High levels of $CD34^{+}CD38^{low/-}CD123^{+}$ blasts are predictive of an adverse outcome in acute myeloid leukemia: a *Groupe Ouest-Est des Leucémies Aiguës et Maladies du Sang* (GOELAMS) study

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ABSTRACT

Background
Acute myeloid leukemias arise from a rare population of leukemic cells, known as leukemic stem cells, which initiate the disease and contribute to frequent relapses. Although the phenotype of these cells remains unclear in most patients, these cells are enriched within the $CD34^{+}CD38^{low/-}$ compartment expressing the interleukin-3 alpha chain receptor, CD123. The aim of this study was to determine the prognostic value of the percentage of blasts with the $CD34^{+}CD38^{low/-}CD123^{+}$ phenotype.

Design and Methods
The percentage of $CD34^{+}CD38^{low/-}CD123^{+}$ cells in the blast population was determined at diagnosis using flow cytometry. One hundred and eleven patients under 65 years of age with *de novo* acute myeloid leukemia and treated with intensive chemotherapy were retrospectively included in the study. Correlations with complete response, disease-free survival and overall survival were evaluated with univariate and multivariate analyses.

Results
A proportion of $CD34^{+}CD38^{low/-}CD123^{+}$ cells greater than 15% at diagnosis and an unfavorable karyotype were significantly correlated with a lack of complete response. By logistic regression analysis, a percentage of $CD34^{+}CD38^{low/-}CD123^{+}$ higher than 15% retained significance with an odds ratio of 0.33 (0.1-0.97; P=0.044). A greater than 1% population of $CD34^{+}CD38^{low/-}CD123^{+}$ cells negatively affected disease-free survival (0.9 *versus* 4.7 years; P<0.0001) and overall survival (1.25 years *versus* median not reached; P<0.0001). A greater than 1% population of $CD34^{+}CD38^{low/-}CD123^{+}$ cells retained prognostic significance for both parameters after multivariate analysis.

Conclusions
The percentage of $CD34^{+}CD38^{low/-}CD123^{+}$ leukemic cells at diagnosis was significantly correlated with response to treatment and survival. This prognostic marker might be easily adopted in clinical practice to rapidly identify patients at risk of treatment failure.

Key words: leukemic stem cells, prognosis, acute myeloid leukemia, $CD34^{+}CD38^{low/-}CD123^{+}$ blasts.

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The online version of this article has a Supplementary Appendix.

Introduction

Acute myeloid leukemia (AML) is a clonal hematologic malignancy arising from a small population of leukemic cells that initiate and propagate the disease. These cells are known as leukemic stem cells and are derived from normal hematopoietic stem cells or from more mature myeloid progenitors. Leukemic stem cells differentiate into leukemic progenitors and then into non-clonogenic blast cells that are blocked from further differentiation and thereby acquire a proliferative and survival advantage.[1,2] Currently, the combination of an anthracycline and cytarabine is still considered to be the gold standard induction therapy for younger patients with AML, resulting in complete remission rates of 50 to 75%.[3] However, relapses frequently occur and the 5-year overall survival rate is less than 40% even after high-dose chemotherapy and stem cell transplantation.[4] In recent years, the characterization of new molecular prognostic tools has led to a tailored therapeutic approach. This is particularly true for the most heterogeneous group of AML patients with a normal karyotype who account for 40% to 50% of the cases. Many molecular abnormalities in different genes, including *FLT3*, *NPM1*, *RUNX1*, *CEPBA*, *IDH1/2*, and *WT1* mutations, have now been identified in this disease and have been reported to have a significant effect on the clinical outcome of such patients.[5]

Relapses from AML are thought to originate from the outgrowth of a leukemic subpopulation with both self-renewal and chemoresistance properties and that likely resides in particular niches of the bone marrow.[6,7] This leukemic sub-population was initially characterized in sub-lethally irradiated non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice and leukemic stem cells were found to be enriched in the $CD34^+CD38^{low/-}$ compartment.[8,9] Taussig *et al.* revealed a bias in experimental conditions and demonstrated that the leukemic stem cell phenotype was more heterogeneous than previously thought.[10] Moreover it was recently demonstrated that although all immature AML subpopulations may contain leukemic stem cells in a different model of human AML cell transplantation in NOD/SCID/IL2$r\gamma^{null}$ mice,[11-13] these rare cells are generally found enriched within their $CD34^+CD38^{low/-}$ compartment, in cells expressing the interleukin-3 alpha chain receptor (α–IL3-R or CD123). This marker enables discrimination in the $CD34^+CD38^{low/-}$ compartment between normal hematopoietic stem cells that do not express CD123 ($CD123^-$) and leukemic stem cells that are positive for this marker ($CD123^+$).[14] More importantly and regardless of their 'stemness' properties, it has been demonstrated both *in vitro* and *in vivo* that $CD34^+CD38^{low/-}$ cells are significantly more resistant than the leukemic bulk population to classical chemotherapeutic agents.[13,15]

Previous studies have emphasized the correlation between the enrichment of the $CD34^+$ or $CD34^+CD38^{low/-}$ phenotype in AML or acute lymphoblastic leukemia cells at diagnosis and a high level of residual disease after treatment.[16-18] However, the prognostic value of the percentage of $CD34^+CD38^{low/-}CD123^+$ cells in the blast population has not been specifically addressed in AML. In the present study it was found that a percentage of leukemic $CD34^+CD38^{low/-}CD123^+$ cells greater than 1% was strongly correlated with decreases in both disease-free and overall survival in AML patients.

Design and methods

Patients

One hundred and eleven patients under 65 years old with *de novo* AML were included in the present study. Patients with a history of myelodysplastic syndrome or therapy-related AML were not included. All the patients were treated according to the LAM-2001,[19] LAM-SA 2007 (ClinicalTrial: NCT00590837), LAM-IR 2006 (ClinicalTrial: NCT00860639), and the LAM-CBF 2006 (ClinicalTrial: NCT00428558) trials by the *Groupe Ouest-Est des Leucémies et Autres Maladies du Sang* (GOELAMS). The present study was approved by the GOELAMS Institutional Review Board and signed informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

Flow cytometry

Blast cells were isolated from bone marrow aspirates that were collected at diagnosis and also at relapse from some patients, by Ficoll-Hypaque gradient density centrifugation. The experiments were done on fresh cells (in 15% of cases) or retrospectively on cells frozen with 10% dimethyl sulfoxide (Sigma-Aldrich, Saint Louis, MO, USA) (in 85% of the cases). We did not observe any differences in the staining pattern for five patients using frozen and fresh cells (*Online Supplementary Figure S1*). After thawing, a total of 5×10^5 primary AML blast cells were stained with the following conjugated antibodies: CD38-PE, CD123-PC5, CD45-FITC and CD34-PC7, or isotypic controls of the corresponding fluorochromes in accordance with the manufacturer's instructions (Beckman–Coulter Inc., Brea, CA, USA). Isotypic controls were used to define the threshold for positive-staining cells. Patients with CD34 expression of less than 1% were not included given the fact that in most of these cases $CD34^+$ cells are not malignant cells.[20] Analyses were performed on an FC 500 flow cytometer with CXP software (Beckman-Coulter Inc., Brea, CA, USA) or on an LSRII flow cytometer with BD FacsDiVa software (BD Biosciences, Franklin Lakes, NJ, USA) in two institutional hematology laboratories in Cochin Hospital (Paris, France) and in Toulouse (France). We analyzed 11 AML samples with the two flow cytometers to validate the reproducibility of the results (*Online Supplementary Figure S1*).

Statistics

Complete response and relapse rates were defined according to the Cheson criteria.[21] The best thresholds for continuous variables were calculated using receiver operating characteristic (ROC) curves. Pair-wise comparisons between patients' characteristics (covariates) were performed using the Mann-Whitney test or Kruskal-Wallis test for continuous variables and with the Fisher's exact test for categorical variables. A multivariable logistic model was used to analyze associations between presenting features and response to induction therapy. Overall survival and disease-free survival rates were measured from the date of diagnosis until death and from the date of complete remission until death or relapse, respectively. Patients alive in complete remission were censored at the time of last contact. Overall and disease-free survival rates were estimated by the Kaplan-Meier method and compared using the log-rank test. Hazard ratios are given with 95% confidence intervals (95% CI). All calculations were performed using GraphPad Prism software, version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Survival-time data (disease-free survival and overall survival) and covariates (age, leukocyte count, karyotype, *NPM-1* and *FLT3* mutations and percentage of $CD34^+CD38^{low/-}CD123^+$ cells) were analyzed using the method of backward Cox proportional hazards regression.

Results

The percentage of CD34+CD38low/-CD123+ leukemic cells is highly variable in patients with acute myeloid leukemia

CD45 staining and side scatter properties were used to isolate the leukemic cell populations, referred to as the bulk of the leukemia and usually defined by weak CD45 expression ($CD45^{dim}$) and low side scatter (SS^{low}). For AML samples with monocytic differentiation in which blast cells can be found in the monocytic gate, the gating strategy was made in agreement with the morphological study. The percentage of $CD34^+CD38^{low/-}CD123^+$ cells was then quantified as the ratio between the numbers of $CD34^+CD38^{low/-}CD123^+$ cells and $CD45^{dim}/SS^{low}$ cells. Results for representative patients with high (sample #24) and low $CD34^+CD38^{low/-}CD123^+$ expression (sample #87) in the blast population are illustrated in Figure 1. The percentage of $CD34^+CD38^{low/-}CD123^+$ cells was evaluated in 111 primary AML samples at diagnosis. The median expression of CD34 was 63.7% (range, 1.3-99.7%). In 21 patients (19%), the CD34 expression was between 1 and 19%. The median expression of CD123 was 76.7% (range, 0.4-99.5%). The percentage of $CD34^+CD38^{low/-}CD123^+$ cells at diagnosis ranged from 0.01 to 67% (median, 2.8%). Fourteen patients were also analyzed at the time of their first relapse and the median percentage of $CD34^+CD38^{low/-}CD123^+$ cells was 4.5% and 3.4% at diagnosis and relapse, respectively, in these cases. Among this subgroup, the percentage of $CD34^+CD38^{low/-}CD123^+$ cells was found to be increased in six patients (median fold-increase, 4.95; range, 2.8-195), stable in two patients and decreased in six patients (median fold-decrease, 5.6; range, 2-6.7).

A high level of CD34+CD38low/-CD123+ cells correlates with a poor response to induction chemotherapy

The main characteristics of patients, including cytogenetic risk group, *FLT3* and *NPM1* mutational status, response to induction chemotherapy and type of consolidation, are reported in Table 1. ROC curve analyses were used to evaluate the best thresholds of $CD34^+CD38^{low/-}CD123^+$ cells to predict the achievement of complete remission (15%) or an adverse event (1%). Three groups of patients were classified according to the percentage of $CD34^+CD38^{low/-}CD123^+$ cells: below 1%, between 1 and 15% and above 15%, representing 36%, 46% and 18% of all patients, respectively. No differences were found between these three groups in terms of age, sex, leukocyte

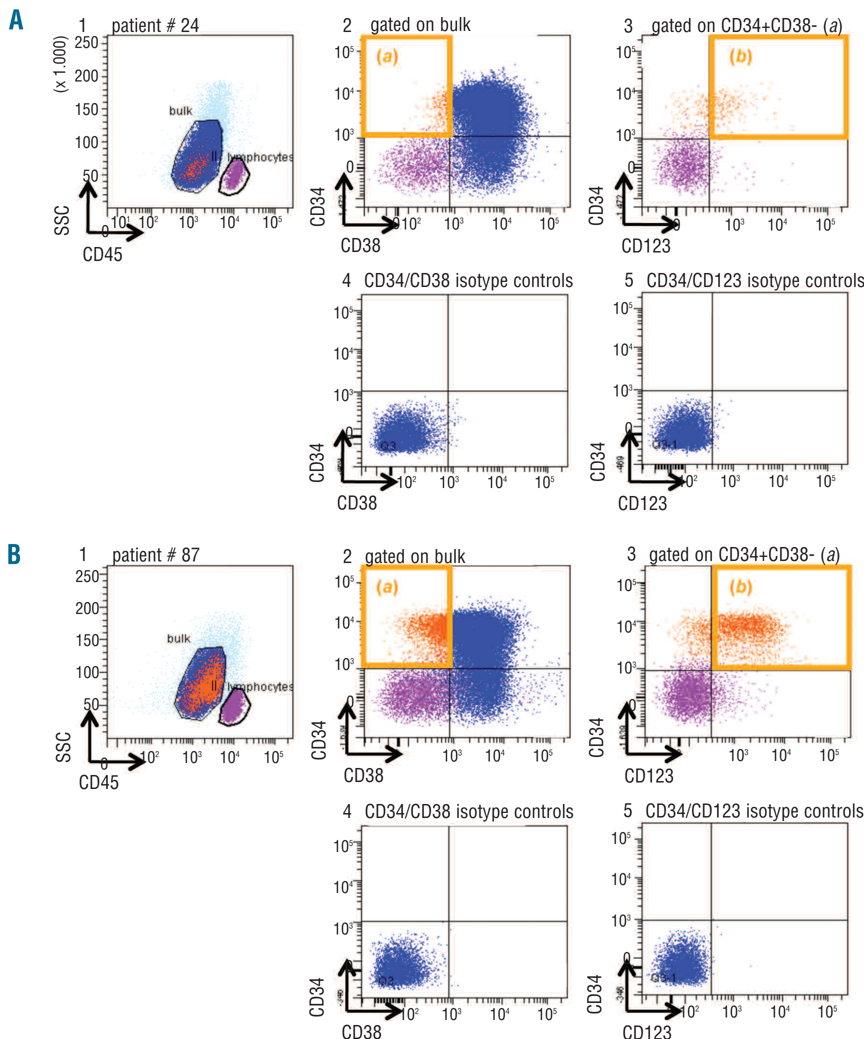

Figure 1. The percentage of $CD34^+CD38^{low/-}CD123^+$ leukemic cells is highly variable in AML patients. Blasts and mononuclear cells from 111 AML samples were analyzed by flow cytometry. First, bulk cells (blue and orange) and lymphocytes (purple) were gated according to CD45 intensity in a CD45/sidescatter dot plot: dot plot 1 in patient #24 (A) and patient #87 (B); CD45 intensity of AML blasts is low/dim whereas it is bright on lymphocytes. Blast expression of CD34 and CD38 was then assessed in a second dot plot (dot plot 2). Isotype controls were used as negative controls. Gate *a* focus on bulk cells with $CD34^+CD38^{low/-}$ phenotype. Finally, CD123 expression on $CD34^+CD38^{low/-}$ cells (in orange, gate *a*) was assessed in a third dot plot (dot plot 3 in patients #24 and #87) in order to exclude normal $CD34^+CD38^{low/-}$ hematopoietic stem cells that do not express CD123. Percentages of $CD34^+CD38^{low/-}CD123^+$ leukemic cells (gate *b*) for patients #24 and #87 are 0.78% and 10.81%, respectively. Isotype controls are shown in dot plots 4 and 5.

count, overall CD34 and CD123 expressions, *NPM1* and *FLT3* mutational status, or type of consolidation received (autologous or allogeneic stem-cell transplantation). Moreover, age, leukocyte count, and *NPM1* and *FLT3-ITD* mutational status had no impact on the achievement of complete remission (Table 1). An unfavorable karyotype was found to be associated with a complete remission rate of 68%. As expected, this was significantly lower than the 80% and 100% complete remission rates achieved in the intermediate and favorable karyotype groups, respectively (P=0.0217).[22] Patients with over 15% of $CD34^+CD38^{low/-}CD123^+$ cells had a lower response rate to induction chemotherapy, with a complete remission rate of 65% *versus* 85% for patients with a population of less than 15% of these cells (P=0.049). By logistic regression analysis, a $CD34^+CD38^{low/-}CD123^+$ percentage of over 15% retained significance for achievement of complete remission, with an odds ratio of 0.33 (0.11-0.97; P=0.044). Covariates associated with complete remission are presented in Table 2.

A level of $CD34^+CD38^{low/-}CD123^+$ of over 1% negatively affects disease-free and overall survival in patients with acute myeloid leukemia

Overall, the median disease-free and overall survival was 1.4 and 1.6 years, respectively, with a median follow-up of 1.25 years in our AML cohort. A higher than 1% level of $CD34^+CD38^{low/-}CD123^+$ cells (0.9 *versus* 4.7 years; P<0.0001) and the presence of an *FLT3*-ITD mutation negatively affected disease-free survival (0.8 *versus* 1.5 years; P=0.03), whereas the leukocyte count, age, karyotype, *NPM1* mutation status and percentage of CD34 or CD123 cells had no impact on disease-free survival (Table 3). Moreover, a percentage of $CD34^+CD38^{low/-}CD123^+$ cells of over 1% retained adverse prognostic significance for disease-free survival by multivariate analysis (95% CI, 0.13-0.55; P=0.00027). Age over 50 years, and an *FLT3*-ITD mutation were found to be significantly associated with decreased overall survival, while leukocyte count and the percentage of $CD123^+$ cells had no impact on overall survival (Table 4). In contrast, a $CD34^+CD38^{low/-}CD123^+$ percentage of over 1% was found to be significantly associated with decreased overall survival (Figure 2). Moreover, having more than 1% of $CD34^+CD38^{low/-}CD123^+$ cells retained an adverse prognostic value within the intermediate and favorable karyotype groups (Figure 2) but had no impact on the prognosis in the unfavorable karyotype group (*Online Supplementary Figure S2*). Remarkably, a percentage of $CD34^+CD38^{low/-}CD123^+$ cells of over 1% remained highly predictive for reduced overall survival when submitted to multivariate analysis (95% CI, 0.11-0.49; P=0.00011, Table 4). A favorable karyotype and *NPM1* mutation were also significantly associated with a better overall survival by multivariate analysis.

Table 1. Characteristics of the 111 AML patients at diagnosis.

	All patients N=111	$CD34^+CD38^-CD123^+$ <1% N=40	$CD34^+CD38^-CD123^+$ 1-15% N=51	$CD34^+CD38^-CD123^+$ > 15% N=20
Gender, male/female	45/66	19/21	24/27	6/14
Age, median	48 (20-65)	47 (20-65)	51 (20-64)	50 (21-65)
White cell count, median (×10^9/L)	33,2 (1-254)	13,8 (1-237)	41 (1.3-254)	32.9 (2.3-193)
Favorable cytogenetics	23	11	8	4
Intermediate cytogenetics	69	24	37	8
Unfavorable cytogenetics	19	5	6	8
NPM1 mutation	28/95	7/31	16/45	5/19
FLT3-ITD	33/100	7/34	21/47	5/19
Complete response	91(82%)	36 (90%)	42 (81.4%)	13 (65%)
Relapse	53 (58%)	13 (36%)	30 (71%)	10 (77%)
Autologous-stem cell transplantation	18 (20%)	7 (19%)	10 (24%)	1 (8%)
Allogeneic-stem cell transplantation	33 (36%)	11 (31%)	15 (36%)	7 (54%)

Table 2. Analysis of covariates associated with the complete remission rate.

	Univariate analysis			Logistic regression		
	P	OR	95% CI	*P*	OR	95% CI
Age > 50 years	0.81	0.82	0.31-2.16	> 0.1	-	-
NPM-1 mutation	0.76	0.62	0.16-2.39	> 0.1	-	-
FLT3-ITD	1.00	1.07	0.36-3.22	> 0.1	-	-
Good karyotype	0.02	12.28	0.70-214.6	> 0.1	-	-
Adverse karyotype	0.36	0.55	0.18-1.71	> 0.1	-	-
White cell count count > 50×10^9/L	1.00	0.98	0.35-2.74	> 0.1	-	-
$CD34^+CD38^-CD123^+$ > 15%	0.049	0.31	0.10-0.92	0.04	0.33	0.11-0.97

P of the univariate analysis is the P value of Fisher's exact test. OR is the value of the odds ratio. 95% CI is the 95% confidence interval of the odds ratio. Data from 95 patients were complete and were included in the logistic regression.

Table 3. Analysis of covariates associated with disease-free survival.

	Univariate analysis P	HR	95% CI	Cox regression P	HR	95% CI
Age > 50 years	0.12	1.49	0.90-2.49	> 0.1	-	-
NPM-1 mutation	0.95	1.02	0.57-1.84	> 0.1	-	-
FLT3-ITD	0.03	1.97	1.07-3.64	> 0.1	-	-
Good karyotype	0.43	0.78	0.43-1.43	> 0.1	-	-
Adverse karyotype	0.24	1.58	0.73-3.38	> 0.1	-	-
White blood count count > 50×10^9/L	0.49	1.21	0.71-2.07	> 0.1	-	-
$CD34^+CD38^-CD123^+$ < 1%	<0.0001	0.34	0.20-0.57	0.0003	0.27	0.13-0.55

P of the univariate analysis is the P value of the log rank test. HR is the value of the hazard ratio. 95% CI is the 95% confidence interval of the hazard ratio. Data from 78 patients were complete and were included in the Cox proportional-hazards regression.

Table 4. Analysis of covariates associated with overall survival.

	Univariate analysis P	HR	95% CI	Cox regression P	HR	95% CI
Age > 50 years	0.0097	1.88	1.17-3.03	>0.1	-	-
NPM-1 mutation	0.79	0.93	0.54-1.61	0.07	0.56	0.30-1.05
FLT3-ITD	0.02	1.91	1.10-3.32	>0.1	-	-
Good karyotype	0.10	0.62	0.35-1.10	0.04	0.47	0.23-0.95
Adverse karyotype	0.09	1.81	0.92-3.57	>0.1	-	-
White blood count count > 50×10^9/L	0.29	1.30	0.80-2.12	>0.1	-	-
$CD34^+CD38^-CD123^+$ < 1%	<0.0001	0.36	0.23-0.58	0.0001	0.24	0.11-0.49

P of the univariate analysis is the P value of the log rank test. HR is the value of the hazard ratio. 95% CI is the 95% confidence interval of the hazard ratio. Data from 95 patients were complete and were included in the Cox proportional-hazards regression.

Discussion

AML is a clinically and biologically heterogeneous disease for which prognostic factors have become increasingly important for the choice of the appropriate therapy. Although cytogenetic findings at diagnosis provide vital prognostic information, important subgroup definitions are now based on the mutational status of genes such as *FLT3*, *NPM-1*, *CEBPA*, *IDH1/2* and *c-KIT*, which are among the most relevant for this disease.[23] However, new prognostic tools based on biological analyses need to be developed. They must be accurate and easily available in a clinical setting. Flow cytometry is one such interesting technique as results can be obtained quickly (within a day) and with a high degree of sensitivity.

Guzman *et al.* showed that the leukemic cell subpopulation bearing the $CD34^+CD38^{low/-}CD123^+$ phenotype was in a quiescent state and highly resistant to both idarubicin and cytarabine *in vitro*.[24] Moreover, Ishikawa *et al.* elegantly demonstrated that, when injected into newborn NOD/SCID/IL2rγ^{null} mice, this subpopulation resides preferentially in the bone-marrow endosteal region where it is protected from cytarabine-induced apoptosis.[15] These results strongly argue that an enrichment of chemoresistant cells, from which relapses might arise, occurs within the $CD34^+CD38^{low/-}CD123^+$ leukemic subpopulation. Both studies proposed a strong rationale for targeting and sensitizing $CD34^+CD38^{low/-}CD123^+$ cells to chemotherapy using oxidative stress-inducing agents, inhibitors of NF-κB or PI3 kinase, or mobilizing agents from their niches with growth factors.

However, no study had assessed the clinical relevance of the percentage of $CD34^+CD38^{low/-}CD123^+$ cells in AML patients treated with intensive chemotherapy. We show that this leukemic subpopulation is not so rare in patients as we found up to 67% of $CD34^+CD38^{low/-}CD123^+$ cells in the bulk of blasts. Flow cytometry is the best technology for detecting subpopulations as it can easily discriminate less than 0.01% of cells.[25] Accordingly, our thresholds, which were a hundred times higher than the accuracy of the technology, can be easily set in daily practice, especially as the percentages of $CD34^+CD38^{low/-}CD123^+$ leukemic cells ranged from 0.01% to 67%.

Although the results of our present study need to be confirmed in a prospective manner and in a larger cohort, our data do show that a high level of $CD34^+CD38^{low/-}CD123^+$ cells at diagnosis is quite predictive of an increased risk of induction failure. This finding may have important implications for early therapeutic intervention because this phenotype can be routinely established within 1 day. The results of cytogenetic and molecular analyses are usually obtained in the month after starting induction chemotherapy and are, therefore, helpful to physicians only at the time of remission to determine the type of consolidation to use (e.g. allogeneic transplantation or not). Hence, the assessment of the level of $CD34^+CD38^{low/-}CD123^+$ cells at diagnosis could help clinicians to quickly identify patients at risk of induction failure and thereby improve the response rate by using different therapeutic strategies. Moreover, a percentage of $CD34^+CD38^{low/-}CD123^+$ cells of more than 1% is strongly associated with early relapses as shown by the disease-free survival curves suggesting that residual disease is likely to be high in these patients. A recent study by

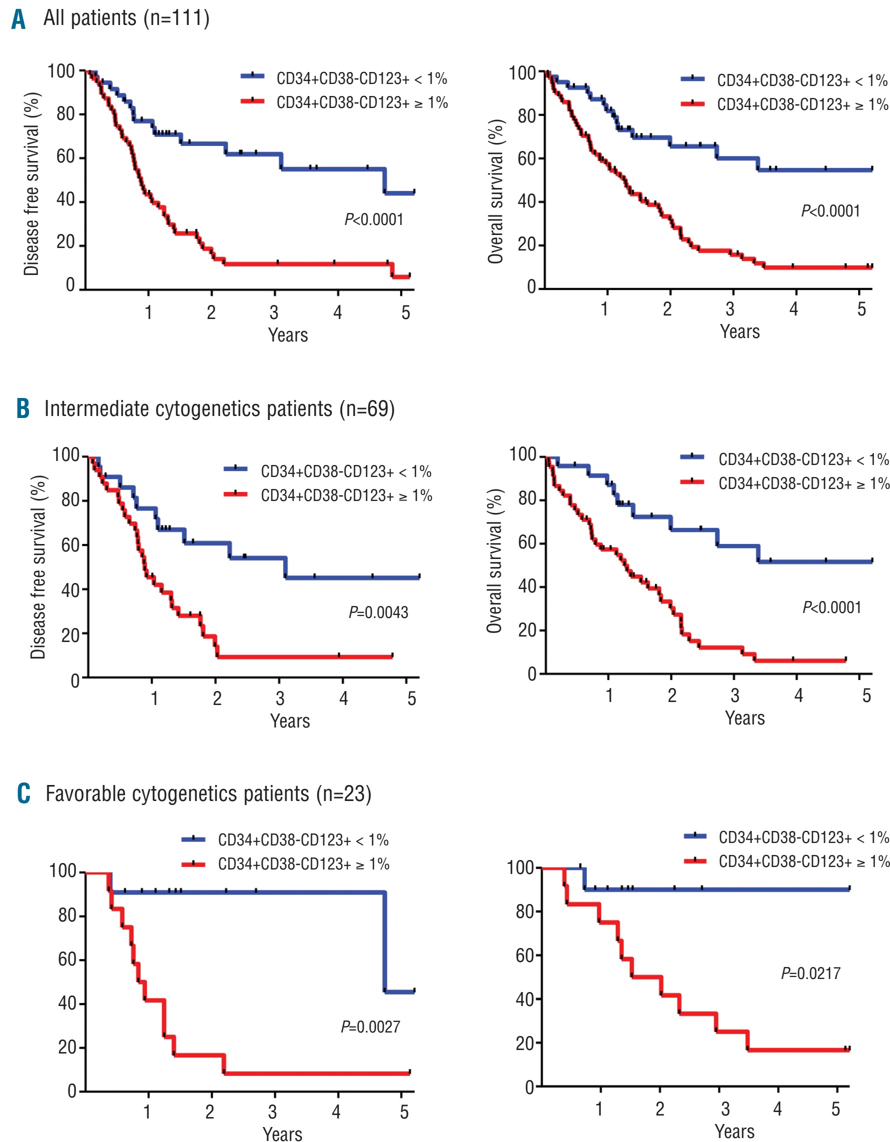

Figure 2. Comparison of disease-free and overall survival of AML patients according to CD34+CD38low/-CD123+ percentage. (A) All patients. (B) Intermediate cytogenetics group. (C) Favorable cytogenetics group.

Witte *et al.* showed similar results regarding the CD34+ CD38low/- population in a cohort of 17 children.[18] In our study we found that the CD34+CD38low/-CD123+ phenotype was more accurate at identifying patients with a worse prognosis (*Online Supplementary Figure S3*).

Finally, a number of recent studies have emphasized CD123 expression as a valuable therapeutic target in AML and several phase I studies assessing anti-CD123 monoclonal antibodies targeting leukemic stem cells in AML are ongoing. Jin *et al.* used an anti-CD123 neutralizing antibody, 7G3, in a NOD/SCID mice model of human AML and showed a reduction of the AML burden in mice with pre-established disease and impaired secondary transplantation.[26] Fey *et al.* also showed the *in vitro* anti-leukemic activity of Fv fragment antibodies directed against CD123, CD33 and CD16 in AML.[27,28] These anti-CD123 targeted therapies should be tested in AML patients, and particularly in cases demonstrating a high CD34+CD38low/-CD123+ population and thus a high risk of relapse.

Overall, our results demonstrate the negative prognostic significance of high levels of CD34+CD38low/-CD123+ leukemic cells detected by flow cytometry at diagnosis in AML patients treated with intensive chemotherapy. These findings will now be pursued prospectively in a larger cohort of patients and, if confirmed, the detection of this cell subpopulation within the leukemic bulk could become a cornerstone of risk-stratified AML clinical trials in the near future.

Authorship and Disclosures

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