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# Clinical significance of chromatin-spliceosome acute myeloid leukemia: a report from the Northern Italy Leukemia Group (NILG) randomized trial 02/06

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## ABSTRACT

Secondary acute myeloid leukemia (sAML) after myelodysplastic or myeloproliferative disorders is a high-risk category currently identified by the clinical history or specific morphological and cytogenetic abnormalities. However, in the absence of these features, uncertainties to identify the secondary nature of some cases, otherwise defined as *de novo* AML, remain. In order to test whether a chromatin-spliceosome (CS) mutational signature might better define the *de novo* AML group, we analyzed a prospective cohort of 413 newly diagnosed AML patients who were enrolled in a randomized clinical trial (NILG AML 02/06) and who provided samples for accurate cytogenetic and molecular characterization. Among clinically defined *de novo* AML, 17.6% carried CS mutations (CS-AML) and showed clinical characteristics closer to sAML (older age, lower white blood cell counts and higher rate of multilineage dysplasia). Outcomes in this group were adverse, more similar to those of sAML as compared to *de novo* AML (overall survival, 30% in CS-AML and 17% in sAML vs. 61% in *de novo* AML,  $P < 0.0001$ ; disease-free survival, 26% in CS-AML and 22% in sAML vs. 54% of *de novo* AML,  $P < 0.001$ ) and independently confirmed by multi-variable analysis. Allogeneic transplant in first complete remission improved survival in both sAML and CS-AML patients. In conclusion, these findings highlight the clinical significance of identifying CS-AML for improved prognostic prediction and potential therapeutic implications. (NILG AML 02/06; clinicaltrials.gov. Identifier: NCT00495287).

## Introduction

According to the current World Health Organization (WHO) classification,<sup>1</sup> secondary acute myeloid leukemia (sAML) is defined either by a previous clinical history of hematological disease, the morphological detection of multilineage dysplasia or specific cytogenetic characteristics; the two latter criteria are additive to the clinical history or may be themselves sufficient to diagnose sAML, even in the absence of a known antecedent myelodysplastic syndrome (MDS) or myeloproliferative neoplasm (MPN).<sup>1,2</sup> However, in clinical practice some uncertainty remains regarding the correct classification of potentially high-risk sAML cases, especially when an antecedent history is not thoroughly documented or does not fully satisfy the diagnostic criteria of MDS. Moreover, morphological examination to assess blast counts and multilineage dysplasia shows inter-observer variability that may impair diagnostic reproducibility.<sup>3</sup> Finally, cytogenetic analysis, which usually takes a long turnaround time of 5–10 days, may not be always informative because of technical failure or a normal result. These features eventually translate into under-recognition of sAML patients and lead to inadequate clinical management, since this high-risk population deems intensive treatment strategies comprising the administration of innovative agents or allocation to clinical trials, which are potentially able to improve the rates of complete remission (CR),<sup>4,7</sup> followed by a rapid consolidation with allogeneic hematopoietic stem cell transplant (alloHSCT).<sup>7,8</sup> Therefore, more accurate diagnostic approaches are warranted. In this regard, studies focusing on the molecular landscape of sAML and preceding conditions have suggested the possibility of defining distinct subtypes of AML based on their mutational profiles. Mutations in genes involved in chromatin regulation (*ASXL1*, *EZH2*, *BCOR*, *STAG2*) and RNA splicing (*SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*) have shown high specificity for sAML after MDS;<sup>9</sup> these mutations, called secondary-type mutations, occur early in leukemogenesis (likely representing the expansion of clones acquired during previous MDS)<sup>10,11</sup> and often persist in clinical remission, presenting as constitutively chemoresistant. Although less characterized, sAML cases progressing after MPN display similar features.<sup>12,13</sup> However, a closely related mutational signature can be identified also in some *de novo* AML cases.<sup>14,15</sup> In the seminal study conducted by Papaemmanuil *et al.*<sup>14</sup> on a large cohort of AML patients, overlapping mutations in genes regulating RNA splicing (*SRSF2*, *SF3B1*, *U2AF1*, and *ZRSR2*), chromatin (*ASXL1*, *STAG2*, *BCOR*, *KMT2A*-PTD, *EZH2*, and *PHF6*), or transcription (*RUNX1*) constituted an independent genomic class, called the chromatin-spliceosome group. Mutations in *RUNX1* and *KMT2A*-PTD frequently co-occurred with other mutations of the signature and, albeit not universally observed in sAML, have been reported as more significantly associated with high-risk than low-risk MDS,<sup>11</sup> and consistently occurred at intermediate time points and not as founding mutations.<sup>11, 14–16</sup> Strikingly, 91% of patients in this group were clinically defined as *de novo* AML; although heterogeneous, mutations in this molecular signature were consistently associated with older age, lower white blood cell and blast counts, lower rates of response to induction chemotherapy and higher relapse rates. While these results warrant prospective validation, it can be hypothesized that the presence of

these mutations represent the trace of a previous, unrecognized MDS or MPN phase. However, a formal comparison between *de novo* AML patients carrying chromatin-spliceosome (CS) mutations and sAML patients defined by standard criteria, along with associated outcomes, is currently lacking.

In order to investigate the clinical significance of the CS mutational signature, we reassessed patients' diagnosis according to the presence of CS mutations in a large cohort of newly diagnosed AML patients enrolled into a prospective trial (NILG AML 02/06) (clinicaltrials.gov. Identifier: NCT00495287).<sup>17</sup> In this study, we report the characteristics and outcomes of initially defined *de novo* AML patients carrying CS mutations as compared with other clinically defined *de novo* AML patients without CS mutations and patients with sAML defined by standard WHO criteria.

## Methods

### Patients, treatment, cytogenetic and molecular analyses

The NILG-AML 02/06 multi-center Italian trial<sup>17</sup> enrolled 574 patients with newly diagnosed AML ( $\geq 20\%$  bone marrow [BM] blasts) or high-risk MDS (10–19% BM blasts) between 2007 and 2012. All participants were randomized to receive induction with standard-dose idarubicin, cytarabine and etoposide (ICE) or high-dose cytarabine and idarubicin (sHD). Patients not responding to first induction underwent an intensified re-induction with sHD. Consolidative alloHSCT was performed in high-risk patients based on study-specific risk stratification, as previously reported.<sup>17</sup> Written informed consent for inclusion in the clinical trial and genetic analyses was provided by all patients. Study protocols were in accordance with the Declaration of Helsinki and approved by the Institutional Review Boards of each participating center.

Informative karyotype was locally obtained at diagnosis for 413 patients. Molecular analyses were centrally performed on samples collected at diagnosis (see the *Online Supplementary Methods*). *NPM1*, *FLT3*-ITD and point mutations, *RUNX1*-*RUNX1T1*, *CBFB*-*MYH11*, biallelic *CEBPA* and *KMT2A*-PTD mutations were tested on all patients using polymerase chain reaction (PCR), Sanger sequencing and/or fragment analysis. Targeted next-generation sequencing (NGS) was performed on 196 normal karyotype patients using an amplicon-based method (Trusight Myeloid, Illumina, San Diego, California, USA) (n=161) amplifying 54 gene regions and a capture-based method (Sophia Myeloid Solution, Sophia Genetics SA, Saint Sulpice, Switzerland) (n=35) selecting 30 gene regions (*Online Supplementary Tables S1* and *S2*).<sup>18</sup>

In order to confirm our results in an independent cohort, we also evaluated a single-center series of AML patients (n=50) treated at ASST Ospedale Papa Giovanni XXIII between 2012 and 2020.

### Definition of acute myeloid leukemia categories

The following AML categories were defined among patients enrolled into the trial: i) CS-AML: with the specific goal of validating the CS mutational signature, clinically defined *de novo* AML patients were included in this category based on the presence of at least one variant described according to Papaemmanuil *et al.*<sup>14</sup> including *ASXL1*, *STAG2*, *BCOR*, *EZH2*, *PHF6*, *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *RUNX1* and *KMT2A*-PTD (or cytogenetic alterations in *KMT2A* gene in the 11q23 cytogenetic region), excluding patients with concurrent WHO-recurrent

rent abnormalities;<sup>1</sup> ii) sAML: patients with a documented clinical history of MDS, MDS/MPN or MPN and/or cytogenetic WHO criteria of AML with MDS-related changes;<sup>1</sup> iii) *de novo* AML: none of the above. In order to avoid any ambiguous interpretation, morphological WHO criteria of AML with MDS-related changes were not considered in the definition of sAML. Patients with MDS, therapy-related AML or not provided with a cytogenetic and/or molecular characterization were excluded from the analysis.

### Statistical analysis

The clinical endpoints of the study were defined according to standard criteria.<sup>19</sup> Comparisons between baseline characteristics and AML categories were analyzed using the Mann-Whitney U test for continuous and Chi-squared or Fisher's exact test for categorical variables. Overall survival (OS) and disease-free survival (DFS) were estimated by the Kaplan-Meier method and any differences between AML categories or consolidation treatment were evaluated with log-rank test. Cox models were used to estimate hazard ratios (HR) with 95% Confidence Intervals (CI) in univariate and multivariable analysis on survival outcomes. AlloHSCT was considered as a time-

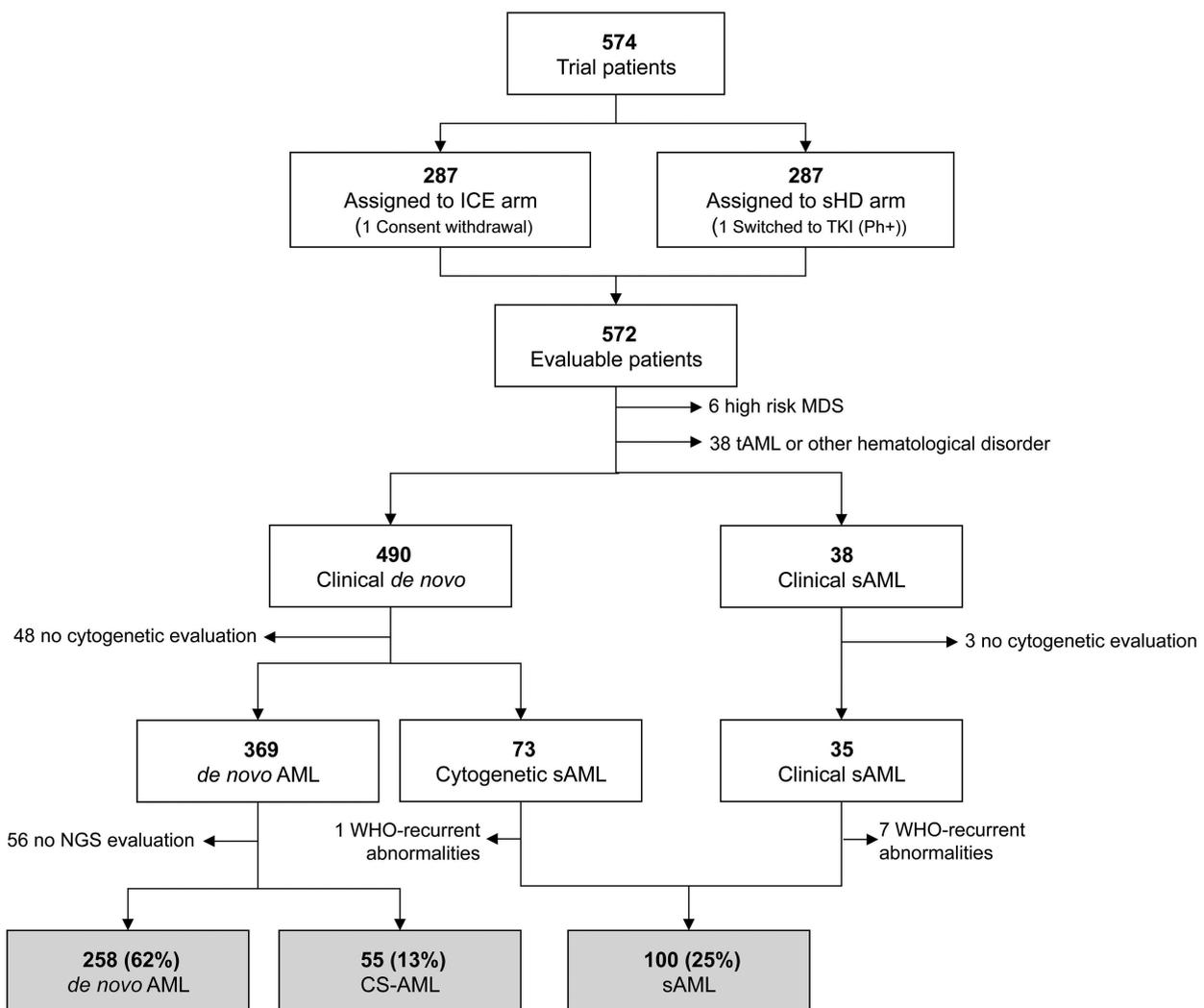
dependent event; outcome data were estimated by the Mantel-Byar method and graphically illustrated by Simon-Makuch plots. All reported *P*-values are two-sided and set at 5% significance level. All analyses were performed with R software, version 3.5.0.

## Results

### Characteristics of patients

Among 574 adult patients enrolled in the NILG-AML 02/06 trial,<sup>16</sup> 413 (72%) with full genetic characterization resulted evaluable and were classified as 55 CS-AML patients, 100 sAML patients (28 defined by clinical history, of which 24 after MDS or MDS/MPN and four after MPN, and 72 defined by cytogenetic criteria) and 258 *de novo* AML patients (Figure 1). The 55 cases reclassified as CS-AML represented 17.6% of otherwise defined *de novo* AML patients and 13% of the whole analyzed cohort.

The main clinical characteristics of patients are reported in Table 1. Compared to *de novo* AML, patients with sAML and CS-AML were similarly older (median age 48,



**Figure 1. CONSORT diagram illustrating patient selection.** ICE: idarubicin, cytarabine, etoposide; sHD: sequential high-dose chemotherapy; TKI: tyrosine-kinase inhibitors; AML: acute myeloid leukemia; tAML: therapy-related AML; sAML: secondary AML; CS-AML: *de novo* AML carrying chromatin-spliceosome mutations; MDS: myelodysplastic syndrome; WHO: World Health Organization; NGS: next-generation sequencing.

59 and 58 years respectively,  $P < 0.0001$ ) and presented at diagnosis with lower white blood cell counts (WBC) ( $P < 0.0001$ ), with no significant differences between the two latter categories. A lower BM blast infiltration was reported in sAML as compared with both *de novo* AML ( $P < 0.0001$ ) and CS-AML ( $P = 0.02$ ). By morphological analysis, multilineage dysplasia was described at diagnosis in a minor proportion of CS-AML patients (11%), close to that of sAML patients (9%,  $P = 0.77$ ) and similarly higher than that of *de novo* AML patients (2%,  $P = 0.0051$ ).

The cytogenetic and molecular characteristics of the cohort are summarized in Figure 2 and the *Online Supplementary Table S2*.

In the CS-AML category ( $n = 55$ ) (Figure 2A and B), the majority of patients (87%) had a normal karyotype; 160 mutations were found in total, with a median of three mutations per patient (range, 1-6). The most frequently reported mutations of the CS signature were in *KMT2A* (*KMT2A*-PTD), *RUNX1* and *ASXL1* genes (respectively 45.5%, 44.4% and 22.2% of evaluable patients), while other mutations accounted for 5-17.5% of cases. While 54.5% of patients in this category presented with a single CS mutation, overlap and significant associations between CS mutations were observed in 45.5% of cases. Other mutations scored in CS-AML patients included *IDH2*, *DNMT3A*, *FLT3*-ITD, *TET2* and *NRAS* (respectively 20%, 15.6%, 14.5% and 11% of evaluable patients), and others (Figures 3 and 4). We also included two patients with previously undescribed variants (UPN 633, *STAG2* p.Met135Ile and UPN 753, *SRSF2* p.Val18Leu) in the CS-AML category due to their predicted pathogenicity. One more patient harboring the *RUNX1* p.Ala60V variant (UPN 3) was also included. This variant has been

found in familial platelet disorder associated to myeloid malignancy and is reported as such in the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/variation/V/CV000463986>).

Among the 100 sAML patients (Figure 2C and D), a huge proportion carried a complex karyotype (58%) and abnormalities of chromosome 7 (12%) or 5 (5%). Nevertheless, 13% of patients in this category had a normal karyotype; 62 mutations were reported in total (median 0, range 0-8), mostly involving CS genes.

The *de novo* AML category ( $n = 258$ ) (Figure 2E and F) included 25% of patients with a core binding factor AML, a high prevalence (65%) of patients with normal karyotype and 10% of patients with other non WHO-recurrent cytogenetic abnormalities. A total of 607 mutations were scored (median two per patient, range 0-15), the most frequently represented being *NPM1* (60.7% of patients), *DNMT3A* (49.3%) and *FLT3*-ITD (27.5%).

### Outcomes after intensive induction

Patients with *de novo* AML, sAML and CS-AML were equally distributed among the randomly assigned induction regimen (conventional ICE or high-dose sHD) and showed a comparable performance status (Table 1).

After the first induction cycle, a lower proportion of sAML patients achieved CR (51 of 100, 51%) as compared to both *de novo* AML (228 of 258, 88.4%;  $P < 0.0001$ ) and CS-AML (43 of 55, 78.2%;  $P = 0.002$ ). However, also CS-AML showed a trend toward an inferior CR rate as compared to *de novo* AML ( $P = 0.07$ ), although this did not reach statistical significance, eventually due to the relative small number of patients in this group. Interestingly, no significantly worse CR rate was observed between

**Table 1. Demographic and clinical characteristics of patients by acute myeloid leukemia category.**

Clinical characteristics	CS-AML N=55	$P^*$	sAML N=100	$P^{**}$	<i>de novo</i> AML N=258	$P^{***}$
Age [years], median (range)	58 (20-72)	0.5542	59 (22-72)	<0.0001	48 (16-73)	0.0001
<60, n(%)	33 (60)	0.4015	53 (53)	<0.0001	207 (80.2)	0.0013
≥60, n(%)	22 (40)		47 (47)		51 (19.8)	
Sex, n (%)		0.0349		0.0076		0.8048
M	25 (45.5)		63 (63)		122 (47.3)	
F	30 (54.5)		37 (37)		136 (52.7)	
ECOG PS, n (%)		0.5674		0.4207		0.9903
0-1	49 (89.1)		92 (92)		230 (89.1)	
2-3	6 (10.9)		8 (8)		28 (10.9)	
Hepatomegaly, n (%)	5 (9.1)	0.5216	6 (6)	0.4916	21 (8.1)	0.7897
Splenomegaly, n (%)	2 (3.6)	0.2150	10 (10)	0.3159	36 (14)	0.0334
Extramedullary involvement, n (%)	6 (10.9)	0.5458	7 (7)	0.0267	41 (15.9)	0.3477
Hemoglobin [g/dL], median (range)	9.3 (5.1-13.8)	0.6334	8.8 (4.3-13.7)	0.1905	9.3 (3-15.8)	0.7040
WBC count [ $\times 10^9/L$ ], median (range)	8.1 (1.1-252)	0.1794	4.8 (0.8-237)	<0.0001	22.3 (0.5-282)	0.0004
Platelets, median (range)	77 (12-815)	0.1441	57 (2-338)	0.3649	52 (5-852)	0.0151
BM blast cells (%), median (range)	80 (8-100)	0.0227	64 (2-100)	<0.0001	80 (0-100)	0.2067
AML with multilineage dysplasia, n (%)	6 (10.9)	0.7786	9 (9)	0.0041	5 (1.9)	0.0051
Induction treatment, n (%)		0.7613		0.6852		0.5065
ICE	25 (45.5)		48 (48)		130 (50.4)	
sHD	30 (54.5)		52 (52)		128 (49.6)	

CS-AML: chromatin-spliceosome acute myeloid leukemia; sAML: secondary acute myeloid leukemia; *de novo* AML: *de novo* acute myeloid leukemia; ECOG PS: Eastern Cooperative Oncology Group Performance Status; WBC: white blood cell count; BM: bone marrow; ICE: idarubicin, cytarabine and etoposide; sHD: sequential high-dose chemotherapy cytarabine and idarubicin.  $P$ -values refer to: \*CS-AML vs. sAML; \*\*sAML vs. *de novo* AML; \*\*\**de novo* AML vs. CS-AML. Hepatomegaly was defined as lower liver edge >2 cm from costal margin. Splenomegaly was defined as spleen >1 cm from costal margin, confirmed by ultrasound scan with longitudinal axis >12 cm. Extramedullary AML was defined as AML presenting with central nervous system involvement or mass lesions.

these latter groups ( $P=0.14$ ) when accounting for both first induction (performed in all patients) and intensified re-induction (performed in patients who did not respond after first cycle). By contrast, sAML patients did not improve their CR rate (59%) even after undergoing re-induction, as compared with both *de novo* AML (92.6%,

$P<0.0001$ ) and CS-AML (85.5%,  $P=0.001$ ). In addition, early death more frequently occurred in sAML patients (14 of 100, 14%) than in *de novo* AML (11 of 258, 4.3%;  $P=0.003$ ) and CS-AML (three of 55, 5.5%;  $P=0.17$ ). Globally, 41% of sAML patients, 14.5% of CS-AML patients and 7.3% of *de novo* AML patients did not

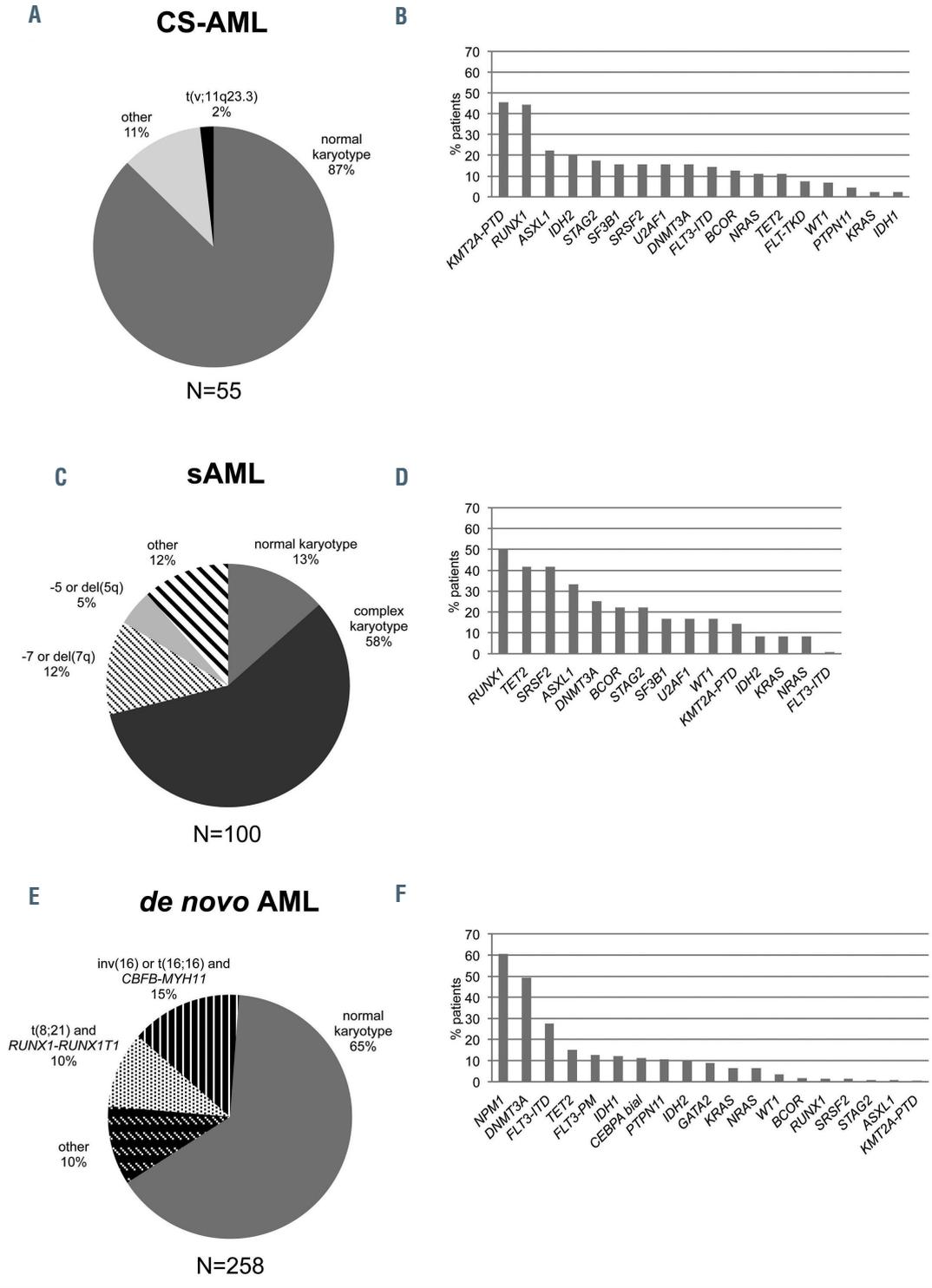


Figure 2. Cytogenetic and molecular characteristics of acute myeloid leukemia categories. For each acute myeloid leukemia (AML) category, pie charts depict the distribution of chromosomal abnormalities, while histograms show the frequency of individual mutations. (A and B) CS-AML (*de novo* AML carrying chromatin-spliceosome mutations), (C and D) secondary AML (sAML) and (E and F) *de novo* AML. The label “other” includes: for the CS-AML category, abnormalities of chromosome 11 [(other than t(v;11q23.3) and del(11q)] and +8; for the sAML category, del(11q), +8, del(12p), t(5q;12p), t(5q;12p), t(1p;3q), t(3q;5q) and -Y; for the *de novo* AML category, +8, del(9q), +21, monosomy 21, +13, t(8q;11q), inv(3), monosomy X, -Y, del(16q), add(4q), add(6p), t(13p;17p).

achieve CR because of early death or chemoresistance; these patients had a dismal 1-year OS (17%) (Online Supplementary Figure S1).

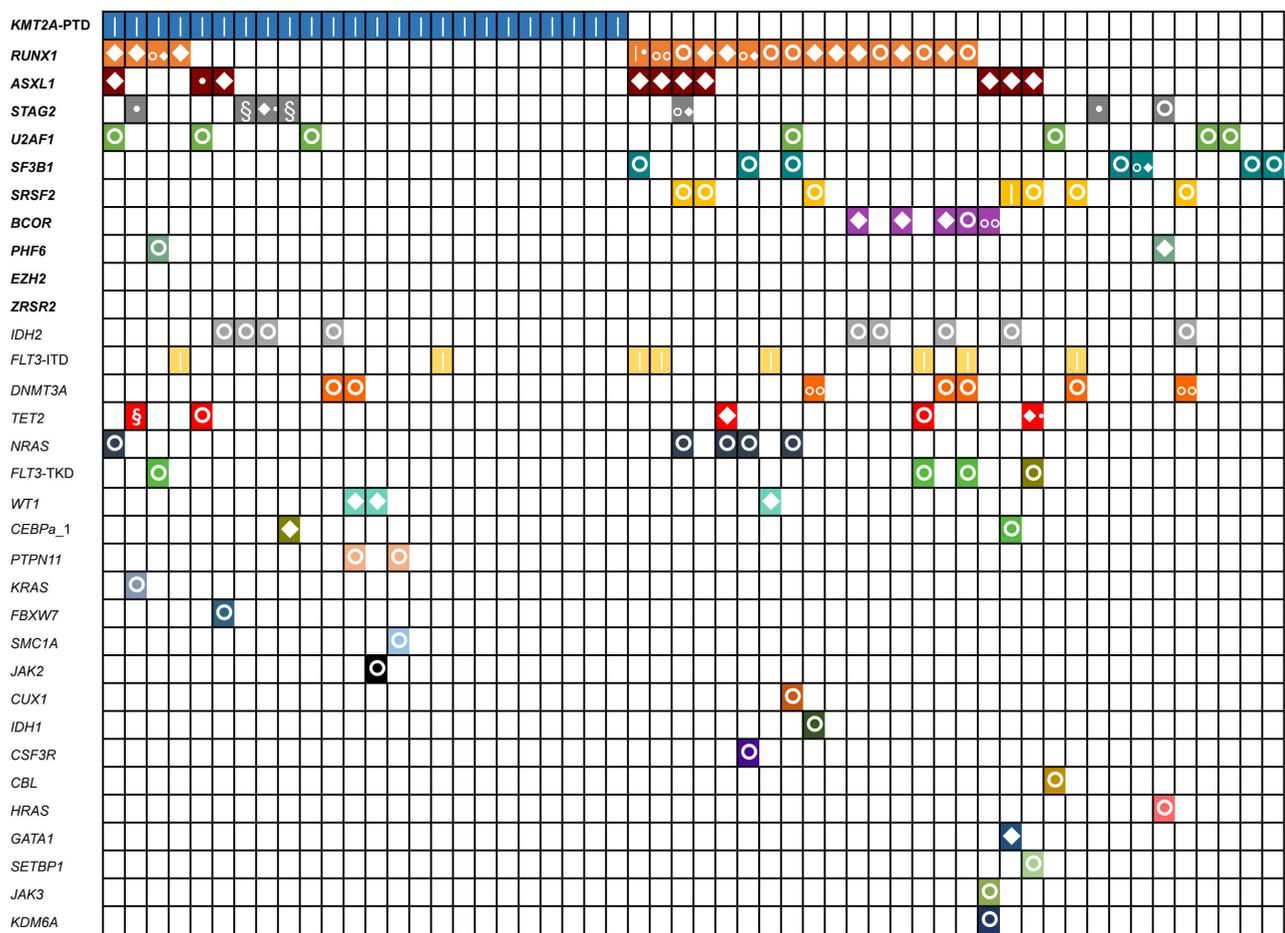
We investigated factors affecting the probability of achieving CR (accounting for both induction cycles) by

performing univariate analysis within each AML category. In *de novo* AML, a significantly negative impact was observed for advanced age, impaired performance status, high WBC and the presence of *FLT3*-ITD mutations. Apart from the presence of *SRSF2* mutations among CS-

**Table 2. Multivariable analysis for complete remission, overall survival and disease free survival on the whole patient cohort.**

All patients Characteristics	Complete remission		Overall survival		Disease-free survival	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age [years] ≥60	0.38 (0.21-0.7)	0.0017	1.67 (1.25-2.23)	0.0005	1.32 (0.95-1.83)	0.1030
ECOG PS 2-3	0.25 (0.11-0.57)	0.0010	2.32 (1.55-3.47)	<0.0001	1.46 (0.87-2.43)	0.1502
WBC count [x10 <sup>9</sup> /L] ≥50	0.5 (0.24-1.03)	0.0582	1.69 (1.23-2.3)	0.0010	1.59 (1.14-2.21)	0.0063
Induction arm ICE	0.87 (0.48-1.57)	0.6486	1.27 (0.97-1.66)	0.0856	1.27 (0.95-1.7)	0.1046
AML category						
sAML	0.09 (0.05-0.19)	<0.0001	3.71 (2.69-5.12)	<0.0001	2.54 (1.76-3.67)	<0.0001
CS-AML	0.51 (0.2-1.37)	0.1608	2.2 (1.48-3.25)	0.0001	1.89 (1.27-2.81)	0.0018

WBC: white blood cell; ECOG PS: Eastern Cooperative Oncology Group Performance Status; sAML: secondary acute myeloid leukemia; ICE: idarubicin, cytarabine and etoposide; HR: hazard ratio; CI: Confidence Interval; CS: chromatin-spliceosome; CS-AML: *de novo* AML carrying the CS mutations.



**Figure 3. Mutational profile of acute myeloid leukemia patients carrying the chromatin-spliceosome mutation.** Each column represents an individual acute myeloid leukemia patient carrying a chromatin-spliceosome mutation (CS-AML patient), while each row represents a single gene mutation out of the list at the left. Colored bars indicate the presence of one or more mutations of each gene. Variant types are specified according to the legend at the bottom of the figure.

AML patients (HR 0.16; 95% CI: 0.02-1.02;  $P=0.05$ ), no other relevant clinical or biological factors (including the intensity of induction regimen) were identified for sAML and CS-AML patients (Online Supplementary Table S3).

### Survival outcomes

The median follow-up for survival analysis was 4.9 years (range, 0.2-8.4). *De novo* AML patients showed a markedly better 5-years OS (61%) and DFS (54%) than CS-AML (OS 30%, DFS 26%;  $P<0.0001$  and  $P=0.0009$  respectively) and sAML (OS 17%, DFS 22%;  $P<0.0001$  for both comparisons) (Figure 5A and B). Patients with sAML had a significantly worse OS as compared to CS-AML ( $P=0.02$ ) (Figure 5A); however, possibly due to the high mortality rate of non-responding patients, no difference was observed between the two categories when considering only patients achieving CR, who showed an overlapping 5-years DFS (26% and 22% respectively,  $P=0.32$ ) (Figure 5B). A similar trend of OS according to the respective categories was confirmed on a smaller ( $n=50$ ), independent single-center cohort (Online Supplementary Figure S2; for demographic and clinical characteristics of this cohort, see the Online Supplementary Table S6).

According to trial risk stratification criteria, consolidative alloHSCT in first CR was administered in 18 of 47 (38.3%) CS-AML, 31 of 59 (52.5%) sAML and 80 of 239 (33.5%) *de novo* AML patients, at a median age of 49.5, 52 and 44.5 years, respectively. By time-dependent analysis, the administration of alloHSCT in first CR carried a 5-years survival advantage in each AML category (CS-AML: 48% vs. 24%,  $P=0.07$ ; sAML: 38% vs. 8%,  $P=0.0001$ ; *de*

*novo* AML: 75% vs. 59%,  $P=0.01$ ) (Figure 6A to C).

By multivariable analysis performed on the whole patients' cohort accounting for age, performance status, WBC count at diagnosis and induction arm (standard vs. high-dose regimen), the markedly unfavorable prognosis of the sAML category was evident for each considered outcome (CR: HR 0.09, 95% CI: 0.05-0.19,  $P<0.0001$ ; OS: HR 3.71, 95% CI: 2.69-5.12,  $P<0.0001$ ; and DFS: HR 2.54, 95% CI: 1.76-3.67,  $P<0.0001$ ). Although to a lesser degree, also the CS-AML category was independently associated to a negative prognosis, in terms of OS (HR 2.2, 95% CI: 1.48-3.25,  $P=0.0001$ ) and DFS (HR 1.89, 95% CI: 1.27-2.81,  $P=0.0018$ ), but not CR (Table 2). Other factors affecting clinical outcomes included age  $\geq 60$  years, performance status (on CR and OS) and WBC  $\geq 50 \times 10^9/L$  (on OS and DFS).

Since CS mutations frequently co-occurred within individual patients, we sought to investigate whether specific variants of the signature might be independently responsible for the adverse prognosis of CS-AML patients. In a multivariable analysis performed on the CS-AML cohort including CS-mutations and adjusting for the same variables as in previous analysis (Online Supplementary Table S4), only *RUNX1* and *U2AF1* independently affected OS (HR 3.55, 95% CI: 1.28-9.87,  $P=0.01$ ; and HR 6.87, 95% CI: 1.71-27.55,  $P=0.006$ ) and DFS (HR 3.13, 95% CI: 1.1-8.95,  $P=0.03$ ; and HR 16.46, 95% CI: 3.14-86.31,  $P=0.0009$ ). Notwithstanding, even after subtracting *RUNX1* and/or *U2AF1*-mutated patients from the first multivariable analysis (Online Supplementary Table S5), the CS-AML category maintained independently worse OS (HR 1.83, 95% CI: 1.07-3.14,  $P=0.0281$ ) and DFS (HR 1.94, 95% CI: 1.15-3.26,  $P=0.0126$ ) as compared to *de novo* AML.

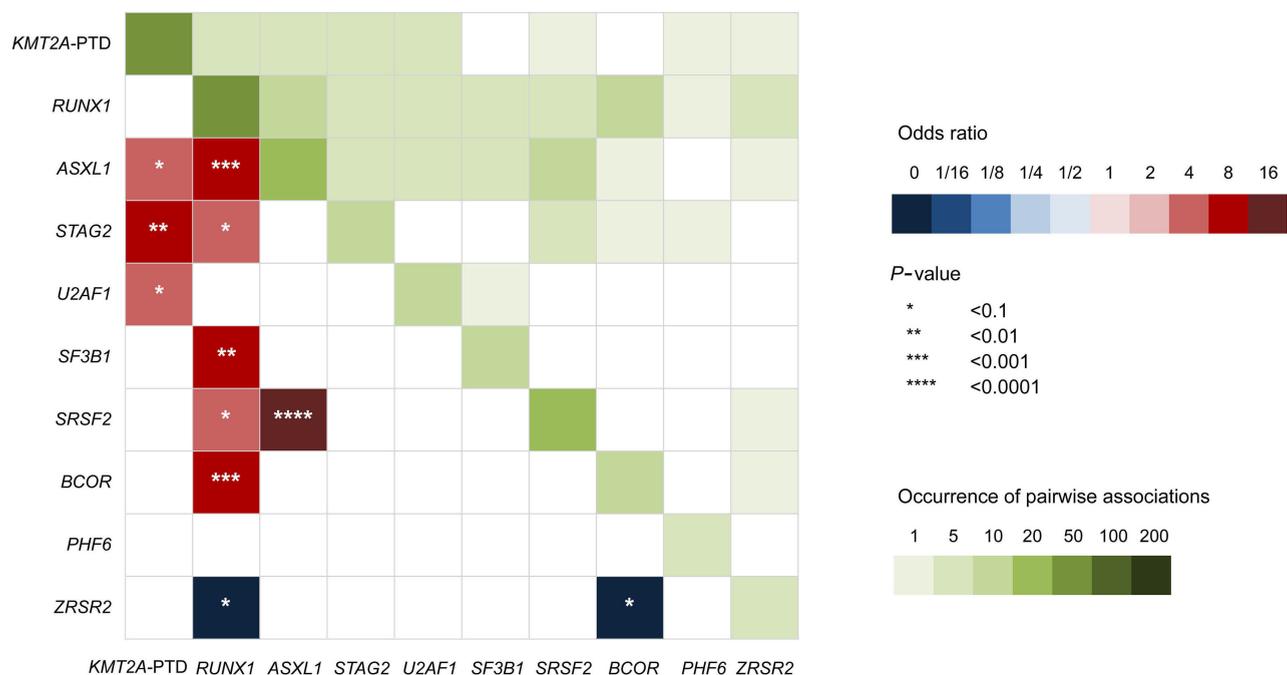


Figure 4. Patterns of co-occurrence and mutual exclusivity of gene mutations among 55 CS-AML patients. In the lower triangle are shown pairwise associations between gene mutations. For each pair, odds ratios indicate an increased ( $>1$ ) or decreased ( $<1$ ) probability of co-occurrence between the two mutations as assessed by the Fisher exact test for statistical significance. The odds ratio of the association is color coded and the significance level is indicated by the number of asterisks in each colored square as reported in the legend at the right of the figure. The upper triangle illustrates the absolute number of occurrences of each molecular pair, shown in green gradient and divided in intervals as reported in the legend. The analysis was performed on the whole study cohort ( $n=413$ ), excluding mutations occurring in less than six patients and not defining AML categories.

Discussion

In this study, we challenged a CS-mutational signature<sup>14</sup> against conventionally defined sAML and *de novo* AML patients to seek whether a comparison between related outcomes might provide a basis for implementing current clinical and cytogenetic diagnostic criteria of sAML with molecular information. Importantly, the identification of

patients carrying CS mutations has been allowed by conventional methods and by commercially available NGS solutions, which can be easily and cost-effectively implemented in the routine diagnostic work-up of AML.<sup>20</sup> Since diagnostic uncertainty is higher in cases lacking informative cytogenetics, we focused the search of CS mutations on patients with normal karyotype as these mutations were infrequently associated with abnormalities of chro-

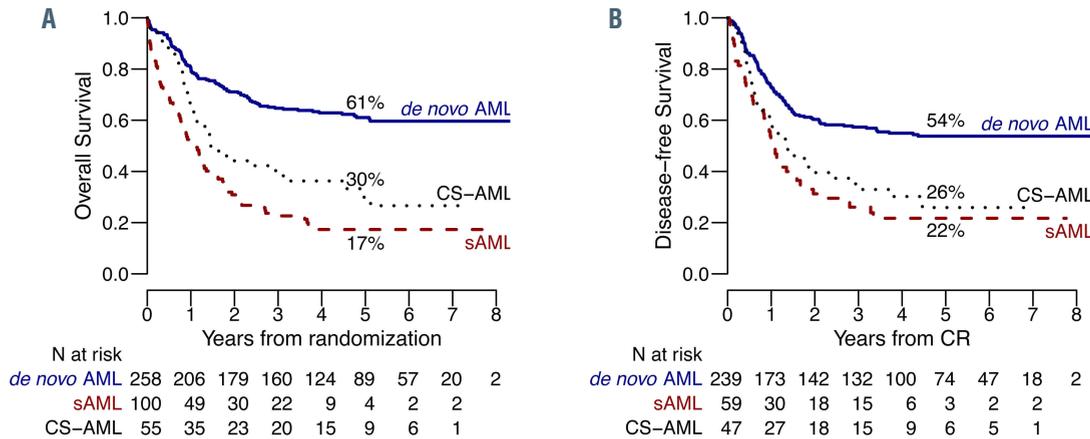


Figure 5. Kaplan-Meier survival analysis according to acute myeloid leukemia category. Survival estimates were calculated at 5 years and not censored at allogeneic transplant. (A) Overall survival; CS-AML vs. *de novo* AML,  $P < 0.0001$ ; sAML vs. *de novo* AML,  $P < 0.0001$ ; CS-AML vs. sAML,  $P = 0.02$ . (B) Disease free survival; CSAML vs. *de novo* AML,  $P = 0.0009$ ; sAML vs. *de novo* AML,  $P < 0.0001$ ; CS-AML vs. sAML,  $P = 0.32$ . CS: chromatin-spliceosome; AML: acute myeloid leukemia; sAML: secondary AML; CS-AML: *de novo* AML carrying the CS mutations.

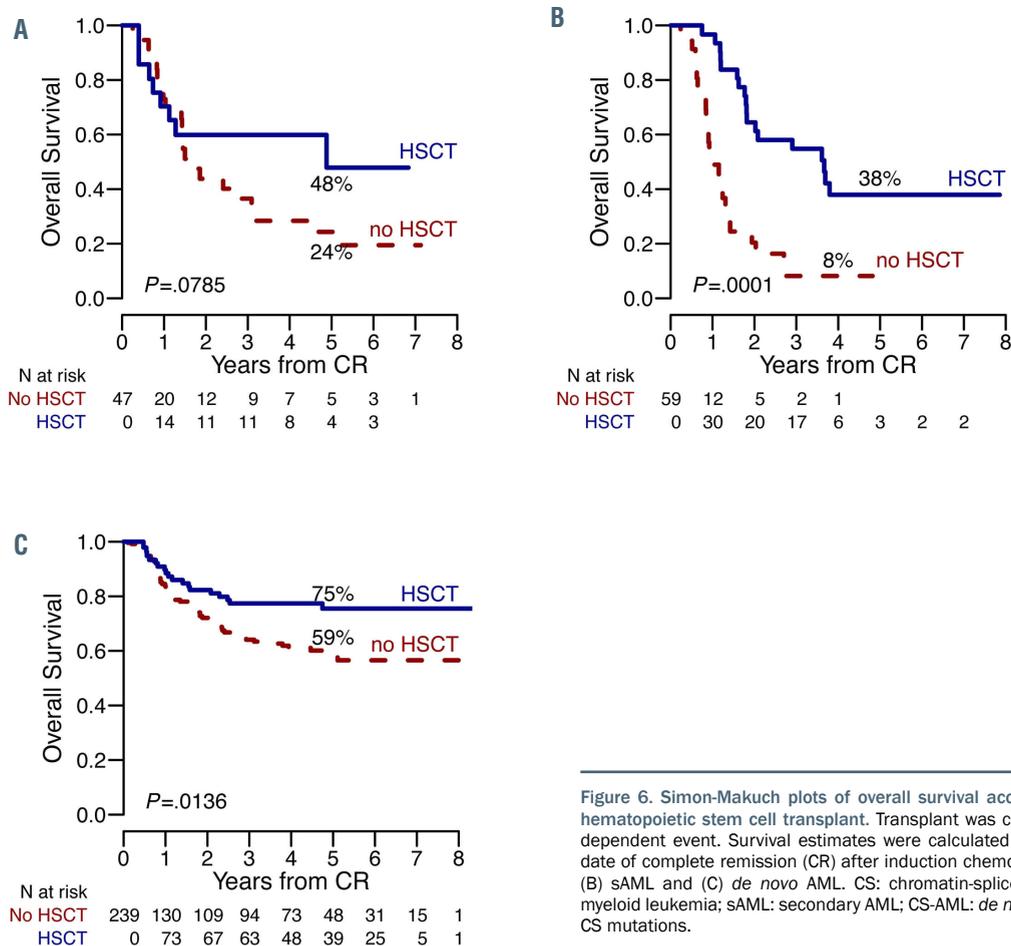


Figure 6. Simon-Makuch plots of overall survival according to allogeneic hematopoietic stem cell transplant. Transplant was considered as a time-dependent event. Survival estimates were calculated at 5 years from the date of complete remission (CR) after induction chemotherapy. (A) CS-AML, (B) sAML and (C) *de novo* AML. CS: chromatin-spliceosome; AML: acute myeloid leukemia; sAML: secondary AML; CS-AML: *de novo* AML carrying the CS mutations.

mosomes 5 and 7 or complex karyotypes in previous studies.<sup>9,21</sup> To the best of our knowledge, this is the first report formally comparing outcomes of patients with *de novo* AML carrying this mutational profile and sAML (defined by clinical and/or cytogenetic criteria) on a large multi-center prospective cohort. Patients included in the study showed a broad age range representative of the real life population, were homogeneously treated with intensive chemotherapy within a prospective clinical trial and had a long duration of follow-up, which allowed to effectively study the prognostic relevance of CS mutations. As an additional feature, we evaluated the impact of alloHSCT in each AML category.

In keeping with previous observations, the identification of a CS-mutational signature revealed markedly high-risk features in about 18% of otherwise defined *de novo* AML patients in our study,<sup>9,14,15</sup> which represents a significant proportion of the whole analyzed cohort, quite consistent with other studies.<sup>14,22</sup> Apart from mutations in *KMT2A-PTD*, *RUNX1* and *ASXL1*, we showed that also mutations in *U2AF1* carry an independent prognostic impact; of note, the adverse significance of CS mutations was maintained independently from *RUNX1* and *U2AF1*, suggesting that the full signature might be further evaluated for the assignment to the high-risk group of the European LeukemiaNet (ELN) stratification model.<sup>7</sup> Based on these data, it remains unclear whether *RUNX1*-mutated AML, albeit constituting the most represented subgroup within this category, accounts for a separate clinical and prognostic entity.

In addition, we have highlighted that CS-AML more closely resembles sAML than *de novo* AML, in terms of clinical characteristics and outcomes. When considering OS, however, CS-AML stands as an intermediate category showing slightly but significantly better survival than sAML; this appears to be the consequence of the high rates of early chemoresistance and related mortality in sAML patients. A possible explanation for this difference resides in the negative impact of complex cytogenetics in the sAML group. Alternatively, this category might contain a mixture of both sAML and true *de novo* cases. As such, the role of the CS signature to accurately diagnose sAML remains not fully elucidated. However, from a practical point of view, the definition of CS-AML as a prognostically homogeneous group might be more clinically significant than a merely ontogenetic classification, as recently shown also for secondary-type mutations.<sup>23</sup> In fact, since we observed a survival advantage in both sAML and CS-AML patients to whom alloHSCT was offered in first CR, patients with a CS molecular profile might be considered for intensive treatment strategies comprising rapid allocation to alloHSCT. The main challenge in this setting, however, would be the improvement of remission rates and depth by means of innovative therapeutics, possibly overcoming the inherent long-term chemoresistance of CS-AML and extending the access to a potentially curative alloHSCT. In this regard, considering the closer similitude between sAML and CS-AML might facilitate the optimization of available treatment

strategies as well as the design of dedicated clinical trials. Among potentially useful agents, CPX-351 has been recently approved by the Food and Drug Administration and European Medicines Agency specifically for the treatment of AML with MDS-related changes or therapy-related AML and might provide a similar benefit in fit CS-AML patients.<sup>4</sup> Furthermore, in a large phase Ib trial the anti-BCL-2 agent venetoclax in association with hypomethylating agents has provided promising CR and survival rates even in sAML patients or AML with poor cytogenetics,<sup>5</sup> while spliceosome modulators<sup>24,25</sup> and DOT1L inhibitors<sup>26</sup> may represent a rational candidate for functional targeting of CS-AML and are currently evaluated in clinical trials involving myeloid neoplasms. Finally, in our dataset *IDH2* mutations were reported in 20% of CS-AML patients, confirming previous observations<sup>14</sup> and representing another potentially important therapeutic target in this population.<sup>27</sup>

The bottom line is that an accurate cytogenetic and molecular characterization is required at the diagnosis of AML, making it reasonable to wait for these data in order to perform the best treatment decision or enrollment into clinical trials; this approach has recently demonstrated to be safe in clinically stable patients.<sup>28</sup> In this context, although conventional cytogenetics are still needed for a correct risk stratification,<sup>7</sup> NGS technologies may overcome its limitations and long turnaround time,<sup>20</sup> also providing additional information with improved cost effectiveness.

In conclusion, we have assessed the impact of a CS-mutational signature on a large prospective cohort of AML patients employing a standardized, easily implementable NGS method and highlighting the need to detect this signature at diagnosis for an accurate risk prediction, with potentially relevant implications for the clinical management of AML patients.

### Disclosures

No conflict of interest to disclose.

### Contributions

CC, FL and AR designed the research, analyzed and interpreted data, supervised the study and wrote the manuscript; FD, EO, TI, AG, GG, MF, DF, EA, ET, LDP, GR, EB, IC, MT, AMS, DM, PC, LC, FC, MB, ET, AG, BF, RB performed research and collected data; genetic studies were performed by RC, KB, LE, PZ, AM; SS and OS performed and supervised genetic studies, collaborated on data interpretation, revised the manuscript and approved the final version; CP analyzed and interpreted data, performed statistical analysis and wrote the manuscript. All authors revised the manuscript and approved the final version before submission.

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