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ACUTE MYELOID LEUKEMIA

CD34+CD38- leukemia stem cells predict clinical outcomes in acute myeloid leukemia patients treated non-intensively with hypomethylating agents

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TO THE EDITOR:

Patients diagnosed with acute myeloid leukemia (AML) who are ineligible for intensive treatment with high-dose chemotherapy (IC) and subsequent stem cell transplantation commonly receive non-intensive treatment with hypomethylating agents (HMA) [1]. HMA monotherapy is not considered curative but prolongs survival and is given until progressive disease [1].

The European LeukemiaNet (ELN) risk classification, determined for the intensive treatment setting [1], does not apply to HMA-treated patients [2]. Recently, a risk classification for non-intensive patients was established, but this was largely based on venetoclax-based regimens [3].

An additional prognostic factor in patients undergoing intensive therapy, is the level of residual leukemic cells after treatment (measurable residual disease (MRD)) [4]. The clinical relevance of MRD in non-intensively HMA-treated patients remains to be determined. In the initial analysis of patients treated with decitabine in the HOVON-SAKK135 trial, MRD was not significantly associated with survival [5].

For intensively treated patients, leukemia stem cells (LSC) have prognostic value at diagnosis, after induction therapy [6, 7], and in the peri-transplantation setting [8, 9]. In this study, we aimed to determine the prognostic relevance of LSC measured using multi-parameter flow cytometry in AML patients aged 66 years or older receiving decitabine and the experimental drug ibrutinib or placebo [5] in the HOVON-SAKK135 trial. LSC was defined as CD34+CD38- [10], and the presence of an aberrant marker, among others CD45RA [11] or CD123 [12]. Detailed information on patients, flow cytometry, and statistics can be found in Supplementary Methods.

A total of 144 patients were enrolled in the HOVON-SAKK135 trial. LSC load was assessed in 113 (78%) individuals at diagnosis (Fig. S1). A third cycle of therapy was received by 87 (60%) patients, with LSC measurement after three cycles available in 49 (56%) patients, of which 38 (78%) exhibited a morphological response, defined as complete remission (CR), CR with incomplete blood count (CRi), or morphological leukemia-free state (MLFS). After a third cycle, 69 (79%) went on to receive a fourth cycle.

In this cohort, only 8 patients were CD34neg at diagnosis (CD34% <1% and absence of LSC; Fig. S2), and these patients were excluded from the survival analysis at diagnosis.

The median LSC percentage at diagnosis was 0.006 (IQR: 0.0007–0.07) and with the previously assessed cutoff of 0.03% [6] we observed that LSCpos patients have a shorter, not significant, overall survival (OS) (HR 1.47 (0.96–2.24); $p = 0.08$; Fig. S3A), but significant shorter event-free survival (EFS) (HR 1.54 (1.03–2.31); $p = 0.035$; Fig. S3B) compared to LSCneg patients. LSCpos patients showed a higher incidence of relapse than LSCneg patients, but the incidence of not reaching CR or CRi after three cycles of HMA (incidence of treatment failure) was comparable (Fig. S3C, D). As the cutoff was established for intensively treated patients, we determined a new cutoff using the maximally selected ranked statistics method. The optimal cutoff for these HMA-treated patients was 0.01% and receiver operating curve (ROC) analysis showed an area under the curve of 0.58 (Fig. S4).

When applying this revised cutoff, we observed that CD34neg and LSCneg patients are more often *NPM1* mutated than LSCpos patients (Table S1 and Table S2). Conversely, LSCpos patients

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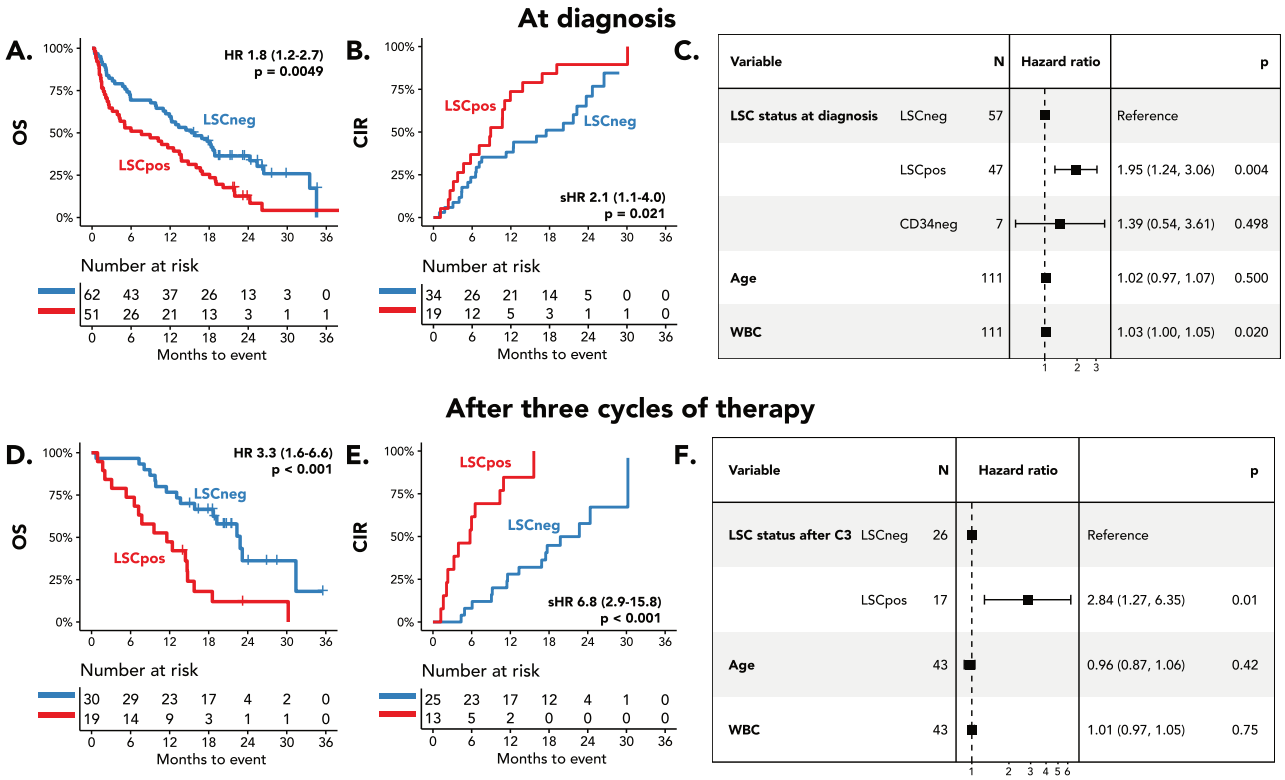

Fig. 1 Prognostic relevance of LSC status at diagnosis, using a cutoff of 0.01% of WBC and after three cycles of therapy, using a cutoff of 0.001% of WBC. A Overall survival (OS) based on LSC status at diagnosis. **B** Cumulative incidence of relapse (CIR) based on LSC status at diagnosis from the time of first CR. **C** Multivariable Cox regression of the overall survival from diagnosis for LSC status at diagnosis. **D** Overall survival based on LSC status after three cycles from the time of sampling. **E** Cumulative incidence of relapse for patients reaching CR/CRi/MLFS from time of sampling based on LSC status after three cycles **F** Multivariable Cox regression for overall survival for LSC status after three cycles (C3).

revealed more often a *RUNX1*, *U2AF1*, or *BCORL1* mutation, all not associated with survival in this group.

We observed that LSCpos patients have significant inferior OS (Fig. 1A) and a significant increased time to relapse after reaching CR, CRi or MLFS (Fig. 1B). After multivariable correction for age and white blood cell count at diagnosis, LSCpos patients still showed worse OS (HR (95% CI): 2.0 (1.2–3.1); $p = 0.004$; Fig. 1C). Furthermore, LSCpos patients had significant shorter EFS than LSCneg patients (Fig. S5A; HR (95%CI): 1.8 (1.2–2.7); $p = 0.0022$) and higher incidence of treatment failure (Fig. S5B; sHR (95%CI): 1.8 (1.1–2.9); $p = 0.015$). LSCneg patients were more likely to reach CR, CRi, or MLFS (LSCpos: 37%, LSCneg: 55%; $p = 0.03$). The higher incidence of relapse lost statistical significance in multivariable correction (sHR (95% CI): 2.0 (0.9-4.5); $p = 0.1$; Fig. S5C).

The median LSC percentage after three cycles of therapy was 0.0003% (IQR: 0.00006–0.004). The cutoff to assess post-induction LSC status in intensively treated patients is 0.0000% [6] with 50% of LSCpos patients. We applied this cutoff in this patient population and observed that 14% of patients were classified as LSCneg, not resulting in a prognostic difference (Fig. S6). Therefore, we determined a revised cutoff for the context of HMA treatment. Using maximally selected ranked statistics, this cutoff was set at 0.001% (Fig. S7A). As a different cutoff reflects differences in remission depth, this indicates that decitabine is not as effective as two cycles of intensive chemotherapy. To assess the potential predictive value of LSC, we performed an ROC analysis for relapse after 1 year and found an area under the curve of 0.84 (Fig. S7B). The numbers were too small to find differences in patient characteristics and mutation status based on LSC status in follow-up (Table S1).

LSCpos patients had significantly shorter OS compared to LSCneg patients (Fig. 1D). Furthermore, LSCpos patients had significantly higher incidence of relapse, having almost all (85%) relapsed within one year (Fig. 1E). When performing multivariable analysis, LSC status remained an independent prognostic factor (HR (95% CI): 2.8 (1.3–6.4); $p = 0.01$; Fig. 1F). In multivariable Fine-Gray analysis, LSC status remained an independent prognostic factor (sHR (95% CI): 4.9 (2.0–12.1); $p < 0.001$; Fig. S8) when adjusting for age and WBC count at diagnosis. After three cycles of HMA treatment, LSCpos patients were able to receive a median of additional 4 cycles (IQR: 1.5–11), compared to 14 cycles (IQR: 9.25–17; $p < 0.001$) in LSCneg patients.

To determine if the combined LSC and MRD results could further stratify prognosis, we performed survival analysis based on the combined LSC and MRD results. We observed that patients positive for both LSC and MRD all relapsed within 6 months (Fig. S9). In the remaining groups i.e. LSCposMRDneg, LSCnegMRDpos, and LSCnegMRDneg, survival was determined by the LSC result. MRD status alone did not result in survival differences (Fig. S10).

To determine how HMA reduced LSC load, we examined LSC kinetics between diagnosis and after three cycles of HMA therapy. From the LSCpos patients at diagnosis, 15 had an evaluable sample after cycle 3. Of these patients, 9 (60%) reached LSC negativity, showing that HMA can eradicate LSC (Fig. 2A). Eleven of the 27 (41%) LSCneg patients at diagnosis with a suitable sample after cycle 3 had an LSCpos result after cycle 3. Furthermore, we categorized patients in descending, stable, or ascending LSC load based on a log difference between the LSC percentage at diagnosis and the LSC percentage after three cycles

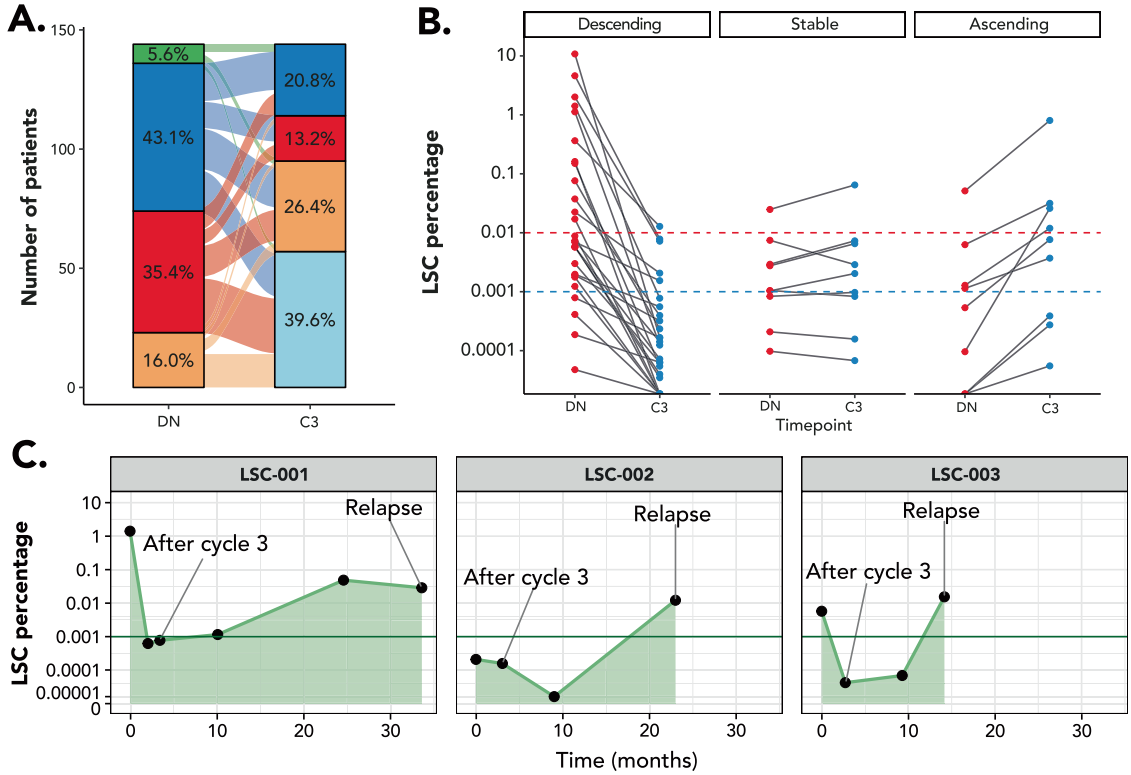

Fig. 2 LSC kinetics between diagnosis (DN) and third cycle (C3) and until relapse. A Proportion of patients categorized as CD34neg (green), LSCneg (dark blue), LSCpos (red), no material or unsuitable sample (orange) and did not reach the third cycle (light blue) at diagnosis (DN) or after three cycles of HMA treatment (C3). **B** LSC percentages at diagnosis and after three cycles for descending, stable, and ascending trajectories, defined as log increase, log decrease or no log difference between diagnostic and follow-up samples. **C** Individual LSC kinetics of three representative patients that relapsed with an LSCneg result after three cycles (other patients can be found in Fig. S11). Dark green line represents LSC cutoff after three cycles (0.001%).

(Fig. 2B). We found that after three cycles, the LSC load had descended in 60% of patients (27/45).

To investigate if the quantification of the LSC load can be used for monitoring, we investigated the trajectory of relapsed patients who were LSCneg after three cycles of therapy and had follow-up samples or a relapse sample available. We identified 10 patients for whom this was the case and observed in all but one (90%) that the LSC load was higher in the relapse sample than measurement after three cycles or had an LSCpos result (cutoff: 0.001%) prior to relapse (Fig. 2C, Supplementary Fig. S11). These kinetics show that LSC are present at relapse. In one relapsed patient (LSC-009) no LSC was present at diagnosis nor at relapse, suggesting that the relapse-initiating cells were not present in our defined LSC phenotype. Despite this exception, LSC could be useful for monitoring HMA-treated patients for upcoming relapse.

In the context of HMA monotherapy, this is the first report demonstrating that residual LSC showed prognostic value. As shown previously, MRD was not associated with prognosis [13] or the prognostic effect was small [14]. We found that LSC status at diagnosis and especially after HMA therapy has high prognostic value. After three cycles of therapy, the area under the curve of the ROC indicated that LSC is a predictive factor for relapse within 1 year.

How LSC measurements can be implemented remains to be determined, as salvage therapy with high-dose chemotherapy or stem cell transplantation is unfeasible. In LSCneg patients after three cycles of HMA treatment, the treatment is deemed effective and thus treatment should be continued. Regarding LSCpos patients, a median of 4 cycles of HMA treatment could still be given before treatment failure. However, other treatment options include stopping treatment, additional targeted treatments or enrollment in a clinical trial. The current standard treatment for IC-ineligible patients is HMA with venetoclax [15]. Whether LSC load has the same effect in the context of venetoclax treatment has to be evaluated. It has been reported that venetoclax prior to allogeneic transplantation reduced LSC proportions [9].

In conclusion, HMA can reduce LSC load and LSC status is significantly associated with prognosis in non-intensively HMA-treated patients.

DATA AVAILABILITY

Data and code are available on request from the corresponding author, Jacqueline Cloos (j.cloos@amsterdamumc.nl).

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AUTHOR CONTRIBUTIONS

Sample collection was done by TP, SKK, GS, LG, DB, DvLV, RB, GJO, AAvdL, GH, DCdL in the HOVON-SAKK135 trial; experiments and/or analysis of flow cytometry LSC data was performed by TR, LLN, DdH, JCH, MMHEF, WJS; Statistical analysis was performed by TR and checked by DAC and TRM; CB, DCdL and JC supervised the study; the manuscript was written by TR and revised by LLN, JCH, WJS, TRM, DAC, GJO, AAvdL, CB, DCdL, JC and the results were reviewed and the manuscript was approved by all the authors.

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COMPETING INTERESTS

LG holds membership on an entity's board of directors or advisory committees for Miltenyi Biomedicine. AAvdL has received honoraria from Amgen, Novartis, Celgene/BMS, and Takeda and has received research funding from Alexion. DCdL participates in the sponsored speaker's bureau of Servier, Roche and AbbVie; is part of the scientific advisory board of Takeda and Servier. GJO serves as a consultant for Novartis, Pfizer Inc, Celgene, Janssen, AGIOS, Amgen, Gilead, Astellas, Roche, Jazz Pharmaceuticals, and Merus; has received honoraria from Novartis, Celgene, AGIOS, Gilead, and Astellas; received research funding from Novartis; and holds membership on an entity's board of directors for Roche. JC receives royalties from Navigate and BD Biosciences; participated in the sponsored speaker's bureau of Astellas; and has received research funding from Takeda, DC-one, Genentech, Janssen, Novartis, and Merus. The remaining authors declare no competing financial interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Patients signed informed consent and the study was conducted according to the declaration of Helsinki. The study was approved by the medical ethics committee of the University Medical Center Groningen (number: 2015.550).

ADDITIONAL INFORMATION

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