Different features of acute myeloid leukemia stem cell quantification in intensively treated patients

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Abstract

In acute myeloid leukemia, the burden of CD34$^+$CD38$^-$ leukemia stem cells (LSC) has prognostic value at diagnosis and after induction chemotherapy. Since different methods of LSC quantification have been proposed, we determined the prognostic value on overall survival and incidence of relapse of these methods across European LeukemiaNet (ELN) 2017 risk groups, using data from the HOVON-SAKK132 trial. In addition, we evaluated the optimal number of acquired white blood cells for accurate LSC detection and the prognostic value of individual LSC markers. Results show that it is essential to acquire at least 1x10^6 white blood cells in order to assess LSC-negativity accurately. Among various LSC markers, CD44 overexpression on CD34$^+$CD38$^-$ cells was the only marker that was not statistically significant in our panel. Testing the impact of several published variations of LSC analysis on prognostic value for overall survival and cumulative incidence of relapse showed only marginal differences, demonstrating the robust prognostic value of LSC burden. For further clinical implementation, the optimal LSC assessment may differ among ELN risk groups. In conclusion, LSC burden is a robust prognostic factor and insight into the different methods of LSC definition can facilitate the clinical implementation. Trial registration EudraCT Number: 2013-002843-26.

Introduction

Measurable residual disease (MRD) after intensive chemotherapy has strong prognostic value in acute myeloid leukemia (AML).[1] However, 30% of MRD-negative patients develop a relapse. Hence, improvement in MRD assessment is warranted. A high risk of relapse may depend on cells with self-renewal capacity that are resistant to therapy. Such features have been described for the low frequency CD34$^+$CD38$^-$ leukemia stem cells (LSC).[2-4] Within the CD34$^+$CD38$^-$ fraction,[5-7] common LSC markers are CLL-1,[8] CD45RA[9] and CD123.[10] The prognostic value of detecting CD34$^+$CD38$^-$LSC marker$^+$ cells has been demonstrated at diagnosis[11,12] and in the follow-up setting[12,13] and has been recently prospectively validated, adding prognostic information to the standard MRD assessment.[14]

Standardization of an assay is crucial in order to ensure reproducible and reliable results, as has been demonstrated for AML samples measured for LSC load at diagnosis.[15] However, standardizing $CD34^+CD38^-$ LSC detection after the second cycle of chemotherapy (C2) remains challenging because of the infrequency of these cells (about 1 per 10^6 cells) in the bone marrow.[2,16] The previously defined cut-off[12,14,17] for the detection of LSC after C2 (>0.00000% LSC of white blood cells [WBC; CD45-expressing cells]) may be too stringent and requires ample guidance during analysis. Furthermore, with the recommendation of requiring $4x10^6$ WBC cells,[12] nonspecific events may also be present, which will complicate the analysis when applying this cut-off.[18]

A standardized and harmonized assay for quantifying LSC in follow-up is not currently being used because of various LSC analysis strategies present in the literature.[11,13,19] Firstly, Li and colleagues[13] demonstrated that by defining a target number of the LSC population with a minimum of 20 $CD34^+CD38^-$ cells and at least 10% LSC marker positivity, their LSC assay showed the best performance. Secondly, different cut-offs have been described by various groups. The 0.00000% cut-off was the optimal cut-off in the HOVON-SAKK 102 (HO102) trial,[12] and this cut-off was prospectively validated in the HOVON-SAKK 132 (HO132) trial.[14] However, this cut-off means that any LSC detected would classify a patient LSC^+ and therefore requires stringent gating to identify specific events. Thirdly, due to the heterogeneity of cells in the WBC compartment and the possibility of hemodilution, changing the denominator to assess LSC based on the $CD34^+$ cells has also been proposed.[19] An advantage of this primitive marker (PM)-based parameter may be that it is less influenced by hemodilution[20,21] as the $CD34^+$ compartment is less influenced by mature cells such as granulocytes.[20,22-24] Lastly, the number of $CD34^+CD38^-LSC^+$ cells is highly dependent on the $CD38^-$ cut-off. In our previous studies, we used a stringent cut-off for CD38 negativity, which was the lower boundary of blank Spherotech beads (CD38 fluorescence intensity $<10^2$).[12,15] Using this stringent CD38 negativity cut-off resulted in lower LSC numbers compared to those in other publications.[11]

In this study, we aimed to evaluate our current assay on the minimum number of cells required for the accurate acquisition of LSC and the prognostic value of individual LSC markers.[13] Furthermore, we aimed to delineate the effect of adjustments to the $CD34^+CD38^-$LSC assay on the prognostic relevance of LSC before and after therapy. Therefore, using the LSC data of the HO132 trial we examined: (i) defining the number of target events to improve sensitivity;[13] (ii) different levels of cut-offs for the assessment of LSC positivity; (iii) changing the denominator to primitive marker positive cells (PM, $CD34^+$ cells) instead of WBC (PM-LSC);[19] and (iv) changing the cut-off of the CD38 negativity threshold of $CD34^+CD38^-LSC^+$ cells.[19] To explore the prognostic value of these adjustments further, we also investigated how these adjustments affected prognostic value in different European LeukemiaNet (ELN) risk groups.

Methods

Patients

Eligible patients in the HO132 study[25] (N=905) were included in the analysis. Patients received "7+3" induction chemotherapy and were randomized to receive additional lenalidomide (15 mg/day for 21 days per cycle without survival benefit). Assessment of MRD, based on quantitative polymerase chain detection of *NPM1* qPCR and/or multiparameter flow cytometry, was used to guide the consolidation treatment after two cycles of chemotherapy in intermediate-risk patients. ELN2017 intermediate-risk patients who were MRD-positive were advised to receive allogeneic stem cell transplantation and ELN2017 intermediate-risk MRD-negative patients were advised not to undergo allogeneic stem cell transplantation. The LSC assay was not reported to the clinic and was not, therefore, used in clinical decision-making. Patients signed written informed consent, and the HO132 study was approved at every center by the local ethical board and performed according to the Declaration of Helsinki.

Flow cytometry leukemia stem cell assay

The one-tube LSC assay[26] was performed during the HO132 trial at diagnosis, and the protocol-defined follow-up time-points: after C2, before second randomization (before maintenance) and 6 months after second randomization. Priority was given to the standard MRD leukemia associated immunophenotype (LAIP) assay over the LSC assay when the sample had insufficient cells for both assays (before lysis: $8x10^6$ cells each for the MRD assay and the LSC assay), as the MRD LAIP assay was reported to the clinic and used for clinical decision-making in the intermediate-risk group. The MRD LAIP assay was measured with the previously published eight-color four-tube assay,[27,28] and the MRD result was determined when a patient was in complete remission (CR) or complete remission with incomplete count recovery (CRi) according to ELN2021 recommendations for MRD assessment. Flow cytometry was performed with a BD FACS Canto II, and the data were analyzed using Infinicyt 1.8 and 2.0 (Cytognos, Salamanca, Spain). LSC were analyzed by gating on $CD45^{dim}CD34^+CD38^-$ cells and then evaluated on LSC marker positivity (CD45RA, CD123, CD33, CD44 or 'combi' containing CD56, CD22, CD11b, CD7, TIM-3, and CLL-1). The gating strategy for LSC in follow-up is described in *Online Supplementary Figures S1* and *S2*. LSC analyses were reviewed during a weekly general consensus sign-off meeting, and the optimal LSC marker was chosen based on the highest frequency of LSC and the best separation between LSC marker positivity and negativity. LSC status at diagnosis was defined in three groups: LSC^{low}, and LSC^{high} based on 0.03% LSC of WBC threshold and as $CD34^-$ defined as <1% $CD34^+$

blasts of WBC, absence of CD34+ LAIP, and absence of LSC. LSC status after C2 was defined as LSC- or LSC+ based on the cut-off of 0.00000% LSC of WBC. CD34- AML was only defined at diagnosis, not during follow-up.[12]

Statistical analysis

Survival analyses were performed with R version 4.2.1[29] with the packages survival,[30] survminer,[31] and cmprsk.[32] The endpoints were overall survival (OS) and cumulative incidence of relapse (CIR), considering death without relapse as a competing event, both measured from the sampling time until the event. Patients could only receive an MRD or LSC result after C2 when they achieved CR or CRi. Survival curves were derived using the Kaplan-Meier method and compared using the log-rank test. CIR was calculated with the cmprsk package[32] and compared using the Gray test. The hazard ratios (HR) for OS were estimated using Cox regression analysis whereas the subdistribution hazard ratios (SHR) for CIR were obtained using the Fine & Gray method.[33] For the optimal cut-off of different methods of LSC quantification, we used the maxstat package based on the event of relapse or death (relapse-free survival). Data were visualized with the packages ggplot2,[34] survival,[30] survminer,[31] and cmprsk.[32]

Results

Evaluation of our current leukemia stem cell assay

In the HO132 trial, we analyzed 764 samples for the presence of LSC at diagnosis. To evaluate the number of cells that are measured for the LSC assay, we summarized the number of WBC. The median numbers of acquired WBC at diagnosis were comparable between groups (Kruskal-Wallis test, *P*=0.801)

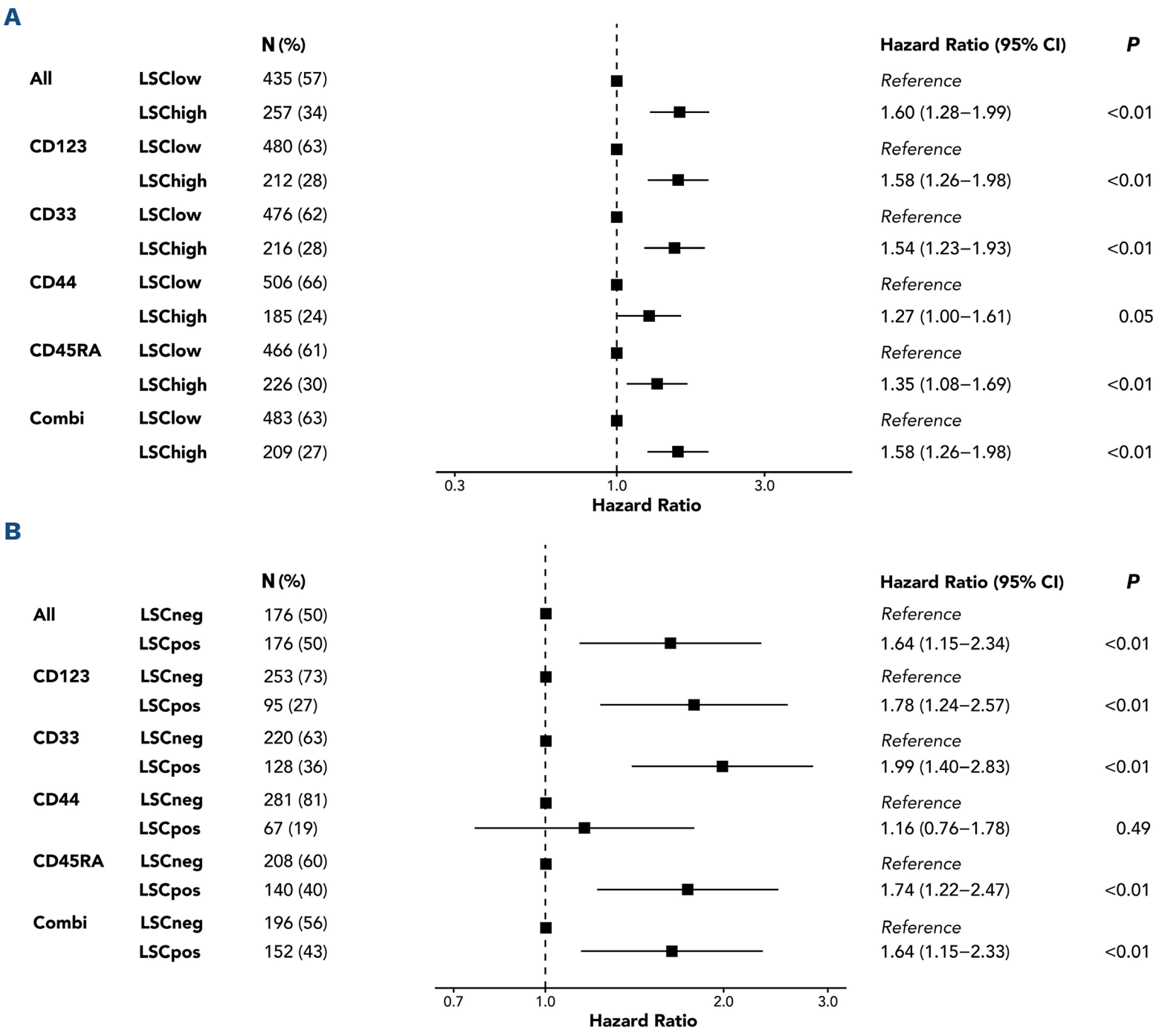

Figure 1. Prognostic value of leukemia stem cells (CD34+CD38-aberrant marker+) when specifically chosen for an aberrant marker. (A) At diagnosis. LSC^{low} patients are the reference. (B) After two cycles of chemotherapy. LSC^{neg} patients are the reference. 95% CI: 95% confidence interval; LSC: leukemia stem cell; Combi: CD56, CD22, CD11b, CD7, TIM-3, and CLL-1; LSCneg: LSC-negative; LSCpos: LSC-positive.

and were around 2x10^6 (*Online Supplementary Table S1*). After C2, the median numbers of acquired WBC were also comparable (Mann Whitney U-test, *P*=0.122), being around 3 x10^6 (*Online Supplementary Table S1*). In the HO132 study, our aim for the one-tube LSC assay was to measure 4x10^6 WBC, which was feasible in 11% (83/764) of the samples at diagnosis and 21.4% (77/359) after C2. The proportions were similar in all the groups at diagnosis and after C2, although 24% (43/180) of LSC$^+$ patients reached the 4x10^6 level after C2 compared to 19% (34/179) of the LSC$^-$ patients.

To determine the minimum number of WBC required for LSC negativity, we investigated the prognostic relevance of LSC after C2 within five groups defined based on their measured WBC: (i) <1x10^6 WBC, (ii) ≥1 x10^6 cells, (iii) ≥2x10^6 cells, (iv) ≥3x10^6 cells, and (v) ≥4x10^6 cells (*Online Supplementary Figures S3* and *S4*). The strongest effect of LSC on OS and CIR was observed in patients in whom ≥4x10^6 WBC were measured, with the highest HR among the different groups. In a small subset of patients in whom <1x10^6 WBC were acquired, we did not observe a survival difference while LSC$^-$ patients seemed to have higher relapse risk. However, when omitting these patients, a benefit of LSC negativity was observed, suggesting that LSC$^-$ patients might be misclassified when fewer than 1x10^6 cells are acquired.

To illustrate that acquiring sufficient WBC for LSC detection is important, we measured samples four times with different numbers of acquired WBC and, after analyzing these measures blindly, we observed that LSC can be missed when <1x10^6 WBC are acquired. Only in the case of a relatively high LSC$^+$ sample (>0.0005%) may a lower amount of WBC be sufficient to assess the LSC burden. Other, less rare, populations such as CD34$^+$ blasts, CD45^{dim} cells and lymphocytes were relatively stable over each amount of WBC (*Online Supplementary Figure S5*).

Evaluation of individual leukemic stem cell markers in the one-tube assay

To identify the most valuable LSC marker and its prognostic relevance, we evaluated each LSC marker separately (Figure 1, *Online Supplementary Figure S6, Online Supplementary Table S2*). We determined LSC status at diagnosis and after C2 based on each LSC marker separately and determined the prognostic value. At diagnosis, LSC status based on the different markers resulted in statistically different survival for all markers except for CD44, and all markers resulted in statistically different times to relapse based on LSC status. After C2, LSC status based on CD44 did not have prognostic relevance regarding OS or CIR and CD123 did not show a statistically significant difference in the CIR.

Aside from the prognostic relevance of the individual LSC markers at specific timepoints, we investigated the kinetics of these markers and the presence of LSC in the relapse samples. We found that the expression of the different LSC markers throughout the treatment of the patients remained

Table 1. Prognostic value based on overall survival for several leukemia stem cell analysis strategies.

Method for refinement	General group			ELN2017 favorable risk			ELN2017 intermediate risk			ELN2017 adverse risk		
	HR (95% CI)	LSC$^-$/LSC$^+$ N/N (%/%)	*P*	HR (95% CI)	LSC$^-$/LSC$^+$ N/N (%/%)	*P*	HR (95% CI)	LSC$^-$/LSC$^+$ N/N (%/%)	*P*	HR (95% CI)	LSC$^-$/LSC$^+$ N/N (%/%)	*P*
Standard	1.64 (1.15-2.34)	176/176 (50/50)	0.007	1.06 (0.57-1.96)	85/77 (52/48)	0.866	1.85 (0.96-3.57)	53/49 (52/48)	0.067	2.02 (1.09-3.7)	38/50 (43/57)	0.024
Define the minimum number of target cells	2.36 (1.46-3.81)	319/33 (91/9)	<0.001	3.04 (1.19-7.77)	153/9 (94/6)	0.02	0.67 (0.16-2.79)	94/8 (92/8)	0.58	2.39 (1.26-4.55)	72/16 (82/18)	0.008
Optimal cut-off for general group	1.86 (1.31-2.64)	233/119 (66/34)	<0.001	1.63 (0.87-3.08)	115/47 (71/29)	0.13	1.38 (0.71-2.68)	69/33 (68/32)	0.345	2.14 (1.21-3.77)	49/39 (56/44)	0.009
Optimal cut-off per ELN risk group	1.55 (1.09-2.20)	191/161 (54/46)	0.015	0.90 (0.48-1.68)	91/71 (56/44)	0.735	1.85 (0.96-3.57)	53/49 (52/48)	0.067	2.29 (1.28-4.07)	47/41 (53/47)	0.005
CD38 cut-off change	1.80 (1.27-2.57)	239/113 (68/32)	0.001	2.26 (1.21-4.24)	120/42 (74/26)	0.01	0.95 (0.48-1.89)	67/35 (66/34)	0.879	1.81 (1.03-3.19)	52/36 (59/41)	0.038
PM-LSC (changing the denominator)	1.86 (1.31-2.64)	206/146 (59/41)	<0.001	1.47 (0.79-2.73)	100/62 (62/38)	0.227	1.92 (1.01-3.66)	61/41 (60/40)	0.048	1.97 (1.11-3.51)	45/43 (51/49)	0.021

ELN: European LeukemiaNet; HR: hazard ratio; 95% CI: 95% confidence interval; LSC: leukemia stem cell; PM-LSC: primitive marker-leukemia stem cell.

similar and that LSC were also detectable in relapse of patients (*Online Supplementary Figure S7*). We show two example patients without mutations in *DNMT3A*, *TET2* or *ASXL1* in *Online Supplementary Figure S8*.

Evaluation of the strategies for analyzing leukemia stem cells

We also aimed to evaluate different analysis strategies for LSC with respect to prognostic relevance after C2, considering the proportion of LSC$^+$ patients and the ELN2017 risk classification. The different analysis strategies we explored were: (i) defining the number of target CD34$^+$CD38$^-$ events for better sensitivity of the assay; (ii) optimizing the cut-off for LSC positivity after C2; (iii) changing the denominator to assess LSC (PM-LSC); and (iv) expanding the CD38$^-$ fluorescent intensity by including CD38^{dim} (defined as CD38^{low}) at diagnosis and after C2 (Table 1, *Online Supplementary Table S3*). For these analyses, we selected patients with known ELN2017 risk groups.

Definition of the number of target CD34$^+$CD38$^-$ events for better sensitivity of the assay

We applied the criteria of Li and colleagues,[13] who defined a minimum of 20 CD34$^+$CD38$^-$ cells and at least 10% LSC marker positivity, to our group of patients. This resulted in higher HR and SHR compared to the standard method in the complete cohort (overall) as well as in the ELN2017 favorable-risk and adverse-risk groups (Table 1, Figure 2). In contrast, the HR and SHR were lower in the intermediate-risk group. The proportion of LSC$^+$ patients declined considerably, e.g., in the general group from 176 (50%) to 33 (9%), and this was also observed in the different ELN risk groups (Table 1).

Optimizing the cut-off of leukemia stem cell positivity after the second cycle of chemotherapy

Based on the standard method, we classified patients at diagnosis into three groups: CD34$^-$ (no LSC), LSC^{low} (<0.03% LSC/WBC) and LSC^{high} (≥0.03% LSC/WBC). After C2, we analyzed 359 samples for the presence of LSC. We classified patients as LSC$^-$ (≤ 0.00000% LSC/WBC) or LSC$^+$ (>0.00000% LSC/WBC). Such a low cut-off is not routinely applied; however, we think that we can apply this cut-off as we use extensive backgating steps and discuss all analyses in a meeting. Furthermore, in normal bone marrow when acquiring more than 4x10^6 cells, we consistently found absence of LSC. When a new cut-off was determined with the maximally selected rank statistics, the test showed an optimal cut-off of 0.000057% (5.7x10^{-5}) LSC of WBC.[35] Yet, this cut-off was not universally applicable across all ELN2017 risk groups (*Online Supplementary Figure S9, Table 1*). Therefore, we also determined the optimal cut-off based on the maximally selected rank statistics for relapse-free survival for each risk group, which were 0.000024% (2.4x10^{-5}) for the favorable-risk group, 0.00000% for the intermediate-risk group, and 0.000045% (4.5x10^{-5}) for the adverse-risk group (Figure 3, *Online Supplementary Figure S10*). Like the standard cut-off, our new cut-off per risk group could not produce a significant prognostic value for the ELN2017 favorable-risk group, while the prognostic value remained in the ELN2017 intermediate- and adverse-risk groups.

Changing the denominator to assess leukemia stem cells

Due to the heterogeneity of cells in the WBC compartment and the possibility of hemodilution, changing the denominator to assess LSC based on CD34$^+$ cells has been proposed.[19] When assessing the maximally selected rank statistics with CD34$^+$CD45^{dim} cells as a denominator, the optimal cut-off for LSC^{high} was 1.42% at diagnosis and LSC$^+$ 0.0074% after C2 (Figure 4). When the prognostic value (HR) regarding OS was compared to that for other possible optimizations, changing the denominator from WBC to CD34$^+$ blasts provided higher HR for OS than the standard LSC assay in the overall and intermediate-risk groups. The prognostic value of LSC in the adverse-risk group remained similar. The ratio between LSC$^+$ and LSC$^-$ patients shifted to more patients being LSC$^-$ (Table 1).

CD38 expression cut-off for defining leukemia stem cells

Because the CD34$^+$CD38$^-$LSC$^+$ are dependent on the CD38 negativity cut-off, we explored another threshold for CD38 negativity. We currently identify a CD38^{verylow} (CD38 fluorescence intensity <10^2) and a CD38^{low} (CD38 fluorescence intensity <10^3; CD38^{dim}) population. These cut-offs were determined based on the lower and higher boundary of the CD38 fluorescence intensity of Spherotech beads, respectively (Figure 5A); however, all these cells are still CD38$^-$. We studied how the LSC assay would perform when we included the CD38^{dim} fraction to allow a higher number of events in the LSC gate for determining LSC positivity. With the higher CD38 fluorescence intensity, we had a higher number of CD34$^+$CD38$^-$ cells. When determining the cut-off for this CD38^{low} population, the optimal cut-off for LSC$^+$ was 3.5% for diagnosis (*Online Supplementary Figure S11*) and 0.0009% after C2, with the maximally selected rank method based on relapse-free survival. After C2, this adjustment increased the HR based on OS and SHR on CIR in the overall group and in the ELN2017 favorable-risk risk. However, this adjustment would not increase the HR based on OS and SHR on CIR in the ELN2017 intermediate-risk group (Figure 5B-I).

When we corrected all adjustments separately in the multivariable analysis for OS, correcting for LSC status at diagnosis, age, ELN2017 risk, WBC at diagnosis, and AML type, LSC after C2 still retained its prognostic value (*Online Supplementary Table S4*).

Discussion

In this study, we aimed to delineate the effect of adjustments to the CD34$^+$CD38$^-$ LSC assay on its prognostic

relevance at diagnosis and after two cycles of intensive chemotherapy. We found that by using different analysis strategies to quantify LSC burden, the LSC assay retained its significance in prognostication of AML patients.

One of the questions regarding the LSC assay is how many cells should be acquired to ensure proper detection of LSC during therapy. In theory, a large number of WBC (>2x10^6) need to be measured in the bone marrow in order to de-

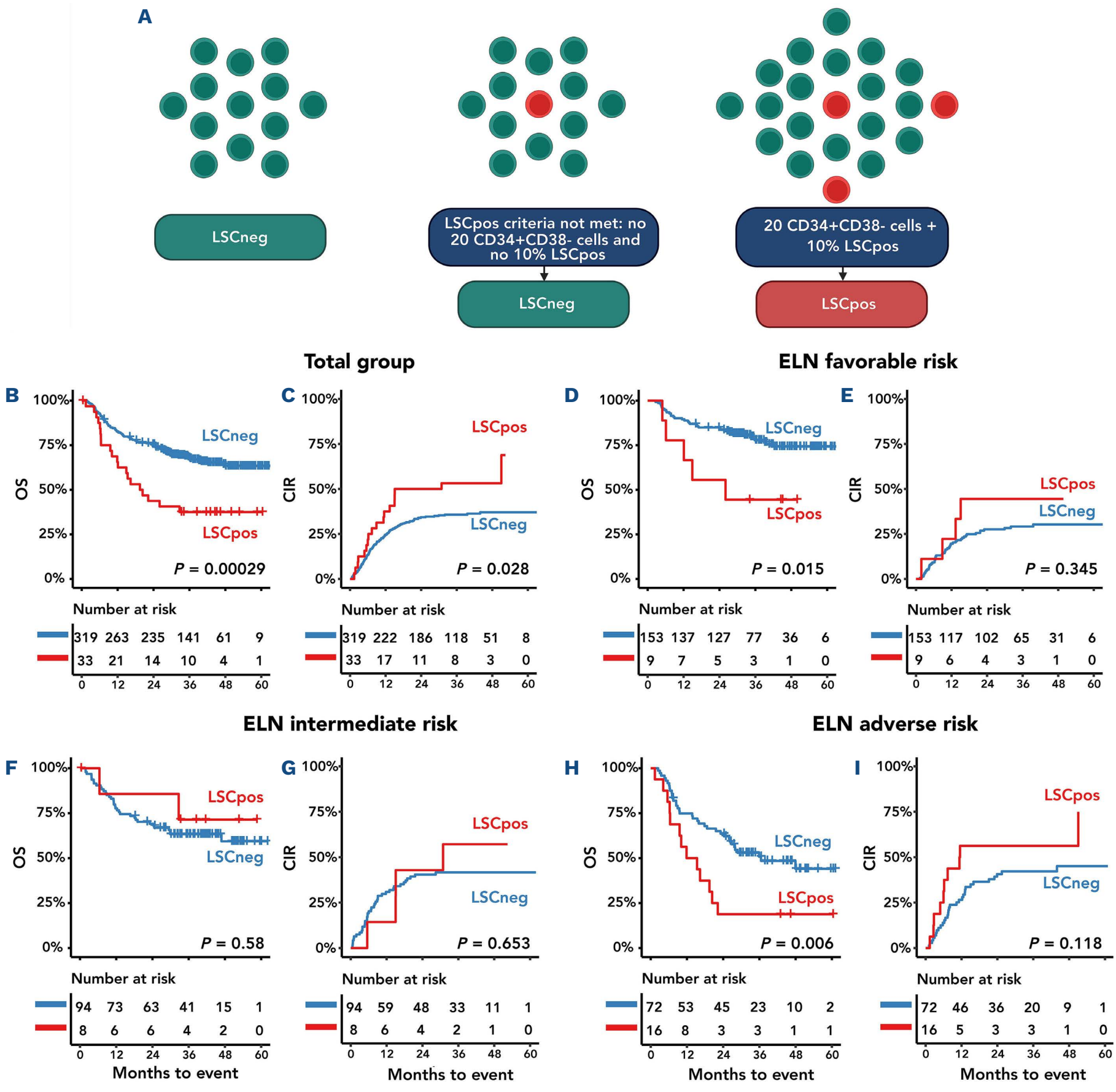

Figure 2. Kaplan-Meier and cumulative incidence of relapse plots showing the prognostic value of leukemia stem cells after two cycles of chemotherapy when a target number of 20 leukemia stem cell events and the 10% leukemia stem cell/hematopoietic stem cell ratio are fulfilled. (A) Scheme depicting the concept of the leukemia stem cell (LSC) adjustment. Patients are classified as LSC$^+$ if there are more than 20 CD34$^+$CD38$^-$ cells of which at least 10% are positive for an LSC marker. (B) Overall survival and (C) cumulative incidence of relapse of the overall group. (D) Overall survival and (E) cumulative incidence of relapse of the favorable-risk group. (F) Overall survival and (G) cumulative incidence of relapse of the intermediate-risk group. (H) Overall survival and (I) cumulative incidence of relapse of the adverse-risk group. LSCneg: LSC-negative; LSCpos: LSC-positive; OS: overall survival; CIR: cumulative incidence of relapse; ELN: European LeukemiaNet.

tect low frequency LSC, which were estimated to be about 1 per 10^6 mononuclear cells.[2] However, in practice, often only a limited volume and/or limited number of cells are available for MRD and LSC measurement. In earlier studies, we recommended measuring $4x10^6$ cells,[12,18] however, since multiparameter flow cytometry assessment of MRD

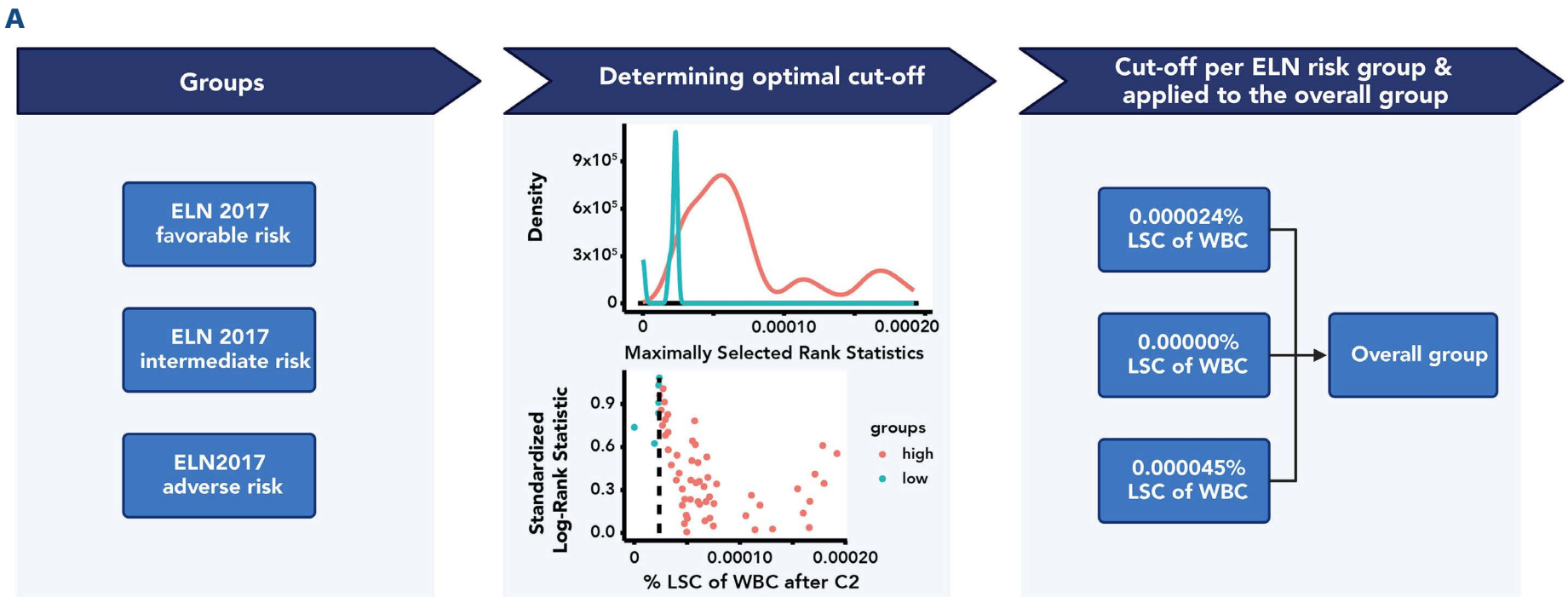

Figure 3. Prognostic value of leukemia stem cells after two cycles of chemotherapy when the optimal cut-off is based on outcome per European LeukemiaNet risk group. (A) Scheme of the adjustment. The overall group was divided into the different risk groups and the optimal cut-off of each, based on event-free survival, was determined with maximally selected rank statistics. Then the risk-based cut-off was applied to the overall group. (B) Overall survival and (C) cumulative incidence of relapse of the whole population with prognostic value based on all individual risk group levels. Favorable risk: 0.000024%; intermediate risk: 0.00000%; adverse risk: 0.000045%. (D) Overall survival and (E) cumulative incidence of relapse of the favorable-risk patients. Cut-off 0.000024%. (F) Overall survival and (G) cumulative incidence of relapse of the intermediate-risk patients. Cut-off 0.00000%. (H) Overall survival and (I) cumulative incidence of relapse of the adverse-risk patients. Cut-off 0.000045%. ELN: European LeukemiaNet; LSC: leukemia stem cells; WBC: CD45-expressing white blood cells; C2: cycle 2 of chemotherapy; OS: overall survival; CIR: cumulative incidence of relapse; LSCneg: LSC-negative; LSCpos: LSC-positive.

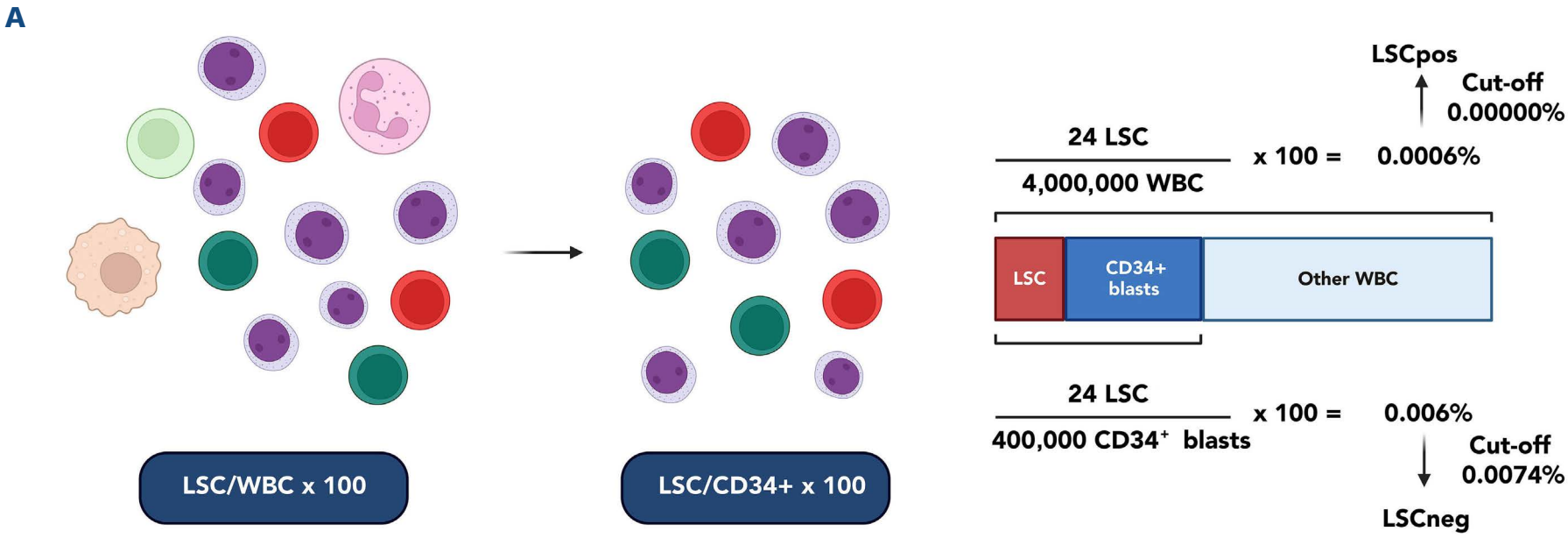

Figure 4. The prognostic value of leukemia stem cells after two cycles of chemotherapy when the readout is percentage of leukemia stem cells of CD34 blasts (PM-LSC). (A) Scheme depicting the concept of the leukemia stem cell (LSC) adjustment. The denominator in our standard assay for the readout is white blood cells. When we switch to CD34$^+$ blasts (primitive marker) and determine the optimal cut-off with the maximally ranked statistics (0.0074%), patients can still have LSC$^+$ cells but not have LSC positivity with the stringent 0.00000% cut-off when the denominator is white blood cells (created with Biorender). (B) Overall survival and (C) cumulative incidence of relapse of the whole population show prognostic value; only 11% are LSC$^+$. (D) Overall survival and (E) cumulative incidence of relapse of the favorable-risk patients. (F) Overall survival and (G) cumulative incidence of relapse of the intermediate-risk patients. (H) Overall survival and (I) cumulative incidence of relapse of the adverse-risk patients. The prognostic value of LSC is still observed in the intermediate- and adverse-risk groups. WBC: CD45-expressing white blood cells; LSCpos: LSC-positive; LSCneg: LSC-negative; OS: overall survival; CIR: cumulative incidence of relapse; ELN: European LeukemiaNet.

has priority and due to trial logistics (morphology, use of in-house assays), acquiring 4x10^6 cells for the LSC assay is challenging. We found that when patients have a relatively high LSC load (>0.0001%), the LSC will be present even when a low number of WBC is acquired (<500,000). However, for patients with a minimal amount of residual LSC, acquiring fewer than 1x10^6 WBC increases the risk of false-negative results. Therefore, we recommend measuring at least 1x10^6

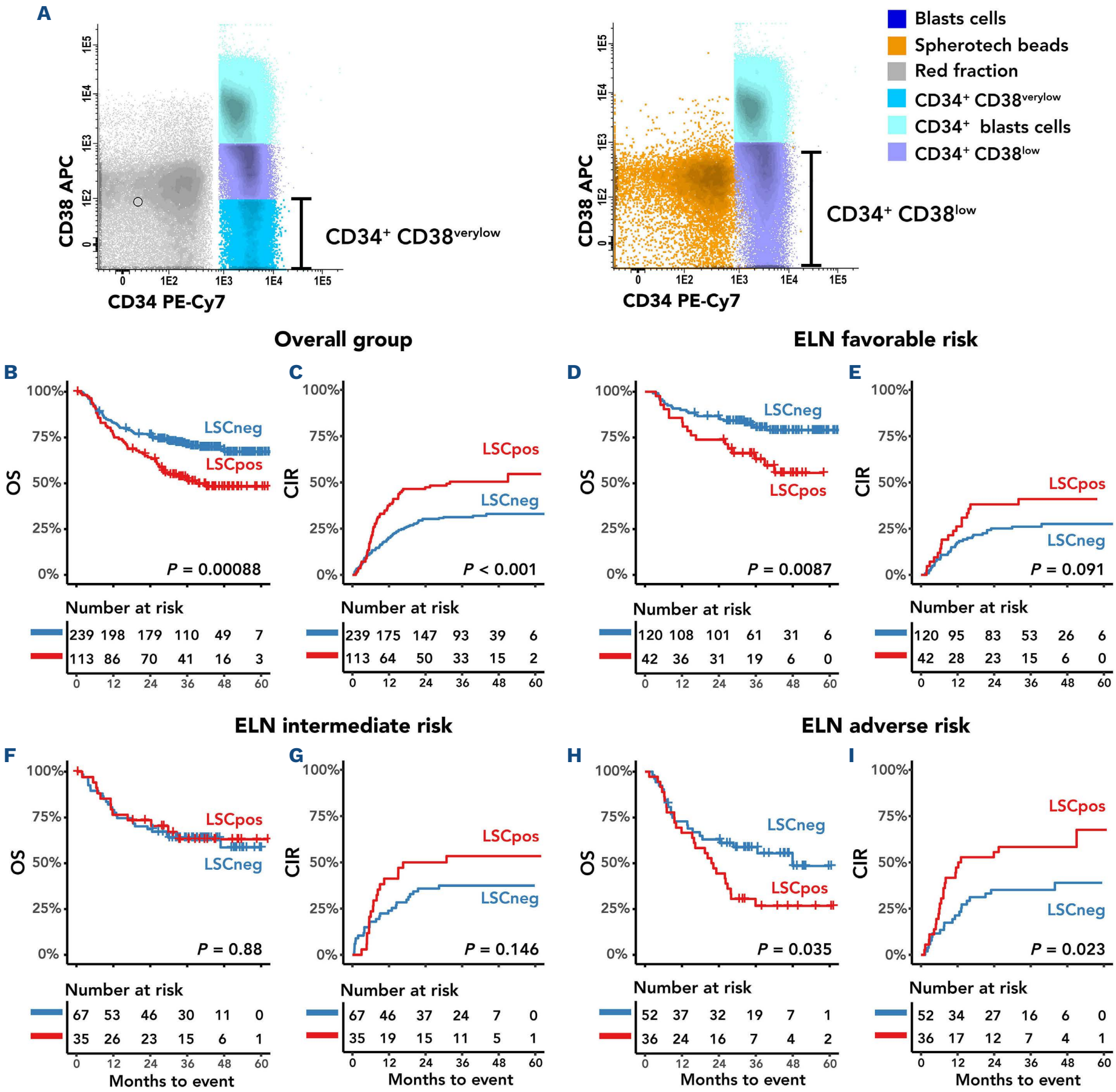

Figure 5. Prognostic value of CD38low leukemia stem cells after two cycles of chemotherapy. (A) Flow cytometry dot plots depicting the change of CD38– cut-off (0.0009%) from CD38$^{very\ low}$ to CD38^{low} (including the CD38^{dim}) (from the *Online Supplementary Materials* of Cloos *et al.*[27] (B) Overall survival and (C) cumulative incidence of relapse of the population show prognostic value; only 11% are LSC$^+$. (D) Overall survival and (E) cumulative incidence of relapse of the favorable-risk patients. (F) Overall survival and (G) cumulative incidence of relapse of the intermediate-risk patients. (H) Overall survival and (I) cumulative incidence of relapse of the adverse-risk patients. The prognostic value of leukemia stem cells is seen in the favorable- and adverse-risk groups but not in the intermediate-risk group. APC: allophycocyanin; PE-Cy7: phycoerythrin-cyanine7; OS: overall survival; LSCneg: leukemia stem cell-negative; LSCpos: leukemia stem cell-positive; CIR: cumulative incidence of relapse; ELN: European LeukemiaNet.

WBC but preferably 4x10^6 WBC for assessing LSC negativity during treatment. For diagnosis, fewer events are necessary because of the higher cut-off. Therefore, we recommend measuring preferably 2x10^6 WBC at diagnosis while keeping the minimum of 1x10^6 events.

By exploring separate LSC markers, we observed that in our panel there is no universal LSC marker that can detect all LSC. However, it should be noted that other markers described to identify LSC, such as GPR56[36,37] and IL1RAP [38-40] were not included in this LSC assay. Furthermore, we explored the prognostic relevance of the separate markers and found a good concordance of LSC positivity between the markers, except for CD44. CD44 overexpression is a difficult marker for gating as most hematopoietic cells express CD44,[41] which makes distinguishing normal from abnormal expression of CD44 difficult. Therefore, CD44 overexpression is a suboptimal marker for detecting residual LSC in a follow-up setting. In addition, we showed that LSC are enriched at relapse, which is in line with the findings in the study by Haubner and colleagues.[42]

Any assay that is used for clinical decision-making should be both technically and clinically validated, and this has also been done for MRD assays.[43,44] For LSC there are several different gating strategies and target events, and each LSC assay has its own cut-off and readouts. We, therefore, investigated several gating and analysis strategies in the whole cohort of patients. When evaluating the different analysis LSC strategies in the general group, all strategies had prognostic value since they had statistically significant HR based on LSC status. This shows that independently of gating and analysis methods, LSC load has robust prognostic importance.

While identifying one or a few LSC may be prone to error because of nonspecific events, some centers prefer to define a minimum number of events in the stem cell gate. Similar to what Li and colleagues[13] documented, a high HR for OS is found when a patient is considered LSC$^+$ if there are at least 20 CD34$^+$CD38$^-$ cells of which at least 10% are LSC. However, using this method on our very low CD38$^-$ cut-off, the proportion of LSC$^+$ patients was low (<10%), and many LSC$^+$ patients with dismal outcome were missed (*Online Supplementary Figure S12*). Only in the ELN favorable-risk group may this method be of benefit, compared to the standard LSC method, by identifying some patients with very poor outcome.

Based on our analysis of defining an optimal cut-off per ELN risk group, we could not find a common optimal cut-off that was applicable on the overall group. In the favorable risk group, the current LSC method does not result in a prognostic difference between LSC$^-$ and LSC$^+$ patients. This is also visible in *Online Supplementary Figure S12*, showing LSC$^+$ patients with good prognosis. However, LSC assessment in this group may improve when increasing CD38 antigen expression negativity (CD38$^{very\ low}$) by including the CD38^{dim} fraction (defined as CD38^{low}). Increasing the CD38 cut-off may indicate that progenitor-like relapse-initiating cells, similar to LSC, might be of more clinical importance in favorable-risk patients, who are often *NPM1* mutated.[45] It would be interesting to validate this method in order to explore clinical applicability of LSC assessment in this group.

Another interesting strategy for LSC analysis would be PM-LSC. We observed that for the intermediate-risk group, the HR increased by using PM-LSC, so changing the WBC denominator to CD34$^+$ cells. This led to a slight decrease in LSC$^+$ proportion from 50% to 41% because of the changing cut-off. An advantage of this method is that it may circumvent the effects of hemodilution as the CD34$^+$ compartment is less influenced by cells such as granulocytes.[20,22-24] Furthermore, PM-LSC may have potential when the LSC burden needs to be assessed on frozen and Ficoll-separated samples containing only mononuclear cells as WBC. With the advancement in spectral flow cytometry and thereby larger panels, inclusion of CD117 could be explored in future studies.

In conclusion, our data based on LSC detection in the CD34$^+$CD38$^-$ cell population in the HO132 study show robust prognostic importance of LSC irrespective of different ways of quantifying the LSC burden. By investigating these different analysis methods for each ELN risk group, LSC assessment will be more individualized, paving the way for personalized diagnostics. This would require comprehensive guidelines for end-users. However, to prepare LSC assessment for clinical decision-making more research is warranted, in particular for specific ELN risk groups. It needs to be emphasized that part of the intermediate-risk group was guided by standard MRD, diminishing the prognostic relevance of MRD in this group. The fact that LSC load is still prognostic is interesting, but it remains to be investigated how this information could be clinically implemented in this group of patients. Based on our analysis at follow-up, we recommend measuring at least 1x10^6 cells to ensure that LSC$^-$ results are adequate, and to be careful when using CD44 overexpression as a LSC marker. The fact that data are robust and comparable between different analysis approaches may enable the combination of data from other platforms and facilitate common external quality control.

Disclosures

BTG serves as a consultant for BerGenBio, Pfizer Inc. and Novartis and holds stock options in Alden Cancer Therapy and KinN Therapeutics. LG is a member of the board of directors and advisory committees for Miltenyi Biomedicine. KP has received honoraria from Pfizer, Novartis, Incyte, Bristol-Myers Squibb, Astellas and AbbVie and has received research funding and honoraria from Celgene/Bristol-Myers Squibb, Incyte, Pfizer and Novartis. AAvdL has received honoraria from Amgen, Novartis, Celgene/BMS and Takeda and has received research funding from Alexion. MGM serves as a consultant for CDR-Life Inc; holds stock options in CDR-Life Inc; and has a patent licensed to the University of Zurich. BL serves as a consultant, has received honoraria from Clear Creek Bio and is a member of an entity's board of directors

or advisory committees for Celgene, Bristol-Myers Squibb, Catamaran Bio Inc, Astellas, AbbVie and F. Hoffmann La Roche. DCdL participates in sponsored speakers' bureaus for Servier, Roche and AbbVie and is part of scientific advisory boards for 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; has received research funding from Novartis; and is a member of the board of directors of Roche. JC serves as a consultant for Novartis; receives royalties from Navigate and BD Biosciences; and has received research funding from Takeda, DC-one, Genentech, Janssen, Novartis and Merus. The remaining authors have no conflicts of interest to disclose.

Contributions

Samples were collected by DAB, TF, BTG, LG, GJ, AAvdL, JAM, MGM, TP, JRP, KP, BL and GJO in the HOVON-SAKK132 trial. Experiments and/or analysis of flow cytometry LSC data were performed by LLN, TR, DH, FJ, JC-H, LO-vH, MMHEF, MAMH, AK, WJS, ANS, and PJMV. The statistical analysis was performed by LLN and TR and checked by PG. The manuscript was written by LLN and revised by JC, TR, GJO, DH, CB, JMT, BL, AAvdL, DCdL, DAB, TF, BTG, LG, GJ, JAM, MGM, TP, JRP, KP and PJMV. The results were reviewed and the manuscript was approved by all the authors.

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Data-sharing statement

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

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