# Prognostic impact of white blood cell count in intermediate risk acute myeloid leukemia: relevance of mutated *NPM1* and *FLT3*-ITD

Hendrik J.M. de Jonge,[1]* Peter J.M. Valk,[2]* Eveline S.J.M. de Bont,[1] Jan Jacob Schuringa,[3] Gert Ossenkoppele,[4] Edo Vellenga,[3] and Gerwin Huls[3]

[1]Division of Pediatric Oncology/Hematology, Department of Pediatrics, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, the Netherlands, [2]Department of Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands, [3]Department of Hematology, University Medical Center Groningen, Groningen, the Netherlands, and [4]Department of Hematology, VU University Medical Center, Amsterdam, the Netherlands

## ABSTRACT

### Background
High white blood cell count at presentation is an unfavorable prognostic factor for treatment outcome in intermediate cytogenetic risk acute myeloid leukemia. Since the impact of white blood cell count on outcome of subgroups defined by the molecular markers *NPMc*$^+$ and *FLT3*-internal tandem duplication (ITD) is unknown, we addressed this issue.

### Design and Methods
We studied the effect of white blood cell count on outcome in a clinically and molecularly well-defined cohort of 525 patients with acute myeloid leukemia using these molecular markers. In addition, since an increased white blood cell count has been associated with an increased *FLT3*-ITD/*FLT3* (wild-type) ratio, we investigated whether the effect of white blood cell count on outcome could be explained by the *FLT3*-ITD/*FLT3* ratio.

### Results
This analysis revealed that white blood cell count had no impact on outcome in patients with the genotypic combinations '*NPMc*$^+$ without *FLT3*-ITD' and '*NPM1* wild-type with or without *FLT3*-ITD'. In contrast, white blood cell count had a significant impact on complete remission rate ($P$=0.034), event-free survival ($P$=0.009) and overall survival ($P$<0.001) in patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'. A *FLT3*-ITD/*FLT3* ratio greater than 1 was also associated with a reduced complete remission rate ($P$=0.066) and significantly reduced event-free survival ($P$= 0.001) and overall survival ($P$=0.001) in patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'. Multivariable analysis revealed that white blood cell count and *FLT3*-ITD/*FLT3* ratio were independent prognostic indicators for outcome in the subgroup with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'.

### Conclusions
Our results demonstrate that both high white blood cell count and *FLT3*-ITD/*FLT3* ratio are prognostic factors in patients with acute myeloid leukemia with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'.

Key words: acute myeloid leukemia, prognosis, white blood cell count, NPM1, *FLT3*-ITD.

*Citation: de Jonge HJM, Valk PJM, de Bont ESJM, Schuringa JJ, Ossenkoppele G, Vellenga E, and Huls G. Prognostic impact of white blood cell count in intermediate risk acute myeloid leukemia: relevance of mutated* NPM1 *and* FLT3*-ITD. Haematologica 2011;96(9):1310-1317. doi:10.3324/haematol.2011.040592*



**HJMdJ and PJMV contributed equally to this manuscript.*

*Manuscript received on January 12, 2011. Revised version arrived on May 12, 2011. Manuscript accepted on May 16, 2011.*

*Correspondence:*
*Gerwin Huls,*
*Department of Hematology, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.*
*E-mail: g.huls@int.umcg.nl*

*The online version of this article has a Supplementary Appendix.*

## Introduction

Several prognostic factors related to patients' and disease characteristics have been described for acute myeloid leukemia (AML).[1,2] The karyotype at diagnosis is a powerful prognostic factor for treatment outcome in patients with AML.[3-7] Various recurrent somatically acquired molecular abnormalities have been identified during the last years.[8] Of these molecular abnormalities, mutations in nucleophosmin (*NPM1*) and internal tandem duplications of the fms-related tyrosine kinase 3 gene (*FLT3*-ITD) have strong prognostic impact.

Mutations in *NPM1* are the most frequently observed molecular abnormalities, present in about 50% of AML cases, and are associated with a favorable outcome.[9-13] AML with mutated *NPM1* (*NPMc*$^+$) shows distinctive biological and clinical features, and is, therefore, a provisional entity in the World Health Organization (WHO) 2008 classification of leukemias. *FLT3*-ITD is another frequent molecular abnormality and can be observed in about 20-25% of patients with AML.[14-18] Clinically, AML patients harboring *FLT3*-ITD frequently have high white blood cell (WBC) counts at presentation.[14-18] The presence of *FLT3*-ITD is generally considered as an unfavorable prognostic factor.[14-19] In particular, those cases with a *FLT3*-ITD mutation/*FLT3* wild-type ratio (hereafter referred to as the *FLT3*-ITD/*FLT3* ratio) above the median value have a dismal prognosis.[17-19] A high *FLT3*-ITD/*FLT3* ratio points to the absence of the FLT3 wild-type allele.

Mutations in *NPM1* and *FLT3*-ITD are frequently associated. Approximately 40% of patients with *NPM1* mutations also carry *FLT3*-ITD.[9] Various studies have shown that the genotypic combination '*NPMc*$^+$ without *FLT3*-ITD' represents a subgroup with favorable prognosis.[9-13] Nevertheless, the beneficial impact of *NPMc*$^+$ on prognosis was seen in patients with as well as those without *FLT3*-ITD and it appeared that both mutations in *NPM1* and *FLT3*-ITD were significant independent predictors of outcome.[19]

Besides cytogenetic and molecular abnormalities, classically, a high WBC count at presentation is considered to be an independent prognostic factor for poor outcome in both adults and children with AML.[1,20-23] The effect of WBC count at diagnosis is most apparent in AML with favorable cytogenetic risk abnormalities, such as t(8;21) and t(15;17).[24-25] However, the prognostic effect of WBC count is also present in AML patients with intermediate cytogenetic risk abnormalities.[20-23] Multivariable analysis has shown that both WBC count and the genotypic subgroup *NPMc*$^+$ without *FLT3*-ITD are independent predictors of outcome.[11] Nevertheless, the effect of WBC count at diagnosis on outcome of patients within the four subgroups defined by the molecular markers *NPMc*$^+$ and *FLT3*-ITD (within the intermediate cytogenetic risk group) is unknown.

The aim of the present survey was to investigate the prognostic impact of WBC count at diagnosis on outcome within AML subgroups defined by *NPMc*$^+$ and *FLT3*-ITD or the *FLT3*-ITD/*FLT3* ratio. Therefore, within a clinically and molecularly well characterized cohort of 525 patients with *de novo* AML, we compared treatment outcome among patients divided into three groups on the basis of their WBC counts: less than $20\times10^9$/L, 20 to $100\times10^9$/L, and above $100\times10^9$/L.

## Design and Methods

### Patients

The study cohort consisted of 525 consecutive adult patients with AML who were treated according to the sequential HOVON/SAKK AML-04, -04A, -29, -32, -42, -43 protocols (available at *http://www.hovon.nl*) and for whom molecular data on *NPMc*$^+$ and *FLT3*-ITD status were available.[26-29] All patients in this study were newly diagnosed with AML and the diagnosis was established according to WHO criteria. Cell specimens were collected at the time of diagnosis. All patients provided written informed consent in accordance with the Declaration of Helsinki, and the study was approved by all participating institutional review boards. Patients were divided into cytogenetic risk groups (favorable, intermediate, or unfavorable) in accordance with HOVON/SAKK criteria (Table 1). Cytogenetic risk was defined as favorable in patients with t(8;21)(q22;q22), inv(16)(p13.1;q22), or t(16;16)(p13.1;q22) and t(15;17), and unfavorable in patients with complex cytogenetic abnormalities (i.e. three or more distinct clonal abnormalities), -7, -5, del 5q or del 7q, abnormalities of the long arm of chromosome 3 (abn 3q), t(6;9)(q23;q34), t(9;22)(q34;q11), or abnormalities of the long arm of chromosome 11 (abn 11q23). All other cytogenetic abnormalities and AML without cytogenetic abnormalities were considered to indicate an intermediate cytogenetic risk. The median overall survival of the whole cohort was 16.3 months, and the median follow-up of survivors was 61.4 months.

Guided by thresholds of WBC counts which are often clinically used for risk stratification, the 525 patients were divided into three groups: those with a WBC count below $20\times10^9$/L (n=221), those with a WBC count between 20 and $100\times10^9$/L (n=205) and those with a WBC above $100\times10^9$/L (n=99). In the current HOVON/SAKK AML study (HOVON102) the following classifications are also used; patients with t(8;21) and WBC greater than $20\times10^9$/L are considered at intermediate risk (instead of favorable risk) and patients with a normal karyotype (or only loss of sex chromosomes) and a WBC greater than $100\times10^9$/L are considered at unfavorable risk (instead of intermediate risk).

Within our cohort 82 patients had the molecular combination *NPMc*$^+$ with *FLT3*-ITD. Six patients of this subgroup had unclassified cytogenetics, and one patient had 5(q)/-7(q) cytogenetics. Therefore, 75 patients with both confirmed intermediate-risk cytogenetics and the genotypic markers *NPMc*$^+$ with *FLT3*-ITD were studied.

### FLT3-*ITD*/FLT3-*wildtype ratio*

Amplification of the *FLT3*-ITD mutations was performed using primers 11F and 11R, as described by Nakao *et al.*[30] Ratios were determined after agarose gel electrophoresis of the quantitative polymerase chain reaction products.

### Statistical analyses

Statistical analyses were performed with SPSS software, release 16.0. Actuarial probabilities of overall survival (with death due to any cause) as well as event-free survival (with failure in case of no complete remission or relapse or death) were estimated according to the Kaplan–Meier method. For quantitative parameters overall differences between the cohorts were evaluated using an F-test (or Student's-t test in the case of two groups) for normally distributed variables or a Kruskal-Wallis test (or Mann-Whitney-U test in the case of two groups) for variables with a skewed distribution. For qualitative parameters, overall group differences were evaluated using a $\chi^2$ test. Cox regression analysis was applied to determine the association of WBC and overall and event-free survivals with

adjustment for possible confounding factors such as age at diagnosis, cytogenetic risk profile (i.e. favorable, intermediate or unfavorable), *FLT3*-ITD, *NPMc*$^+$ and the transcription factor *CCAAT/enhancer binding protein* α (*CEBPA*). All tests were two-tailed, and a *P* value of less than 0.05 was considered statistically significant.

## Results

### Patients

The clinicopathological, demographic, and molecular data of the 525 patients with AML in the study cohort are presented in Table 1. Guided by thresholds of WBC counts which are often clinically used for risk stratification, the 525 patients were divided into three groups: those with a WBC count below 20×10$^9$/L (n=221), those with a WBC count between 20 and 100×10$^9$/L (n=205) and those with a WBC above 100×10$^9$/L (n=99). The frequency of patients with a WBC count greater than 100×10$^9$/L (often designated as hyperleukocytosis) appeared to be higher in the group of AML patients with intermediate cytogenetic risk abnormalities (21%) than in the group with unfavorable cytogenetic risk abnormalities (9%). Within this study cohort, 49 of 143 (34%) patients with *FLT3*-ITD and 45 of 159 (28%) patients with *NPMc*$^+$ presented with hyperleukocytosis (WBC >100×10$^9$/L) at diagnosis.

### Impact of white blood cell count on complete remission rate, event-free survival and overall survival

When considering all 525 patients, hyperleukocytosis was significantly associated with a lower complete remission rate (67% *versus* 84%; *P*<0.001), shorter event-free survival (median 6.8 months *versus* 11.6 months; *P*=0.001) and shorter overall survival (median 8.9 months *versus* 17.2 months; *P*=0.002) (*Online Supplementary Figure S1A,B*). Furthermore, within the subgroup of patients with cytogenetically intermediate-risk AML, hyperleukocytosis was

**Table 1.** Patients' characteristics.

| Characteristics | All patients | WBC <20x10$^9$/L | WBC 20-100x10$^9$/L | WBC >100x10$^9$/L |
|---|---|---|---|---|
| N. of patients | 525 | 221 | 205 | 99 |
| Median age, years (range) | 47 (15-77) | 46 (16-76) | 48 (15-77) | 46 (16-74) |
| Median white blood cell count ×10$^9$/L (range) | 26 (0.3-510) | 5.3 (0.3-19.7) | 43 (20-99.5) | 135 (101.6-510) |
| Median blast percentage (range) | 65 (1-99) | 58 (1-99) | 67 (2-99) | 79 (3-97) |
| Median platelet count ×10$^9$/L (range) | 56 (3-998) | 62 (3-998) | 54 (5-931) | 51 (13-687) |
| Cytogenetics | | | | |
| *Favorable* | *89 (17%)* | *37 (17%)* | *38 (19%)* | *14 (14%)* |
| t(8;21) | 34 | 22 | 11 | 1 |
| t(15;17) | 20 | 11 | 8 | 1 |
| inv16 | 35 | 4 | 19 | 12 |
| *Intermediate* | *331 (63%)* | *129 (58%)* | *133 (65%)* | *69 (70%)* |
| normal karyotype | 218 | 74 | 91 | 53 |
| + 8 | 25 | 13 | 10 | 2 |
| -9q | 7 | 3 | 4 | 0 |
| other | 81 | 39 | 28 | 14 |
| *Unfavorable* | *85 (16%)* | *50 (23%)* | *27 (13%)* | *8 (8%)* |
| 11q23 | 11 | 3 | 5 | 3 |
| complex | 20 | 13 | 5 | 2 |
| -5(q)/-7(q) | 42 | 29 | 10 | 3 |
| other | 12 | 5 | 7 | 0 |
| *Not available* | *20 (4%)* | *5 (2%)* | *7 (3%)* | *8 (8%)* |
| Molecular genotype | | | | |
| *NPMc*$^+$ without *FLT3*-ITD | 77 (15%) | 25 (11%) | 38 (18%) | 14 (14%) |
| *NPMc*$^+$ with *FLT3*-ITD | 82 (15%) | 17 (8%) | 34 (17%) | 31(31%) |
| *NPM1* wild type without *FLT3*-ITD | 305 (58%) | 164 (74%) | 105 (51%) | 36 (37%) |
| *NPM1* wild type with *FLT3*-ITD | 61 (12%) | 15 (7%) | 28 (14%) | 18 (18%) |
| *CEBPA* wild type *versus* mutant[$] | 486/38 | 203/17 | 190/15 | 93/6 |
| Allogeneic stem cell transplantation | 140# | 64 | 57 | 19 |
| Autologous stem cell transplantation | 68 | 22 | 33 | 13 |
| Cycles to complete remission, n (%) | | | | |
| 1 | 297 (57%) | 126 (57%) | 124 (61%) | 47 (48%) |
| 2 | 111 (21%) | 52 (24%) | 44 (21%) | 15 (15%) |
| 3 | 8 (2%) | 3 (1%) | 2 (1%) | 3 (3%) |
| >3 | 5 (1%) | 0 (0%) | 4 (2%) | 1 (1%) |
| no complete remission | 104 (20%) | 40 (18%) | 31 (15%) | 33 (33%) |
| Relapse, n (%) | 202 (39%) | 81 (40%) | 84 (41%) | 37 (37%) |
| Death, n (%) | 316 (60%) | 124 (56%) | 123 (60%) | 69 (70%) |

NPM1*: nucleophosmin 1;* FLT3*: fms-related tyrosine kinase 3; ITD: internal tandem duplication;* NPMc$^+$*, mutated nucleophosmin 1; CEBPA, CCAAT/enhancer binding protein* α*; Cytogenetic risk group classification (favorable, intermediate, and unfavorable) is described in the Design and Methods section.* [$]CEBPA *status is unknown for one patient.* # *indicates 12/140 patients who underwent allogeneic stem cell transplantation after non-myeloablative conditioning.*

significantly associated with a lower complete remission rate ($P$<0.001), shorter event-free survival (median 7.3 months *versus* 13.2 months; $P$=0.009) and shorter overall survival (median 9.2 months *versus* 19.1 months; $P$=0.001) (*Online Supplementary Figure S1C,D*). As expected, in the subgroup of patients with favorable cytogenetic risk, a WBC count below 20×10$^9$/L appeared to be significantly associated with a higher complete remission rate ($P$=0.024), improved event-free survival (median 77.2 months *versus* 9.7 months) and improved overall survival (median 85.5 months *versus* 28.9 months; $P$=0.001) (*Online Supplementary Figure S1E,F*). Finally, in the subgroup of patients with cytogenetically unfavorable risk AML, WBC count did not affect the complete remission rate ($P$=0.593), event-free survival ($P$=0.717) or overall survival ($P$=0.672) (*Online Supplementary Figure S1G,H*).

### Prognostic value of white blood cell count in the context of additional known risk factors for the whole group of patients with acute myeloid leukemia

The impact of hyperleukocytosis on event-free and overall survival in all 525 patients with AML was confirmed in univariate analysis. Age at diagnosis, *NPMc*$^+$, *FLT3*-ITD, mutated *CEBPA* and cytogenetic risk group (*i.e.* unfavorable, intermediate and favorable risk) also correlated with event-free and overall survival (*data not shown*). When we subsequently considered these variables in a multivariable analysis, hyperleukocytosis maintained its independent prognostic value for both event-free survival (HR: 1.56, 95% CI: 1.16-2.11; $P$=0.004) and overall survival (HR: 1.67, 95% CI: 1.21-2.30; $P$=0.002) (Table 2).

### Prognostic value of white blood cell count in the context of the molecular markers NPMc$^+$ and FLT3-*ITD* within the intermediate cytogenetic risk group

The impact of WBC count on complete remission rate, event-free survival and overall survival was evident in the intermediate cytogenetic risk group, which contained 63% of the patients of this study cohort. Multivariate analysis established that hyperleukocytosis, *NPMc*$^+$ and *FLT3*-ITD were independent predictors for event-free survival as well as overall survival (WBC count >100×10$^9$/L: HR: 1.39, 95% CI: 1.01-1.92, $P$=0.042 for event-free survival: HR: 1.59, 95% CI: 1.14-2.21, $P$=0.006 for overall survival; *FLT3*-ITD: HR: 1.58, 95% CI: 1.18-2.13, $P$=0.002 for event-free survival: HR: 1.80, 95% CI: 1.32-2.45, $P$<0.001 for overall survival; *NPMc*$^+$: HR: 0.61, 95% CI: 0.46-0.82, $P$=0.001 for event-free survival: HR: 0.59, 95% CI: 0.43-0.80, $P$=0.001 for overall survival). Furthermore, there was a significant positive interaction between *NPMc*$^+$/*FLT3*-ITD and WBC in the multivariable model both for event-free and overall survival ($P$=0.018 and $P$=0.002, respectively).

Since we aimed to investigate the impact of WBC count on outcome within the four subgroups defined by the molecular markers *NPMc*$^+$ and *FLT3*-ITD (within the intermediate cytogenetic risk group), we subsequently focused on these subgroups. In the most favorable genotypic subgroup, '*NPMc*$^+$ without *FLT3*-ITD', WBC count did not significantly affect complete remission rate ($P$=0.451), event-free survival ($P$=0.932) or overall survival ($P$=0.400) (Figure 1A,B). Furthermore, also when WBC count was analyzed as a continuous variable, no significant association was found between WBC count and event-free and overall survival in the subgroup '*NPMc*$^+$ without *FLT3*-ITD' (*data not shown*). Interestingly, however, in the subgroup with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' WBC count did have a significant impact on complete remission rate ($P$=0.034), event-free survival ($P$=0.009) and overall survival ($P$<0.001) (Figure 1C,D). Within this particular subgroup, it appeared that patients with a WBC count less than 20×10$^9$/L or between 20-100×10$^9$/L had a relatively favorable prognosis with a median overall survival of 21 and 15 months, respectively, and estimated 5-year overall survival rates of 50% and 51%, respectively. In contrast, patients with a WBC greater than 100×10$^9$/L evidently had a poor prognosis with a median overall survival of 7 months and an estimated 5-year overall survival rate of 9%. So, based on WBC count (i.e. <100 *versus* >100×10$^9$/L) patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' could be divided into two groups, one with a relatively good prognosis, the other with a very poor prognosis. These results were underscored by univariate Cox regression analyses using WBC count as a continuous variable for event-free survival (HR: 1.006; 95% CI: 1.002-1.010, $P$=0.001) and for overall survival (HR: 1.009; 95% CI: 1.005-1.013,

**Table 2. Multivariable analysis of WBC count as a prognostic marker for event-free survival and overall survival in all 525 patients.**

| Variable | Event-free survival HR (95% CI) | P | Overall survival HR (95% CI) | P |
|---|---|---|---|---|
| WBC 20-100×10$^9$/L[$] | 1.09 (0.85-1.39) | 0.493 | 1.17 (0.90-1.53) | 0.233 |
| WBC >100×10$^9$/L[¶] | 1.56 (1.16-2.11) | 0.004 | 1.67 (1.21-2.30) | 0.002 |
| Intermediate* | 1.73 (1.22-2.46) | 0.002 | 2.18 (1.46-3.26) | <0.001 |
| Poor* | 3.26 (2.22-4.78) | <0.001 | 4.06 (2.64-6.25) | <0.001 |
| Age, decades | 1.08 (1.00-1.17) | 0.070 | 1.14 (1.05-1.24) | 0.003 |
| *FLT3*-ITD[#] | 1.55 (1.19-2.01) | 0.001 | 1.72 (1.31-2.27) | <0.001 |
| *NPMc*$^+$[$] | 0.61 (0.46-0.80) | <0.001 | 0.58 (0.43-0.77) | <0.001 |
| *CEBPA* mutation[^] | 0.57 (0.36-0.89) | 0.015 | 0.50 (0.30-0.83) | 0.007 |

*HR: hazard ratio; CI: confidence interval; intermediate refers to intermediate cytogenetic risk and poor refers to poor cytogenetic risk as defined in the Design and Methods section; WBC: white blood cell count;* FLT3*: fms-related tyrosine kinase 3; ITD,: internal tandem duplication;* NPMc$^+$*, mutated nucleophosmin 1;* CEBPA*, CCAAT/enhancer binding protein α. [$]WBC 20-100×10$^9$/L* versus *WBC <20×10$^9$/L. [¶]WBC >100×10$^9$/L* versus *WBC <20x10$^9$/L. *Cytogenetic risk* versus *favorable cytogenetic risk. [#]*FLT3*-ITD* versus *no* FLT3*-ITD. [$]*NPMc$^+$ versus *no NPMc+. [^]*CEBPA *mutation* versus *no* CEBPA *mutation.*

**Table 3. WBC count and *FLT3*-ITD/*FLT3* ratio in the 75 patients with the genotypic combination '*NPMC*$^+$ with *FLT3*-ITD'**

**A**

| White blood cell count | FLT3 <1 | ITD / FLT3 1 | ratio (n.) >1 |
|---|---|---|---|
| <20×10$^9$/L | 4 | 3 | 9 |
| 20-100×10$^9$/L | 4 | 13 | 16 |
| >100×10$^9$/L | 1 | 12 | 13 |

**B**

| Variable | Event-free survival HR (95% CI) | P | Overall survival HR (95% CI) | P |
|---|---|---|---|---|
| WBC >100×10$^9$/L[¶] | 2.29 (1.30-4.03) | 0.004 | 3.30 (1.83-5.97) | <0.001 |
| *FLT3*-ITD / *FLT3** | 2.84 (1.59-5.08) | <0.001 | 3.19 (1.73-5.89) | <0.001 |
| Age, decades | 1.13 (1.05-1.65) | 0.017 | 1.35 (1.07-1.70) | 0.011 |

***(A)** The number of patients for the three WBC groups with regards to* FLT3*-ITD/*FLT3 *ratio.* FLT3 *fms-related tyrosine kinase 3; ITD: internal tandem duplication. **(B)** HR hazard ratio; CI, confidence interval, [¶]WBC >100×10$^9$/L* versus *WBC <100×10$^9$/L,* *FLT3 *ITD/*FLT3 *ratio >1* versus FLT3*-ITD/*FLT3 *ratio ≤1.*

*P*<0.001).

In the two other subgroups '*NPM1* wild-type with *FLT3*-ITD' and '*NPM1* wild-type without *FLT3*-ITD', WBC count had no evident impact on complete remission rate, event-free survival or overall survival (Figure 1E-H). In addition, when, analyzing WBC count as a continuous variable, no association between WBC count and event-free survival or overall survival was found in the subgroups with the genotypes '*NPM1* wild-type with *FLT3*-ITD' and 'NPM1 wild-type without *FLT3*-ITD' (data not shown). So, when considering the impact of WBC count within the four subgroups defined by the molecular markers *NPMc*$^+$ with *FLT3*-ITD, WBC count only had an impact on outcome in patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'. Of note, within the subgroup of patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' no difference in age distribution was found between patients with a WBC count below $20\times10^9$/L and between $20\text{-}100\times10^9$/L *versus* greater than $100\times10^9$/L (*P*=0.412): median age in years (range); 46 (24-68), 51 (18-77) and 50 (19-68), respectively. *CEBPA* was not taken into account since only two cases had mutated *CEBPA* within the subgroup of patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'.

### *Prognostic value of* FLT3*-ITD/*FLT3*-wildtype ratio among patients with acute myeloid leukemia with the genotypic combination '*NPMc$^+$ *with* FLT3*-ITD'*

It has been shown that the amount of *FLT3* signaling is associated with WBC count.[17-19] Patients who have lost both *FLT3* alleles have higher WBC counts than patients with both wild-type *FLT3* and *FLT3* -ITD alleles.[17-19] We, therefore, wondered whether the gene dosage of wild-type *FLT3* was different in patients with the genotypic

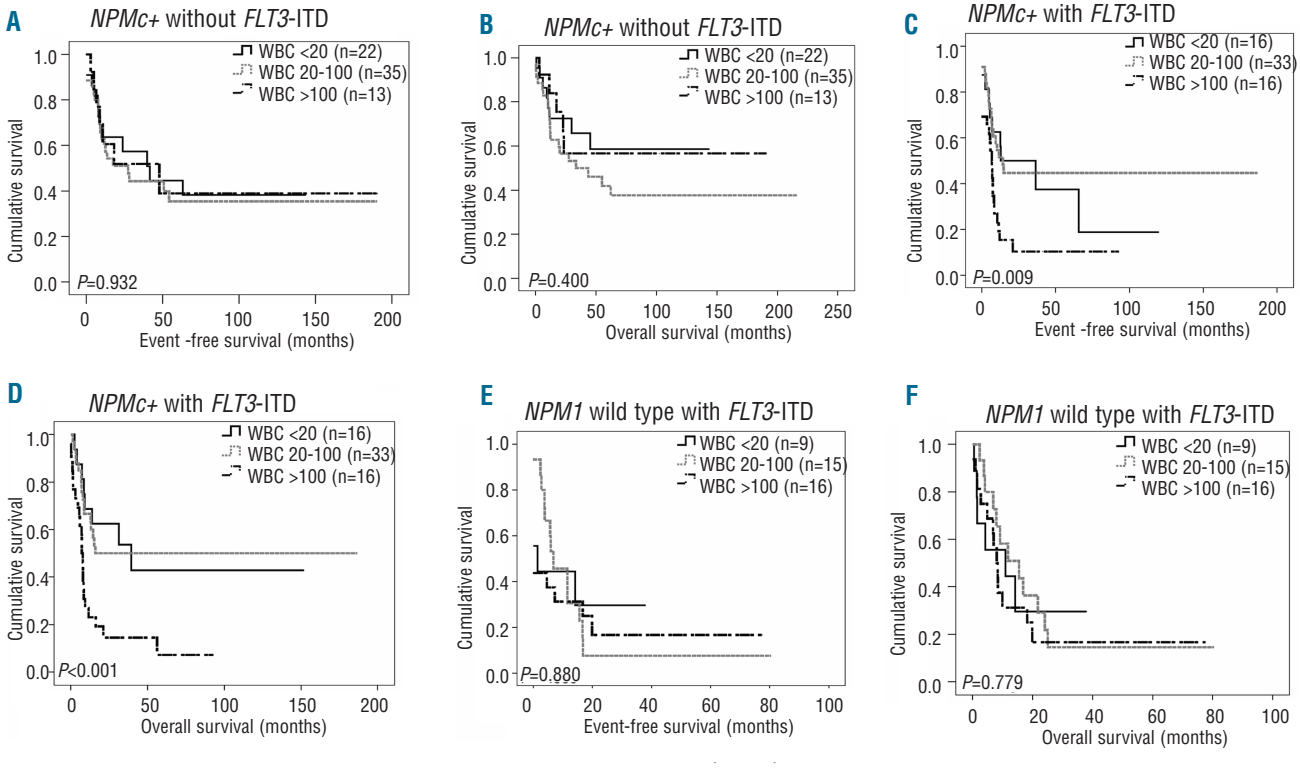


**Figure 1. Impact of WBC count on event-free survival (EFS) and overall survival (OS) in cases with cytogenetically intermediate risk AML defined by the molecular markers *NPMc*+ and *FLT3*-ITD. (A) EFS according to WBC levels in 70 cytogenetically intermediate risk AML cases with *NPMc*$^+$ without *FLT3*-ITD mutation. (B) OS for this group. (C) EFS according to WBC levels in 75 cytogenetically intermediate risk AML cases with *NPMc*$^+$ and *FLT3*-ITD. (D) OS for this group. (E) EFS according to WBC levels in 40 cytogenetically intermediate risk AML cases with wild type *NPM1* and *FLT3*-ITD. (F) OS for this group. (G) EFS according to WBC levels in 146 cytogenetically intermediate risk AML cases with wild-type *NPM1* without *FLT3*-ITD. (H) OS for this group. The *P* value is given for the overall comparison across all three groups.**

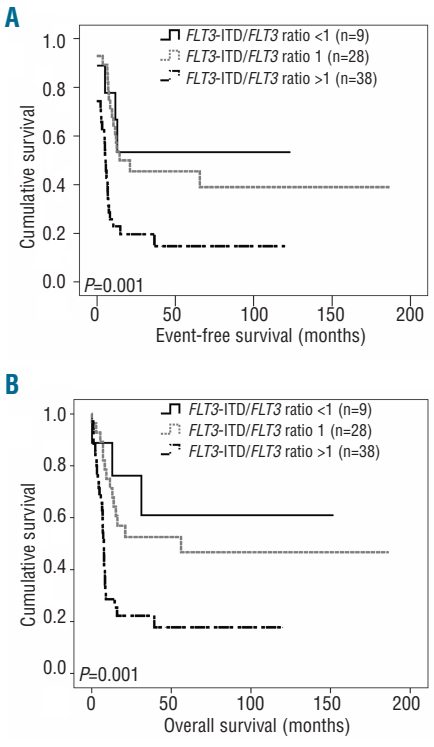


**Figure 2.** Impact of *FLT3*-ITD/*FLT3* ratio on event-free survival (EFS) and overall survival (OS) in cases with cytogenetically intermediate risk AML with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'. (A) EFS and (B) OS according to *FLT3*-ITD/*FLT3* ratio within 75 cytogenetically intermediate cytogenetic risk AML cases with the genotypic combination *NPMc*$^+$ with *FLT3*-ITD. Ratio <1: n=9; ratio 1: n=28; ratio >1: n = 38. The *P* value is given for the overall comparison across all three groups.

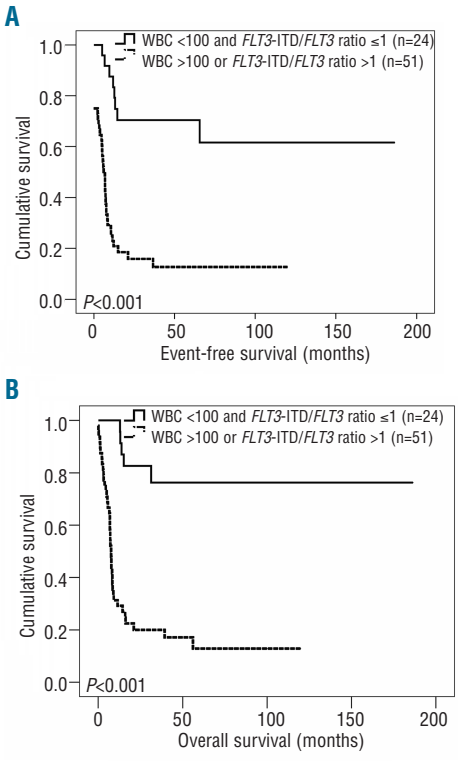


**Figure 3.** Combined effect of WBC count and *FLT3*-ITD/*FLT3* ratio on event-free survival (EFS) and overall survival (OS) in cases with cytogenetically intermediate risk AML with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'. (A) EFS in patients with WBC count <100x10$^9$/L and *FLT3*-ITD/*FLT3* ratio ≤1 (n=24) compared to those with WBC count >100x10$^9$/L or *FLT3*-ITD/*FLT3* ratio >1 (n=51) (B) OS in these cases.

combination '*NPMc*$^+$ with *FLT3*-ITD' depending on whether they had a high or low WBC count. To address this question, we investigated the impact of the *FLT3*-ITD/*FLT3* ratio on clinical outcomes within this particular subgroup of patients. The *FLT3*-ITD/*FLT3* ratio was known for all 75 patients and was categorized as less than 1, 1, or more than 1. Interestingly, the *FLT3*-ITD/*FLT3* ratio was significantly associated with event-free survival ($P$=0.001) and overall survival ($P$=0.001) within this subgroup of patients (Figure 2). In detail, patients with an *FLT3*-ITD/*FLT3* ratio greater than 1 had a significantly shorter event-free survival (median 5.6 months *versus* 15.2 and 13.0 months for patients with a ratio of 1 and <1, respectively) as well as a significantly shorter overall survival (median 7.7 months *versus* 16.5 and 31.3 months for patients with a ratio of 1 and <1, respectively). Thus, it appears that particularly patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' and an *FLT3*-ITD/*FLT3* ratio greater than 1 had a poor outcome.

So far, our data indicate that the group of patients with intermediate cytogenetic risk AML with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' can be further dissected, with the subgroup of patients with hyperleukocytosis or *FLT3* ITD/*FLT3* ratio greater than 1 having an unfavorable prognosis. However, interestingly, not all patients with hyperleukocytosis had an *FLT3*-ITD/*FLT3* ratio greater than 1 and vice versa (Table 3A). To investigate whether a *FLT3*-ITD/*FLT3* ratio greater than 1 and hyperleukocytosis are indeed independent prognostic factors for patients with AML with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD', a multivariable analysis was performed. This analysis established that *FLT3*-ITD/*FLT3* ratio greater than 1, WBC count greater than 100×10$^9$/L and age are independent prognostic factors. In detail, with regards to *FLT3*-ITD/*FLT3* ratio greater than 1, the hazard ratio for event-free survival was 2.84 ($P$<001) while that for overall survival was 3.19 ($P$<0.001); for hyperleukocytosis the hazard ratio for event-free survival was 2.29 ($P$=0.004) and for overall survival 3.30 ($P$<0.001), and finally, for age (in decades), the hazard ratio for event-free survival was 2.13 ($P$=0.017) and that for overall survival was 1.35 ($P$=0.011) (Table 3B).

These data prompted us to propose a model in which patients with intermediate cytogenetic risk AML with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' were considered to have an unfavorable prognosis if they had a WBC count greater than 100×10$^9$/L or an *FLT3*-ITD/*FLT3* ratio greater than 1. Consequently, patients with a WBC count below 100×10$^9$/L and an *FLT3*-ITD/*FLT3* ratio of 1 or less were considered to have a favorable prognosis. Within this model, patients with hyperleukocytosis or an *FLT3*-ITD/*FLT3* ratio greater than 1 (n=51) compared unfavorably with patients with a WBC count below 100×10$^9$/L and an *FLT3*-ITD/*FLT3* ratio of 1 or less (n=24), with regards to complete remission rate ($P$=0.010), event-free survival ($P$<0.001) and overall survival ($P$<0.001)

(Figure 3). In detail, patients with a WBC count below $100\times10^9$/L and an *FLT3*-ITD/*FLT3* ratio of 1 or less had median event-free and overall survivals of 24 and 33 months, and estimated 5-year event-free and overall survival rates of 71% and 79%, respectively. In contrast, patients with hyperleukocytosis or an *FLT3*-ITD/*FLT3* ratio greater than 1 had median event-free and overall survivals of 7 and 8 months, and estimated 5-year event-free and overall survival rates of 16% and 18%, respectively. Of note, the median age at diagnosis and the number of patients who had undergone allogeneic stem cell transplantation was not different between these two groups ($P$=0.55 and $P$=0.17, respectively).

## Discussion

It is generally accepted, and confirmed in this study, that high WBC count predicts an adverse outcome among AML patients with favorable or intermediate cytogenetic risk.[1,2,20-23] In the current study, we have focused on the impact of WBC count at diagnosis on outcome among patients with intermediate cytogenetic risk, divided into subgroups according to the presence of the molecular markers *NPMc*$^+$ and *FLT3*-ITD. It was found that the WBC count had an impact on complete remission rate, event-free survival and overall survival only among the patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'. Importantly, these results were underscored when using WBC count as a continuous variable. Apparently, the impact of WBC count at diagnosis on treatment outcome is dependent on the molecular genotype of AML blasts since the poor prognostic impact of high WBC count can be bypassed by intrinsic molecular abnormalities of the AML blasts, such as mutated *NPM1*. This is consistent with observations that *NPMc*$^+$ AML blasts, independently of *FLT3*-ITD, have good response to chemotherapy *in vivo* and *in vitro*.[19,31-33]

Analysis of a large cohort of young adult AML patients by the MRC study-group showed that both mutations in *NPM1* and *FLT3*-ITD are significant independent predictors of outcome, implying that the beneficial impact of *NPMc*$^+$ on prognosis is also seen in patients with *FLT3*-ITD.[19] Although the prognosis of patients with AML is better in the presence of *NPMc*$^+$, various clinical studies have shown that only patients with the genotypic combination '*NPMc*$^+$ without *FLT3*-ITD' have a favorable outcome.[9-13] Interestingly, our results show that the subgroup of patients with AML with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' can be further divided into subgroups with favorable and unfavorable prognosis based on WBC count and *FLT3*-ITD/*FLT3* ratio. It seems that the less favorable clinical course of patients with *NPMc*$^+$ AML, imposed by the presence of *FLT3*-ITD, does not apply for those patients with WBC counts below $100\times10^9$/L and an *FLT3*-ITD/*FLT3* ratio greater than 1. Indeed, the prognosis of patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' and lower WBC counts and *FLT3*-ITD/*FLT3* ratio greater than 1 appeared comparable to that of patients with the genotypic combination '*NPMc*$^+$ without *FLT3*-ITD'.

A number of studies suggested that patients with AML harboring *FLT3*-ITD have a worse outcome than patients without *FLT3*-ITD.[14-18] However, the number of mutated alleles, rather than its presence or the insertion site of the ITD, has been shown to affect outcome.[17-19,34,35] Likewise, in our study we also found that patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' and high levels of the mutant allele (i.e. an *FLT3*-ITD/*FLT3* ratio >1) had significantly worse long-term outcome. It is likely that higher levels of *FLT3*-ITD might trigger pathways involved in chemoresistance more intensively, for example by enhancing DNA repair and salvage of damaged cells.[36] Our observations confirm the importance of the *FLT3*-ITD/*FLT3* ratio, representing the *FLT3*-ITD allelic burden, with regards to prognosis and demonstrate that WBC count is not a surrogate marker for the *FLT3*-ITD/*FLT3* ratio, but indeed is an independent prognostic factor.

The importance of the WBC count and *FLT3*-ITD/*FLT3* ratio as prognostic factors in AML patients with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD' needs to be confirmed in further cohorts of patients. However, for the future it will be of interest to study whether patients with the genotypic combination 'NPMc$^+$ with *FLT3*-ITD' and low WBC count and an *FLT3*-ITD/*FLT3* ratio less than 1 can be excluded from consolidation therapy with allogeneic hematopoietic cell transplantation. This would extend the work of Schlenk *et al.*,[13] who demonstrated that patients with cytogenetically normal karyotype AML bearing the genotypic combination '*NPMc*$^+$ without *FLT3*-ITD' had no survival benefit from allogeneic hematopoietic cell transplantation, with an overall survival of 50-60%.

In conclusion, the present study demonstrates that high WBC count and *FLT3*-ITD/*FLT3* ratio are important prognostic factors in patients with AML with the genotypic combination '*NPMc*$^+$ with *FLT3*-ITD'.

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

*Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.*

## References


1. Estey E, Dohner H. Acute myeloid leukaemia. Lancet. 2006;368(9550):1894-907.
2. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999;341(14):1051-62.
3. Breems DA, Van Putten WL, De Greef GE, Van Zelderen-Bhola SL, Gerssen-Schoorl KB, Mellink CH, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. J Clin Oncol. 2008;26(29):4791-7.
4. Byrd JC, Mrozek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002;100(13):4325-36.
5. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult

and Children's Leukaemia Working Parties. Blood. 1998;92(7):2322-33.
6. Slovak ML, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS, Mohamed A, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. Blood. 2000;96 (13):4075-83.
7. Wheatley K, Burnett AK, Goldstone AH, Gray RG, Hann IM, Harrison CJ, et al. A simple, robust, validated and highly predictive index for the determination of risk-directed therapy in acute myeloid leukaemia derived from the MRC AML 10 trial. United Kingdom Medical Research Council's Adult and Childhood Leukaemia Working Parties. Br J Haematol. 1999;107 (1):69-79.
8. Dohner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115(3):453-74.
9. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3):254-66.
10. Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF, et al. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood. 2005;106(12):3733-9.
11. Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood. 2006;107(10):4011-20.
12. Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hugens W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood. 2005;106(12):3747-54.
13. Schlenk RF, Dohner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008;358(18):1909-18.
14. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999;93(9):3074-80.
15. Frohling S, Schlenk RF, Breitruck J, Benner A, Kreitmeier S, Tobis K, et al. Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. Blood. 2002;100(13):4372-80.
16. Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. Blood. 2001;98(6):1752-9.
17. Thiede C, Steudel C, Mohr B, Schaich M, Schäkel U, Platzbecker U, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002;99(12):4326-35.
18. Whitman SP, Archer KJ, Feng L, Baldus C, Becknell B, Carlson BD, et al. Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a Cancer and Leukemia Group B study. Cancer Res. 2001;61(19):7233-9.
19. Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, et al. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood. 2008;111(5):2776-84.
20. Dutcher JP, Schiffer CA, Wiernik PH. Hyperleukocytosis in adult acute nonlymphocytic leukemia: impact on remission rate and duration, and survival. J Clin Oncol. 1987;5(9):1364-72.
21. Hug V, Keating M, McCredie K, Hester J, Bodey GP, Freireich EJ. Clinical course and response to treatment of patients with acute myelogenous leukemia presenting with a high leukocyte count. Cancer. 1983;52(5):773-9.
22. Rees JK, Gray RG, Swirsky D, Hayhoe FG. Principal results of the Medical Research Council's 8th acute myeloid leukaemia trial. Lancet. 1986;2(8518):1236-41.
23. Estey E, Smith TL, Keating MJ McCredie KB, Gehan EA, Freireich EJ. Prediction of survival during induction therapy in patients with newly diagnosed acute myeloblastic leukemia. Leukemia. 1989; 3(4):257-63.
24. Burnett AK, Grimwade D, Solomon E, Wheatley K, Goldstone AH. Presenting white blood cell count and kinetics of molecular remission predict prognosis in acute promyelocytic leukemia treated with all-trans retinoic acid: result of the randomized MRC trial. Blood. 1999;93 (12):4131-43.
25. Nguyen S, Leblanc T, Fenaux P, Witz F, Blaise D, Pigneux A, et al. A white blood cell index as the main prognostic factor in t(8;21) acute myeloid leukemia (AML): a survey of 161 cases from the French AML Intergroup. Blood. 2002;99(10):3517-23.
26. Lowenberg B, Boogaerts MA, Daenen SM, Verhoef GE, Hagenbeek A, Vellenga E, et al. Value of different modalities of granulocyte-macrophage colony-stimulating factor applied during or after induction therapy of acute myeloid leukemia. J Clin Oncol. 1997;15(12):3496-506.
27. Lowenberg B, van Putten W, Theobald M, Gmür J, Verdonck L, Sonneveld P, et al. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. N Engl J Med. 2003;349(8):743-52.
28. Lowenberg B, Ossenkoppele GJ, van Putten W, Schouten HC, Graux C, Ferrant A, et al. High-dose daunorubicin in older patients with acute myeloid leukemia. N Engl J Med. 2009;361(13):1235-48.
29. Ossenkoppele GJ, Graveland WJ, Sonneveld P, Daenen SM, Biesma DH, Verdonck LF, et al. The value of fludarabine in addition to ARA-C and G-CSF in the treatment of patients with high-risk myelodysplastic syndromes and AML in elderly patients. Blood. 2004;103(8):2908-13.
30. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia. 1996; 10(12):1911-8.
31. Cilloni D, Messa F, Rosso V, Arruga F, Defilippi I, Carturan S, et al. Increase sensitivity to chemotherapeutical agents and cytoplasmatic interaction between NPM leukemic mutant and NF-kappaB in AML carrying NPM1 mutations. Leukemia. 2008;22(6):1234-40.
32. Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. Blood. 2007;109(3):874-85.
33. Schneider F, Hoster E, Unterhalt M, Schneider S, Dufour A, Benthaus T, et al. NPM1 but not FLT3-ITD mutations predict early blast cell clearance and CR rate in patients with normal karyotype AML (NK-AML) or high-risk myelodysplastic syndrome (MDS). Blood. 2009;113(21):5250-3.
34. Breitenbuecher F, Schnittger S, Grundler R, Markova B, Carius B, Brecht A, et al. Identification of a novel type of ITD mutations located in nonjuxtamembrane domains of the FLT3 tyrosine kinase receptor. Blood. 2009;113(17):4074-7.
35. Kayser S, Schlenk RF, Londono MC, Breitenbuecher F, Wittke K, Du J, et al. Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. Blood. 2009; 114(12):2386-92.
36. Seedhouse CH, Hunter HM, Lloyd-Lewis B, Massip AM, Pallis M, Carter GI, et al. DNA repair contributes to the drug-resistant phenotype of primary acute myeloid leukaemia cells with FLT3 internal tandem duplications and is reversed by the FLT3 inhibitor PKC412. Leukemia. 2006;20(12): 2130-6.