Favorable outcome of patients with acute myeloid leukemia harboring a low-allelic burden \textit{FLT3-ITD} mutation and concomitant \textit{NPM1} mutation: relevance to post-remission therapy

Marta Pratcorona\(^1\), Salut Brunet\(^2\), Josep Nomdedéu\(^2\), Josep Maria Ribera\(^3\), Mar Tormo\(^4\), Rafael Duarte\(^5\), Lourdes Escoda\(^6\), Ramon Guàrdia\(^7\), M\(^\text{a}\) Paz Queipo de Llano\(^8\), Olga Salamero\(^9\), Joan Bargay\(^{10}\), Carmen Pedro\(^{11}\), Josep Maria Martí\(^{12}\),Montserrat Torrebadell\(^1\), Marina Díaz-Beyá\(^1\), Mireia Camós\(^{13}\), Dolors Colomer\(^1\), Montserrat Hoyos\(^2\), Jorge Sierra\(^2\), Jordi Esteve\(^1\).

Hematology Departments of:
\(^1\)Hospital Clínic, IDIBAPS, Barcelona;
\(^2\)Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona;
\(^3\)ICO-Hospital Germans Trias i Pujol, Badalona;
\(^4\)Hospital Clínico, Valencia;
\(^5\)Hospital Duran i Reynals, L’Hospitalet de Llobregat;
\(^6\)Hospital Joan XXIII, Tarragona;
\(^7\)Hospital Josep Trueta, Girona;
\(^8\)Hospital Virgen de la Victoria, Málaga;
\(^9\)Hospital Vall d’Hebron, Barcelona;
\(^10\)Hospital de Son Llàtzer, Palma de Mallorca;
\(^11\)Hospital del Mar, Barcelona;
\(^12\)Hospital Mútua de Terrassa;
\(^13\)Hospital Sant Joan de Déu, Esplugues de Llobregat;
on behalf of the Cooperative Group CETLAM, Spain.

Running title: Outcome of NPM1mut AML with low FLT3-ITD ratio

Key words: AML, prognosis, FLT3-ITD allelic burden, NPM1, post-remission therapy
**Corresponding author:** Jordi Esteve, MD, Hematology Department, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain. Phone: +34932275428. Fax: +34932275484. E-mail: jesteve@clinic.ub.es
Key points:

- In intermediate risk AML, effect of FLT3 burden is modulated by NPM1 mutation, especially in patients with a low ratio.
- Combined evaluation of NPM1 mutation and FLT3-ITD burden might contribute to identify patients who benefit from early allogeneic HSCT.

Abstract

Risk associated to FLT3 internal tandem duplication (FLT3-ITD) in patients with acute myeloid leukemia (AML) may depend on mutational burden and its interaction with other mutations. We analyzed the effect of FLT3-ITD allelic burden in 303 patients with de novo intermediate-risk cytogenetics AML treated in CETLAM trials. Outcome of patients was analyzed according to FLT3-ITD/FLT3 wild-type (FLT3wt) ratio and NPM1 mutation (NPM1mut). Among NPM1mut patients, FLT3wt and low ratio (<0.5) subgroups showed similar overall survival, relapse risk, and leukemia-free survival, whereas high ratio (≥0.5) patients had a worse outcome. In NPM1wt AML, FLT3-ITD subgroups showed a comparable outcome, with higher RR and shortened OS than FLT3wt patients. Allogeneic stem cell transplantation in CR1 was associated with a reduced relapse risk in all molecular subgroups with the exception of NPM1mut AML with absent or low ratio FLT3-ITD. In conclusion, effect of FLT3 burden is modulated by NPM1 mutation, especially in patients with a low ratio.
Introduction

Presence of *FLT3*-ITD is associated with an increased risk of relapse (RR) and inferior overall survival (OS) in patients with normal karyotype AML, arising as one of the main prognostic factors in this group of patients\(^1\)-\(^4\). However, the risk conferred by this mutation has been related to specific characteristics, such as the allelic burden, length of the mutation or specific sequence\(^4\),\(^5\),\(^7\). Thus, the proportion of the mutant allele among leukemic population is considered one of the most important features modulating the prognostic impact of the mutation\(^6\),\(^7\). Nonetheless, the resulting effect of the *FLT3*-ITD is not only a consequence of intrinsic characteristics of the mutation but can also depend on the interaction with other mutations, such as *WT1* or *DNMT3A*, which seem to add an adverse effect in patients with *FLT3*-ITD AML\(^8\)-\(^10\). Moreover, *FLT3*-ITD, considered as a secondary mutation involving signaling cell pathways which confer a proliferation advantage to the leukemic clone (type I mutation), is frequently observed in patients harboring *NPM1* mutations, associated to a favorable prognosis\(^11\)-\(^14\). Therefore, the effect of *FLT3*-ITD burden might depend on the presence or absence of an underlying *NPM1* mutation, as recently suggested\(^15\)-\(^17\). To test this hypothesis we analyzed a large series of patients with a long follow-up from the Spanish cooperative group CETLAM.
Patients and methods

Patients and treatment

Three hundred and three patients up to 60 years old diagnosed with de novo AML of intermediate-risk cytogenetics according to the MRC classification\textsuperscript{18} and with available material at diagnosis were included. Patients were treated according to CETLAM trials since 1994 (AML-94, n=15; AML-99, n=91; and AML-03, n=197) Details of treatment received are provided in Supplementary Table 1.

Analysis of $NPM1$, $FLT3$-ITD, and $FLT3$-ITD/wt allelic burden

All samples were obtained at diagnosis after written informed consent in accordance with the Declaration of Helsinki. All the experiments were approved by the Ethics Committee of each institution. Detection of $NPM1$ and $FLT3$-ITD mutations was performed on genomic DNA (gDNA) as previously described\textsuperscript{4,19} with labeled primers and analyzed by fragment analysis (3130XL Genetic Analyzer, Applied Biosystems). $FLT3$-ITD/wt allelic burden was calculated as the ratio of the area under the curve of mutant and wild-type alleles ($FLT3$-ITD/$FLT3$wt). In cases with more than one mutation, all $FLT3$-ITD were summed-up.
Statistical methods

Characteristics between groups were compared using the $\chi^2$ test and Fisher exact test for categorical variables, and Mann-Whitney test for continuous variables. Median value of continuous variables was used to dichotomize them for prognostic analyses. OS was calculated from diagnosis to death whereas leukemia-free survival (LFS) was calculated from complete response (CR) to death or relapse; both functions were estimated with the method of Kaplan and Meier. RR was estimated using the cumulative incidence (CI) method, defining relapse as main event and death without relapse as a competitive event. For calculating RR, patients who received an allogeneic stem cell transplantation (alloHSCT) in CR1 were censored at date of transplantation. Comparisons of OS and LFS between groups were performed with log-rank test, whereas Gray statistic was used to calculate RR$^{20}$. Multivariate analysis for survival was performed using the Cox proportional hazards model. Variables analyzed in univariate and multivariate analyses for all outcomes included gender and age, haematological parameters at diagnosis (white blood cell count (WBC), platelet, percentage of bone marrow (BM) blasts), and main cytogenetic and molecular features ($NPM1$ and $FLT3$-ITD mutational status). Statistical analyses were performed using software packages SPSS version 18.0 and Windows 95/NT (SPSS Inc Chicago, IL), except RR analysis and univariate and multivariate analyses using time-dependent covariates, which were performed with R version 2.13.1.
Results and discussion

**Characteristics of patients**

Main characteristics of patients are summarized in Supplementary Table 2. *FLT3*-ITD and *NPM1* mutations were determined in 303 patients and were detected in 94 (31%) and 161 (53%) patients respectively, with a significant association between them (65 patients harbored both mutations, \( p < 0.001 \)). Patients with *FLT3*-ITD, compared to *FLT3*wt, presented with higher WBC and BM blast infiltration, lower platelet count, and normal karyotype in a higher proportion of cases. Median value of *FLT3*-ITD/wt ratio was 0.61 (range: 0.033-7.515) without differences between *NPM1*wt and *NPM1*mut groups (0.59 vs 0.652; \( p = 0.4 \)). Of note, WBC at diagnosis increased progressively in patients without *FLT3*-ITD, with a *FLT3*-ITD/wt ratio between 0-0.5, and with a *FLT3*-ITD/wt ratio>0.5 (11.5x10^9/L vs. 39.7x10^9/L, \( p = 0.002 \); and 39.7x10^9/L vs. 90x10^9/L, \( p = 0.01 \), respectively).

**Outcome and prognostic factors**

Overall, CR rate was 85%, and OS and LFS at 5-yr were 43±3% and 46±3%. The only factor predictive of CR achievement was a lower WBC at diagnosis (91% vs 79%; \( p = 0.004 \)). Univariate and multivariate prognostic studies are summarized in Table 1 and Supplementary Table 3. *FLT3*-ITD/wt ratio, analyzed as a continuous variable, showed prognostic value for all endpoints. To further confirm the prognostic value of *FLT3*-ITD/wt ratio, we subdivided our
cohort in three groups: FLT3wt, FLT3-ITD/wt ratio<0.5 (low ratio), and FLT3-ITD/wt ratio≥0.5 (high ratio). Different thresholds of FLT3-ITD/wt ratio have been proposed, although heterogeneity in methods of determination and therapy administered among studies makes their comparison difficult, and probably reflects a continuous effect of FLT3-ITD/wt ratio. Our choice of 0.5 as the cut-off value was based on the maximum clinical prognostic impact derived from this threshold. In the overall series, this cut-off level resulted in a striking different RR in patients with FLT3-ITD, with a CI of relapse of 49±10% and 82±7% in the low and high ratio group, respectively (p=0.0075). These differences, however, did not translate in different OS, probably due to the effect of alloHSCT.

When the analysis was restricted to NPM1mut AML, patients with FLT3wt and low ratio showed similar RR, OS, and LFS (38±6% vs 20±9%, p=NS; 56±5% vs 47±10%, p=NS; 56±6% vs 53±11%, p=NS; respectively). On the contrary, patients with a high ratio compared to low ratio and FLT3wt patients showed an increased RR (79±6% vs. 20±9% vs 38±9% at 5-yr, p=0.000054), and shorter OS and LFS (5-yr OS: 29±7% vs 47±10% vs 56±5%, p=0.017; 5-yr LFS: 32±8% vs 53±11 vs. 56±6%, p=0.025). In contrast, in the NPM1wt cohort, patients with low ratio showed higher RR and shorter OS compared to those lacking FLT3-ITD (5-yr RR: 74±20% vs. 48±6%, p=0.017; 5-yr OS: 20±12% vs. 39±6%; p=0.014) (Figure 1).

We analyzed the potential benefit of alloHSCT in CR1 according to molecular features. First, to avoid a negative bias for patients allocated in the non-alloHSCT arm, we restricted the analysis to patients with a minimum CR duration of three months. In the NPM1mut group, high ratio patients
experienced a significant reduction of relapse after alloHSCT (5-yr RR: 20±13% vs. 80±9%, respectively, p=0.014), which resulted in a longer OS (22±10% vs. 70±14% at 5-yr, p=0.03). On the contrary, no differences in outcome according to post-remission therapy were observed between patients with FLT3wt (5-yr OS, RR, and LFS in patients without an allograft and those undergoing alloHSCT in CR1: 64±7% vs. 79±11%, p=0.296; 35±7% vs. 20±11%, p=0.49; and 58±7% vs. 73±11%, p=0.44, respectively) or low ratio FLT3-ITD/wt (5-yr OS, RR, LFS, and RR: 67±16% vs. 56±17%, p=0.873; 22±15% vs 19±20%, p=0.566; and 67±16% vs. 56±17%, p=0.685, respectively). In the NPM1wt subgroup, alloHSCT in CR1 was associated with a significant reduction of RR in patients with FLT3-ITD (5-year CI: 42±14% vs.100%, p=0.00016), and showed a similar trend in the FLT3wt subgroup (5-yr CI: 25±10% vs. 45±7%, p=0.08), resulting in a better LFS of the entire NPM1wt cohort (5-year LFS: 35±7% vs. 62±8%, p=0.032). The effect of alloHSCT in CR1 was also analyzed as a time-dependent variable in molecular risk categories defined following previous results: a low-risk category comprising NPM1mut patients with FLT3wt or low ratio, and a high-risk category constituted by patients with NPM1wt and/or high ratio FLT3-ITD. Thus, whereas alloHSCT did not modify the outcome of patients within the low-risk category, high-risk patients had a longer OS (HR=0.51, 95% CI: 0.29-0.88, p=0.01) and LFS (HR=0.48, 95% CI: 0.29-0.79, p=0.004)(Supplemental material Figure 1).

Prognostic relevance of FLT3-ITD/wt allelic burden might be based on biological grounds: highest FLT3-ITD/wt ratios correspond to a homozygous state, usually due to a process of uniparental disomy, while FLT3-ITD with a low ratio might have arisen in a minor subclone, perhaps occurring at a later stage.
of the leukemogenic process\textsuperscript{21}. Thus, the prognosis of these latter patients might not be determined as much by constitutional activation of $\text{FLT3}$ pathway as by other relevant leukemogenic mutations, like $\text{NPM1}$ mutation. This prognostic interaction between $\text{NPM1}$ mutational status and $\text{FLT3}$-ITD/wt ratio, confirmed in our series, has only been identified in two previous studies\textsuperscript{15,16}, although $\text{FLT3}$-ITD/wt ratio in these two studies was determined on cDNA instead of usual determination on gDNA.

Despite some controversial results, a general recommendation to perform an early alloHSCT in younger AML patients with $\text{FLT3}$-ITD AML has been established\textsuperscript{6,14,22-25}. Our finding of a subset of patients with $\text{NPM1}$ mutation and low ratio $\text{FLT3}$-ITD/wt with a low RR, even in patients not submitted to alloHSCT, would prompt to refine this general recommendation and consider their inclusion in a category of AML with favorable genotype. Nonetheless, this observation warrants confirmation in prospective studies, given its potential clinical relevance in the design of post-remission therapeutic strategies.
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Author contributions

The study was designed by MP and JE. Laboratory and data analysis was performed by MP, JN, MC, MT, DC, and JE. Clinical samples and data were provided by SB, JN, JMR, MT, MDB, RD, LE, RG, MPQL, OS, JB, CP, JMM, MH, JS and JE. The manuscript was drafted by MP and JE; all authors contributed to the final version.

Conflict-of-interest disclosure

The authors declare to have no competing financial interests.
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Table 1. Summary of prognostic factors for relapse incidence, survival and leukemia-free survival identified in the multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>OS</th>
<th>RR</th>
<th>LFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>CI (95%)</td>
<td>p</td>
</tr>
<tr>
<td>Age*</td>
<td>1.771</td>
<td>1.287-2.436</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBC#</td>
<td>1.429</td>
<td>1.021-2.0</td>
<td>0.038</td>
</tr>
<tr>
<td>FLT3 (ITD vs. wt)</td>
<td>1.854</td>
<td>1.315-2.614</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NPM1 (mut vs. wt)</td>
<td>0.676</td>
<td>0.484-0.944</td>
<td>0.022</td>
</tr>
<tr>
<td>NPM1mut/FLT3wt§</td>
<td>0.601</td>
<td>0.422-0.858</td>
<td>0.005</td>
</tr>
<tr>
<td>FLT3-ITD ratio¥</td>
<td>1.274</td>
<td>1.124-1.444</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

All variables analyzed for all reported outcomes (response to induction, OS, RR, and LFS) are described in the Statistical Methods section.
*below and above the median value, 47 year-old; #below and above the median, 19.9x10^9/L; § NPM1mut/FLT3wt genotype vs all other possibilities; ¥considered as a continuous variable
**Legend for figure**

**Figure 1.** Different prognostic value of *FLT3*-ITD allelic burden according to underlying *NPM1* mutational status. Thus, in patients with mutated *NPM1*, a similar survival (A) and relapse risk (B) were observed between patients without *FLT3*-ITD and low ratio *FLT3*-ITD/wt (<0.5), whereas patients with a high *FLT3*-ITD/wt ratio showed a worse prognosis. In contrast, among wild-type *NPM1* AML patients, the presence of *FLT3*-ITD was associated with a worse outcome, compared to those without *FLT3*-ITD, regardless *FLT3*-ITD/wt mutational load, both in terms of survival (C) and relapse risk (D).
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