunity to salvage patients who relapse [11]. Still, considering its prognostic relevance in patients with CD7+, LOQ positivity could serve as a warning sign, prompting a timely repeated measurement. Although our data

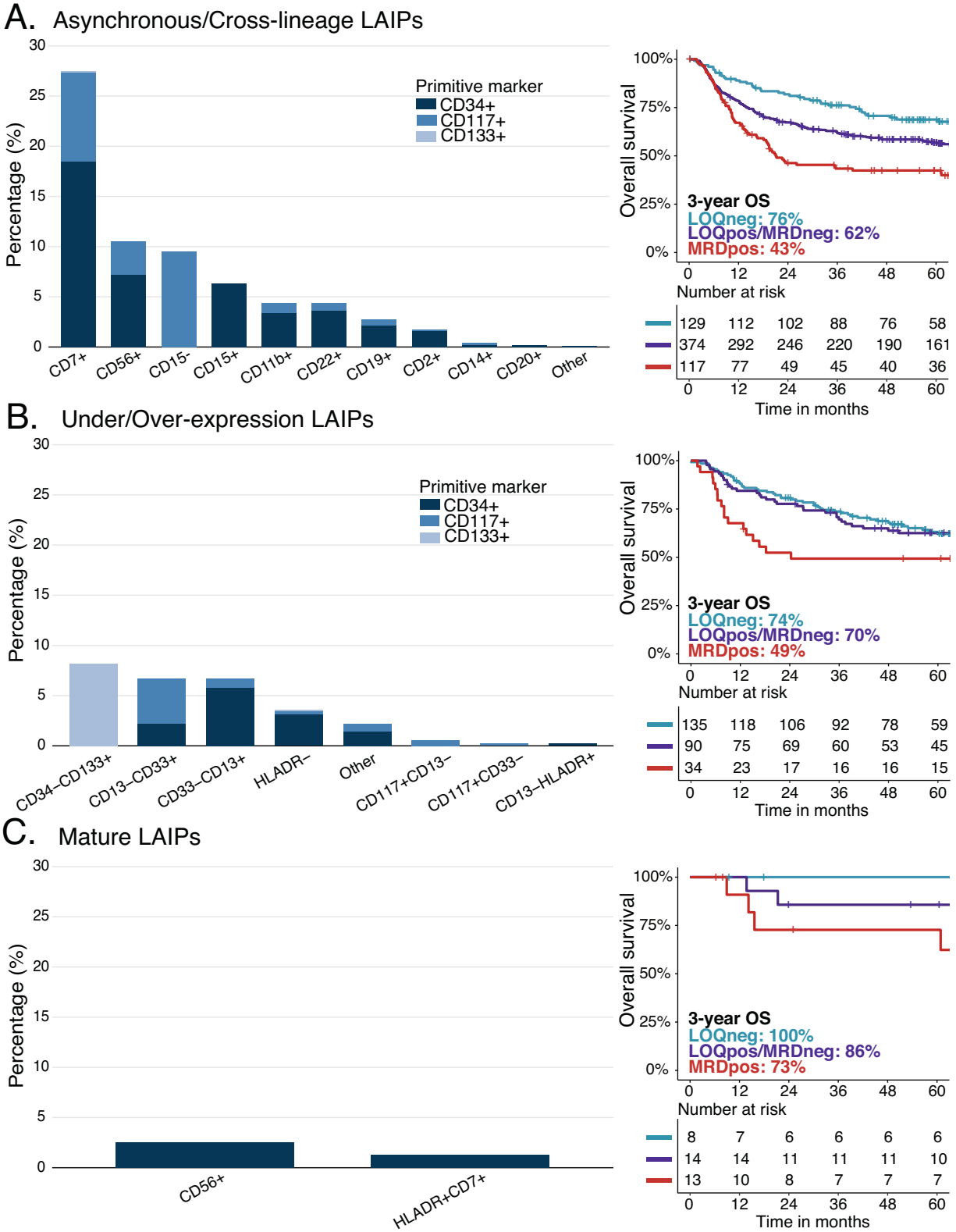

Fig. 1 Prevalence of leukemia-associated immunophenotypes (LAIPs) stratified by LAIP groups and corresponding survival outcomes per measurable residual disease (MRD) category. A Asynchronous/cross-lineage (A/C) LAIPs. **B** Under/over-expression (U/O) LAIPs. **C** Mature LAIPs. Time in months after MRD measurement. The prevalence of LAIPs is shown as a percentage of the total cohort and, for the immature phenotypes (**A** & **B**), stratified per primitive marker (PM). The definition of certain LAIPs depend on the PM: i.e. the A/C-LAIP CD15+ is only aberrant on CD34+ blasts and the U/O-LAIP CD34- only on CD133+ blasts.

should only be interpreted in the context of post-induction, LOQ positivity could potentially also be leveraged post-consolidation, where MRD positivity is strongly associated with relapse [13]. In this setting, low-level MRD could guide more frequent monitoring and MRD sampling and, potentially, early interventions like tapering of immunosuppression and donor lymphocyte infusion.

Although this study includes a large and unique cohort with detailed LAIP-specific MFC-MRD data, certain limitations warrant consideration. Most patients (84%) were included from HOVON trials, which, although accounted for in multivariable analysis, could potentially skew results. Differences in panel design revealed a higher proportion of mature LAIPs in the GIMEMA AML-1310 trial (Fig. S2C) and, although sample analysis was universally performed according to ELN guidelines [14], dissimilarities in gating procedures between institutions might readily impact low-level MRD (Fig. S10). Variability between trials includes differences in treatment protocols and the use of 4- to 10-color flow cytometry panels, although the latter did not affect the observed results (Fig. S11) as the same gating principles were applied. Clinicians in the GIMEMA AML-1310 and HO132 trials were advised to guide consolidation in intermediate-risk patients based on MRD positivity (0.035% and 0.1%, respectively). Future studies should evaluate the prognostic relevance of LOQ in patients treated with non-intensive regimens and novel targeted therapies, and evaluate if results remain similar using current MFC analysis strategies. LAIPs in this study were followed using the LAIP method, while the LAIP-based different from normal (DfN) approach is currently standard practice. Emergence of new LAIPs at low levels detected by the LAIP-based DfN approach might be more challenging to differentiate from background noise caused by regeneration and post-therapy-associated inflammation [15]. Even larger studies, currently being designed within the ELN-DAVID consortium, are required to substantiate the prognostic relevance of LOQ and evaluate the potential of LAIP-specific thresholds.

Our study demonstrates that the relevance of LOQ varies significantly across different LAIPs, highlighting several challenges of low-level thresholds, including assay background levels and LAIP specificity. Considering the strong prognostic relevance for CD7+, an LOQ-positive result might serve as a clinical warning category prompting a timely repeated measurement. The heterogeneous results across LAIP categories using the MFC-MRD gating strategies in this study underline the strength of the current 0.1% cutoff and suggest that a universal low-level threshold is not yet feasible and should be further evaluated.

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DATA AVAILABILITY

Data are available upon reasonable request from the corresponding author, Jacqueline Cloos (j.cloos@amsterdamumc.nl).

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AUTHOR CONTRIBUTIONS

The study was conceptualized by LHH, RP, JMT, FB, and JC; Data were curated by LHH, RP, LN, VA, AJS and LM; Statistical analysis was performed by LHH, TR, and PG; Data and sample collection was done by AV, BL, GJO, MRVI, FB, and JC; Immunophenotypic analysis were performed by AK, WJS, MRVI and FB. The manuscript was written by LHH and revised by RP, TR, JMT, LN, CB, AK, WJS, PG, LM, AV, DCL, MRVI, FB, and JC; The manuscript was approved by all authors.

COMPETING INTERESTS

AV: BMs celgene: Consultancy, Other: invited speaker; Servier: Consultancy, Other: invited speaker; astellas: Consultancy, Other: invited speaker; Jazz: Consultancy, Other: invited speaker, Research Funding; Pfizer: Consultancy, Other: invited speaker; AstraZeneca: Consultancy; Janssen: Consultancy, Other: invited speaker; Glycostem: Consultancy; laboratories Delbert: Consultancy; Beigene: Consultancy; Abbvie: Consultancy, Other: invited speaker; Gilead: Consultancy, Other: invited speaker; Menarini: Consultancy, Other: invited speaker; Istituto Gentili: Consultancy. DCeL: AbbVie: Consultancy; Servier: Consultancy; Immedica: Consultancy Takeda: Membership on an entity's Board of Directors or advisory committees; Daiichi Sankyo: Consultancy; Immedica Pharma: Scientific advisory board. FB: Jazz Pharmaceuticals: Honoraria; Delbert: Honoraria; Servier: Honoraria. JC: Novartis, Genentech: Research Funding; Navigate, BD biosciences: Patents & Royalties: MRD measurement; Astellas: Speakers Bureau; Merus: Research Funding; Genentech: Research Funding.

ETHICAL STATEMENT

The study was approved by the institutional review boards of all included trials (EudraCT numbers for HOVON 42A, HOVON 81, HOVON 92, HOVON 102, HOVON 103, HOVON 132, and GIMEMA AML-1310 are shown in Supplementary Table S1), and by the institutional ethics committee of Hospital Amaral Carvalho for patients included from the AMLSG Brazil (CAAE: 69725623.0.0000.5434). All patients provided written informed consent and the study was conducted according to the Declaration of Helsinki.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41375-025-02825-x.

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