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# Predictive value of pretransplantation molecular minimal residual disease assessment by WT1 gene expression in FLT3-positive acute myeloid leukemia

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**The FMS-like tyrosine kinase 3 (FLT3) mutation in acute myeloid leukemia (AML) is a negative prognostic factor and, in these cases, allogeneic stem cell transplantation (allo-SCT) can represent an important therapeutic option, especially if performed in complete remission (CR). However, it is increasingly clear that not all cytological CRs (cCRs) are the same and that minimal residual disease (MRD) before allo-SCT could have an impact on AML outcome. Unfortunately, FLT3, due its instability of expression, is still not considered a good molecular MRD marker. We analyzed the outcome of allo-SCT in a population of FLT3-positive AML patients according to molecular MRD at the pretransplantation workup, assessed by the quantitative expression evaluation of the panleukemic marker Wilms' tumor (WT1) gene. Sixty-two consecutive AML FLT3-positive patients received allo-SCT between 2005 and 2016 in our center. The median age at transplantation was 55 years. The quantitative analysis of the WT1 gene expression (bone marrow samples) was available in 54 out of 62 (87%) cases, both at diagnosis (100% overexpressing WT1 with a mean of 9747 ± 8064 copies) and before allo-SCT (33 WT1-negative and 21 WT1-positive cases at the pretransplantation workup). Of these cases, 33/54 (61%) were both in cCR and molecular remission (WT1-negative) at the time of transplantation, 13/54 (24%) were in cCR but not in molecular remission (WT1-positive), and 8/54 (15%) showed a cytological evidence of disease (relapsed or refractory). Both post-allo-SCT overall survival (OS) and disease-free survival (DFS) were significantly better in patients who were WT1-negative (WT1 $<250$ copies) at the time of transplantation compared with those who were WT1-positive (WT1 $>250$ copies), with a median OS and DFS not reached in the WT1-negative group and 10.2 and 5.5 months, respectively, in the WT1-positive group (OS log–rank $p = 0.0005$; hazard ratio [HR] = 3.7, 95% confidence interval [95% CI] = 1.5–9; DFS log–rank $p = 0.0001$; HR = 4.38, 95% CI = 1.9–10). Patients with cCR who were WT1-positive had the same negative outcome as those with a cytological evidence of disease. The relapse rate after allo-SCT was 9% (3/33) in pre-allo-SCT WT1-negative cases and 54% (7/13) in WT1-positive cases ($p = 0.002$). At multivariate analysis, WT1 negativity before allo-SCT and grade $<2$ acute graft versus host disease were the only independent prognostic factors for improved OS and DFS. These data show that pre-allo-SCT molecular MRD evaluation through WT1 expression is a powerful predictor of posttransplantation outcomes (OS, DFS, relapse rate). Patients with both cCR and a WT1-negative marker before allo-SCT have a very good outcome with very low relapse rate; conversely, patients with positive molecular MRD and refractory/relapsed patients have a negative outcome. The WT1 MRD stratification in FLT3-positive AML is a valuable tool with which to identify patients who are at high risk of relapse and that could be considered from post-allo-SCT prophylaxis with FLT3 inhibitors or other strategies (donor lymphocyte infusion, tapering of immunosuppression, azacitidine).** 

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The FMS-like tyrosine kinase 3 (FLT3) gene, located on chromosome 13, encodes a tyrosine kinase receptor located on the surface of hematopoietic progenitor cells that is essential for cellular survival and differentiation [1–3]. FLT3 mutations can involve the receptor juxtamembrane internal tandem duplication (ITD) domain or the intracellular tyrosine kinase domain (TKD) and can cause, in most cases, an autophosphorylation with the constitutive activation of the receptor, resulting in a higher proliferation and an increased survival of leukemic cells [1,3,4]. FLT3 mutations are present in 20–30% of acute myeloid leukemia (AML) cases in adults and are associated with a high risk of relapse and a poor prognosis [5–8]. Allogeneic stem cell transplantation (allo-SCT) is an important therapeutic strategy in AML with unfavorable prognostic factors, as well as in FLT3-mutated AML [9].

Even though there exists much data supporting allo-SCT in FLT3-positive patients, in this context, it is still necessary to define risk subcategories (with different transplantation efficacy) based on FLT3-related (such as the FLT3 allelic burden) and FLT3-unrelated parameters (such as the presence of other pretransplantation predictive factors) [8,10–16].

We have analyzed the FLT3-mutated AML cases treated with allo-SCT at our institution's department of hematology in the last 11 years (2005–2016) to identify clinical or biological variables (other than FLT3) that might predict post-SCT outcome in this specific high-risk AML group.

## Methods

The study population included 62 adult AML patients (over 18 years old) with FLT3 mutation at diagnosis who underwent allo-SCT at the Udine Hospital Department of Hematology from January 2005 to March 2016.

FLT3 mutational analyses (ITD and TKD) were performed according to the method described by Gale and colleagues [10] based on RNA extraction from bone marrow samples using automated protocols (RNA mini kit/QiaCube, Qiagen, Europe), a subsequent reverse transcription to cDNA using the random hexamers technique, and a multiplex polymerase chain reaction (PCR) able to determine both the ITD and TKD mutations in the same reaction. The primers used for the ITD mutations detection were juxtaposed to the coding region of juxtamembrane domain, whereas the TKD mutations were determined by the inclusion of a restriction enzyme (EcoRV, Amersham International, UK) and subsequent digestion of amplicons. The PCR products and the EcoRV digestion were then separated via standard electrophoresis (qualitative assessment) or by capillary gel electrophoresis performed using a genetic analyzer (AB3500 DX, Applied Biosystems, Europe) and the obtained electropherograms were analyzed with Genescan software, allowing the determination of the length of amplicons and their relative concentration by fluorescence intensity [10,17].

Wilms' tumor 1 (WT1) gene quantitative analysis was performed on bone marrow samples using the real-time quantitative PCR method. The WT1 ProfileQuant (Ipsogen, Marseille, France) kit, standardized according to the European Leukemia Net guidelines, was used for this analysis [18]. Primers and probes were localized on exons 1 and 2. The WT1 transcript values obtained were normalized with respect to the number of ABL transcripts (control gene) and expressed as WT1 copy number every $10^4$ copies of ABL (WT1/ABL $\times$ $10^4$). The cut-off for bone marrow samples was 250 WT1 copies/$10^4$ ABL copies, as reported by Leukemia NET [18].

The cytological disease status at transplantation was evaluated according to National Cancer Institute criteria and the subsequent international working group review [19]. Cytological complete remission (cCR) was defined as the absence of or $<5\%$ blastic cells in the marrow, polymorphonuclear neutrophil (PMN) cell number $>1 \times 10^9$/L, platelets $>100 \times 10^9$, and transfusion independence. Partial remission (PR) was defined as a blast cell percentage in the marrow ranging between 5% and 15% in the presence of an appropriate cellularity and signs of trilinear cell regeneration. Patients who did not meet cCR or PR criteria were defined as being resistant (RES) or nonresponders (NRs).

The molecular cytogenetic risk at diagnosis was defined according to the European Leukemia Net classification [20]. Comorbidity index at the time of transplantation was calculated according to Sorror criteria and scores [21].

The European Society for Blood and Marrow Transplantation (EBMT) risk score was calculated as established by Gratwohl, considering the following parameters at the time of transplantation: age, stage of disease (early, intermediate, late), interval from diagnosis to transplantation ($<12$ months), type of donor, and donor–recipient combination (sex) [22]. Graft versus host disease (GVHD) was diagnosed and staged according to Przepiorka and Filipovich criteria [23,24]. None of the analyzed patients received a FLT3 inhibitor before or after transplantation.

### *Statistical analysis*

Continuous variables were analyzed using descriptive statistical methods (arithmetic mean, standard deviation, median, range, minimum, maximum). Categorical variables were compared with chi-square test (two-tailed); $p \leq 0.05$ was considered statistically significant. Multivariate analysis was performed using Cox regression. Survival curves were constructed with the Kaplan–Meier method and compared, where indicated, with the log–rank test. Posttransplantation OS was calculated as the period (in months) from the time of transplantation to last follow-up or death from any cause. Posttransplantation DFS was defined as the period (in months) from the reinfusion to posttransplantation relapse or death. Patients who did not relapse were censored at the time of death or at last follow-up. Estimation of relapse probability was performed using the proper estimation of cumulative incidence curves. The follow-up is updated to July 15, 2016. Data were analyzed with MedCalc software (version 12.5.0.0; MedCalc Software bvba, Ostend, Belgium).

## Results

### *Patient characteristics*

Thirty-five percent of the 62 patients with FLT3-mutated AML who received transplantations at our department of hematology from 2005 to 2016 were 60 years old or older. The cytogenetic–molecular risk at diagnosis was: adverse

and intermediate in 17% and 83% of cases, respectively; 81% of the patients (50/62) had an ITD FLT3 mutation, 17% (11/62) had a TKD mutation, and 2% (1 case) had both mutations.

The quantitative analysis of the WT1 gene expression was available in 54/62 (87%) cases at baseline, whereas WT1 was not evaluated in eight cases. WT1 was overexpressed in all evaluable patients (54/54; 100%), with a mean of 9747 ± 8064 copies and a median of 7493.5 copies (range, 454–33563). Eighty-four percent (52/62) of the patients were in cCR at the time of transplantation (48 in first cCR and four in second cCR) and 16% (10/62) had an active disease, either relapsed or RES. Patient characteristics at baseline are shown in Table 1. Patient characteristics at the time of transplantation are shown in Table 2.

*Transplantation characteristics*

The mean period of time between diagnosis and allo-SCT was 7.4 ± 5 months, with a median of 6 months (range, 3–28). The transplantation comorbidity index was: 0–1 in 31% of cases (19/62), 2 in 21% of cases (13/62), 3 in 33% of cases (21/62), and 4–6 in 15% of cases (9/62). The EBMT risk score was: 1–2 in 32% of patients (20/62), 3–4 in 53% (33/62) of cases, and 5–6 in 15% (9/62) of cases. According to the type of donor, 28/62 (45%) received a matched unrelated donor allo-SCT, 27/62 (43%) a sibling allo-SCT, 6/62 (10%) a mismatched SCT, and 1/62 (2%) a SCT from cord blood. Bone marrow was the stem cell source in 23/62 (37%) cases and peripheral blood in the remaining 39/62 (63%) cases. Conditioning regimen was myeloablative in 71% (44/62) of patients and not myeloablative in the other 29% (18/62) (Table 2).

*Outcome after allo-SCT*

After a mean follow-up of 39 ± 40 months (median, 18 months; range, 1–130), 55% (34/62) of the patients were alive (31/34 in cCR) and 45% (28/62) were deceased. Transplantation-related mortality (TRM) was 23% (14/62 cases). The primary causes of death were relapsed/refractory AML (14/28 cases) and TRM (14/28 cases). The causes of death from TRM were: acute (aGVHD) or chronic (cGVHD) GVHD in seven cases, bacterial or fungal infections in seven cases, myocardial infarction in one case, and esophageal carcinoma in one case. Death from TRM occurred within 90 days after allo-SCT in 5/14 patients, between 90 days and 1 year in 4/14 patients, and >1 year after transplantation in the remaining 5/14 cases (Table 3).

aGVHD occurred within the first 3 months after transplantation in 35/62 patients (56%), with a prevalence of grade I–II GVHD (27/35 cases), whereas 8/35 (23%) cases were of grade III or higher. cGVHD was documented in 12 of 51 evaluable cases (24%).

The median post-SCT survival of the study population was 79 months, with an OS probability of 70% and 62% at 12 and 36 months, respectively (Fig. 1A). The median DFS was 58 months, with a DFS probability of 63% at 12 months and 57% at 36 months (Fig. 1B). In univariate analysis, the nine patients with an EBMT score of 5–6 had a worse post-allo-SCT OS compared with the 53 patients with a score <4 (OS at 12 months: 25% vs 76%, respectively; log–rank, $p = 0.001$).

**Table 1.** Patient characteristics

| | |
|---|---|
| Total Number | 62 |
| Sex (F/M) | 36/26 |
| Age, Mean ± SD | 51 ± 12.24 |
| Age, Median (range) | 55 (20–69) |
| Molecular-Cytogenetic Risk | |
| Adverse[a] | 11/62 (17%) |
| Intermediate (I and II) | 51/62 (83%) |
| FLT3 Mutational Status | |
| FLT3 ITD-Positive | 50/62 (81%) |
| FLT3 D835-Positive | 11/62 (17%) |
| FLT3 ITD-Positive and D835-Positive | 1/62 (2%) |
| WT1 at Diagnosis | |
| Available | 54/62 (87%) |
| Not Available | 8/62 (13%) |
| WT1 Overexpression (>250 copies) | 54/54 (100%) |
| Copies, Mean ± SD | 9747 ± 8064 |
| Copies, Median (range) | 7493.5 (454–33563) |

FLT3 = FMS-like tyrosine kinase 3; SD = standard deviation; WT1 = Wilms' tumor.
[a]8/11 complex karyotype, 3/11 del 7.

**Table 2.** Patient characteristics at the time of allo-SCT

| | |
|---|---|
| Age >60 Years at Transplantation | 22/62 (35%) |
| Age <60 Years at Transplantation | 40/62 (65%) |
| Disease Status | |
| AML Relapsed/Refractory | 10/62 (16%) |
| AML CR | 52/62 (84%) |
| First CR | 48/52 (92%) |
| Second CR | 4/52 (8%) |
| EBMT Risk Score | |
| 1–2 | 20/62 (32%) |
| 3–4 | 33/62 (53%) |
| 5–6 | 9/62 (15%) |
| Comorbidity Index | |
| 0–1 | 19/62 (31%) |
| 2 | 13/62 (21%) |
| 3 | 21/62 (33%) |
| 4 | 6/62 (10%) |
| 5–6 | 3/62 (5%) |
| Type of Donor | |
| Matched Unrelated Donor | 28/62 (45%) |
| Sibling | 27/62 (43%) |
| Mismatched | 6/62 (10%) |
| Cord Blood | 1/62 (2%) |
| Conditioning Regimen | |
| Myeloablative | 44/62 (71%) |
| Not Myeloablative | 18/62 (29%) |
| Diagnosis allo-SCT (Months) | |
| Mean ± SD | 7.4 ± 5 |
| Median (Range) | 6 (3–28) |

allo-SCT = allogeneic stem cell transplantation; AML = acute myeloid leukemia; CR = complete remission; EBMT = European Society for Blood and Marrow Transplantation; SD = standard deviation.

**Table 3.** Outcome after allo-SCT ($N$ = 62 patients)

| | |
|---|---|
| Follow-up, Months, Mean ± SD | 39 ± 40 |
| Follow-up, Months, Median (Range) | 18 (1–130) |
| Status | |
| Alive | 34/62 (55%)[a] |
| Deceased | 28/62 (45%) |
| TRM | 14/62 (23%) |
| Cause of Death | |
| AML Relapsed/Refractory | 14/28 |
| TRM[b] | 14/28 |
| <3 Months after SCT | 5/14 |
| 3–12 Months after SCT | 4/14 |
| >12 Months after SCT | 5/14 |

allo-SCT = allogeneic stem cell transplantation; cCR = cytological complete remission; GVHD = graft versus host disease; SD = standard deviation; TRM = transplantation-related mortality.
[a]31/34 in cCR.
[b]Seven GVHD, five infections, one acute myocardial infarction, and one esophageal carcinoma.

### *Pre-allo-SCT WT1 evaluation and post-allo-SCT outcome*

Fifty-four of the 62 patients (87%) had a quantitative WT1 assessment both at diagnosis (100% overexpressing WT1) and at the pre-SCT workup (immediately before starting the conditioning regimen). At the pretransplantation workup, 33 patients were WT1-negative (WT1 <250 copies) and 21 were WT1-positive (WT1 >250 copies).

Overall, the post-allo-SCT OS was significantly better in WT1-negative patients at the time of SCT compared with WT1-positive patients, with a median OS not reached in the WT1-negative group and of 10.2 months in the WT1-positive (log–rank $p$ = 0.0005; HR = 3.7, 95% CI = 1.5–9; Fig. 2A). Similarly, the post-allo-SCT DFS was significantly better in patients WT1-negative at the time of transplantation compared with WT1-positive patients, with a median DFS not reached in the WT1-negative group and of 5.5 months in the WT1-positive group (log–rank $p$ = 0.0001; HR = 4.38, 95% CI = 1.9–10; Fig. 2B).

In addition, after stratification of the patients into three groups, 33 of 54 cases (61%) were both in cCR and molecular remission at the time of transplantation (group 1: cCR and WT1 <250 copies/$10^4$ Abl), 13/54 (24%) cases were in cCR but not in molecular remission (group 2: cCR and WT1 >250 copies/$10^4$ Abl), and 8/54 (15%) cases showed a cytological evidence of disease with resistant or relapsed AML (group 3). At the time of transplantation, the WT1 mean value in these three groups was 76 ± 71 copies, 1632 ± 1918 copies, and 8003 ± 11920 copies, respectively (Table 4). We underscore that, according to this stratification, a significantly lower OS and DFS were observed in patients with active disease (refractory or relapsed) and in those with cCR but not molecular remission (groups 2 and 3) compared with patients with both cCR and WT1-negative (group 1) at SCT (log–rank, $p < 0.001$; Fig. 3A and B).

The probability of post-allo-SCT relapse in the entire patient population at 12 and 24 months was 25% and 30%, respectively and was significantly lower (9%) in patients in cCR + molecular remission (group 1) at the time of transplantation compared with the other two groups (54% in group 2 and 62.5% in group 3; log–rank $p < 0.001$; Fig. 4 and Table 4).

In addition, all group 2 and 3 (both WT1-positive) cases that did not relapse after transplantation (9/24) achieved and maintained WT1 negativity after transplantation. In the 12/24 WT1-positive patients who relapsed, the WT1 trend was different: in 5/12 cases, the WT1 expression remained positive after transplantation until cytological relapse; in another 5/12 cases, the WT1 expression became negative from 1 to 2 months after reinfusion and then went back to positive; in other 2/12 cases, it was not evaluable. In the five patients who reverted to positive after a posttransplantation WT1 negativity, the time from transplantation to WT1 overexpression and the speed of copy number increase of WT1 were extremely variable from case to case.

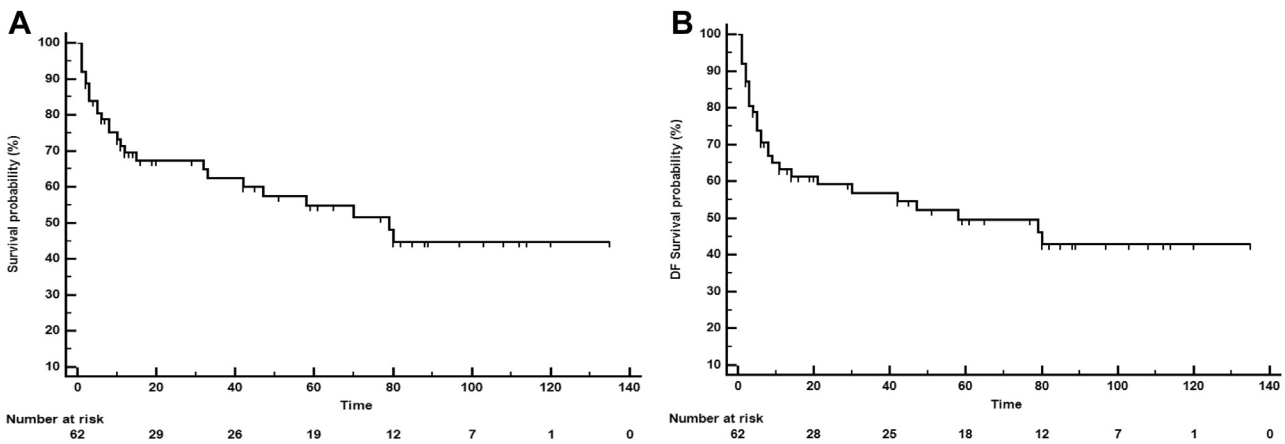


**Figure 1.** (**A**) OS after allo-SCT; median OS = 79 months. Probability of OS at 12 and 36 months = 70% and 62%, respectively. (**B**) DFS after allo-SCT; median DFS = 58 months. Probability of DFS at 12 and 36 months = 63% and 57%, respectively.

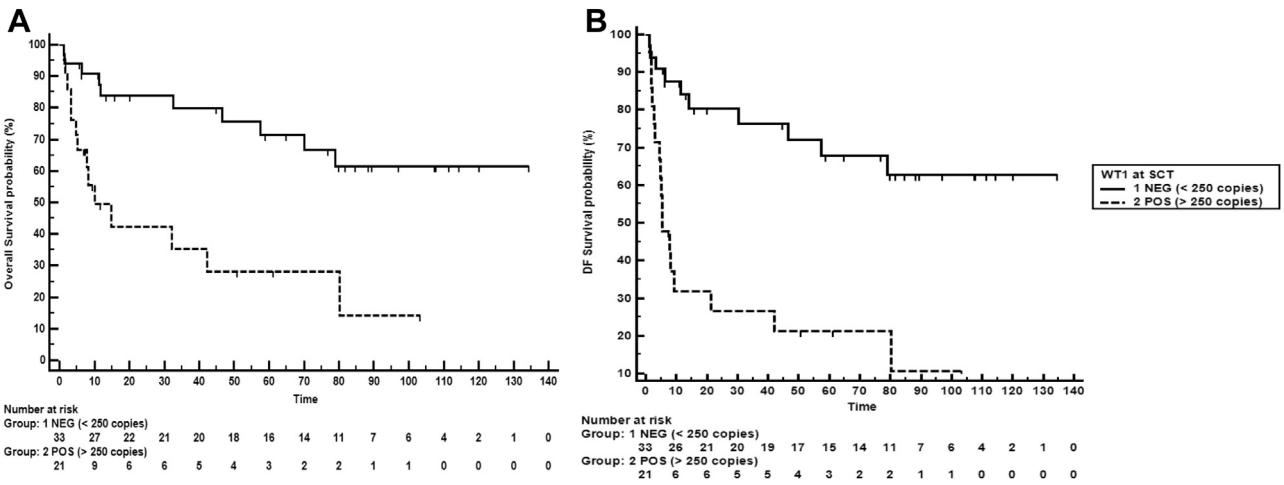


**Figure 2.** (**A**) OS and (**B**) DFS after allo-SCT according to WT1 levels before allo-SCT (WT1-negative vs WT1-positive). For (**A**), WT1-negative patients, median OS was not reached; for WT1-positive patients, median OS = 10.2 months (log–rank $p = 0.0005$, HR = 3.7, 95% CI = 1.5–9). For (**B**), WT1-negative patients, median DFS was not reached; for WT1-positive patients, median DFS = 5.5 months (log–rank $p = 0.0001$, HR = 4.38, 95% CI = 1.9–10); for WT1-negative patients, 5-year probability of OS and DFS: 70% and 67%, respectively.

In the 54 patients with WT1 levels available both at baseline and pre-allo-SCT, the following pre- and post-allo-SCT variables were included in a common multivariate model: cytogenetic–molecular risk (high vs intermediate), FLT3 mutation type (ITD vs TKD-D835), status at transplantation (cCR + WT1 <250 vs cCR+ WT1 >250 or relapse/refractory), EBMT risk score (≤4 vs >4), stem cell source (marrow vs peripheral blood), conditioning regimen (myeloablative vs not myeloablative), aGVHD (grade <II vs grade >II), and cGVHD (presence vs absence). The analysis showed that DFS was affected only by the status of cCR + WT1-negative (WT1 <250 copies) at the time of transplantation ($p = 0.0001$, HR = 6.21, 95% CI = 2.46–15.66), and also by a grade < II aGVHD ($p = 0.011$, HR = 3.35, 95% CI = 1.31–8.52). Similarly, the only two variables found to independently influence the OS after allo-SCT were the status of cCR + WT1-negative at transplantation ($p = 0.0078$, HR = 3.82, 95% CI = 1.43–10.18) and the aGvHD grade <II ($p = 0.028$, HR = 3.05, 95% CI = 1.13–8.21; Table 5).

## Discussion

AML is a highly heterogeneous disease both from a phenotypical and a molecular–genetic point of view. It is now clear that, even in the context of a single patient, AML consists of different leukemic subclones, each with particular biological characteristics and a different proliferation pattern, as well as different survival and sensitivity to chemotherapy [4]. FLT3 mutation represents a clear example of this heterogeneity. In fact, not only is this mutation present in approximately 20–30% of AML cases, but in the context of individual cases, it can also have a different level of intensity from the diagnosis and during the course of the disease due to the expression of leukemic subclones [1,3,4,8]. The presence of the FLT3 mutation is a poor prognostic factor in AML patients with a high risk of relapse and short duration of remission [1,3,4,8]. In addition, some recent studies emphasize the prognostic importance of the FLT3 allelic burden (allelic ratio); in particular, an allelic ratio higher than 0.5–0.8 could be associated with a negative prognostic effect determined by the mutation [2,8,25,26]. It is noteworthy that, even if all of

**Table 4.** Relapse rate after allo-SCT according to WT1 expression and cytologic status at transplantation in 54 cases with quantitative WT1 expression available both at diagnosis and at pre-allo-SCT workup

| | *n* | Mean WT1 ± SD | Median WT1 (range) | Relapse Rate |
|---|---|---|---|---|
| FLT3-Positive Patients with WT1 Overexpression at AML Diagnosis | 54/54 (100%) | 9747 ± 8064 | 7493.5 (454–33563) | 15/54 (28%) |
| AML Status at Allo-SCT | | | | |
| cCR and Molecular Remission (WT1 <250 copies) | 33/54 (61%) | 76 ± 71 | 55 (10–245) | 3/33 (9%)[a] |
| cCR But Not Molecular Remission (WT1 >250 copies) | 13/54 (24%) | 1632 ± 1,918 | 688 (305–6672) | 7/13 (54%)[a] |
| Refractory or Relapsed AML | 8/54 (15%) | 8003 ± 11920 | 2706 (1375–36423) | 5/8 (62.5%) |

allo-SCT = allogeneic stem cell transplantation; AML = acute myeloid leukemia; cCR = cytological complete remission; FLT3 = FMS-like tyrosine kinase 3; SD = standard deviation; WT1 = Wilms' tumor.
[a] $p = 0.0024$ for comparison (Fisher's exact test).

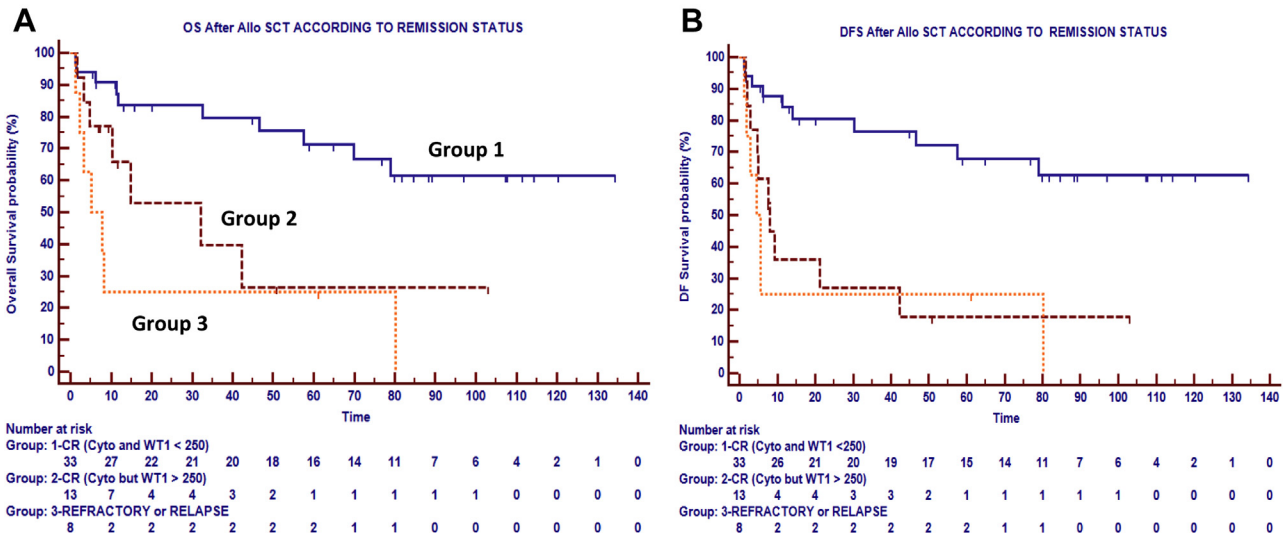


**Figure 3.** (**A**) OS and (**B**) DFS after allo-SCT according to remission status at transplantation (cCR and molecular remission, cCR but not molecular remission, refractory or relapsed AML).

the studies had not confirmed this cut-off, the idea that FLT3-positive AMLs are not all prognostically equivalent (due to different allelic burden, length of mutation, insertion site, etc.) is being consolidated and, in this context, FLT3-positive AMLs with high allelic burden would be associated with a higher risk of relapse [8,26,27]. However, quantitative rather than qualitative assessment of FLT3 has been adopted recently, but it is not yet performed routinely. Even in our cases, especially in patients being diagnosed and receiving transplantations before 2010, only qualitative FLT3 analysis was available because semiquantitative evaluation (allelic burden), calculated by analysis of capillary electrophoresis, was not yet available in our center.

Another important aspect about FLT3 is its possible role as a marker of MRD. Several studies have confirmed that FLT3 is not a good marker of MRD because leukemic relapse can result from a FLT3-negative subclone [8]. In particular, FLT3 is still considered an inadequate MRD marker both because its expression is unstable and because the available detection methods (mainly PCR) have a limited sensitivity (maximum 5% sensitivity); conversely, the most recent and sensitive detection methods (such as next-generation sequencing) are still not widespread and must be validated carefully [8,28]. In absence of newer standardized methods, it is difficult to monitor molecular MRD properly after

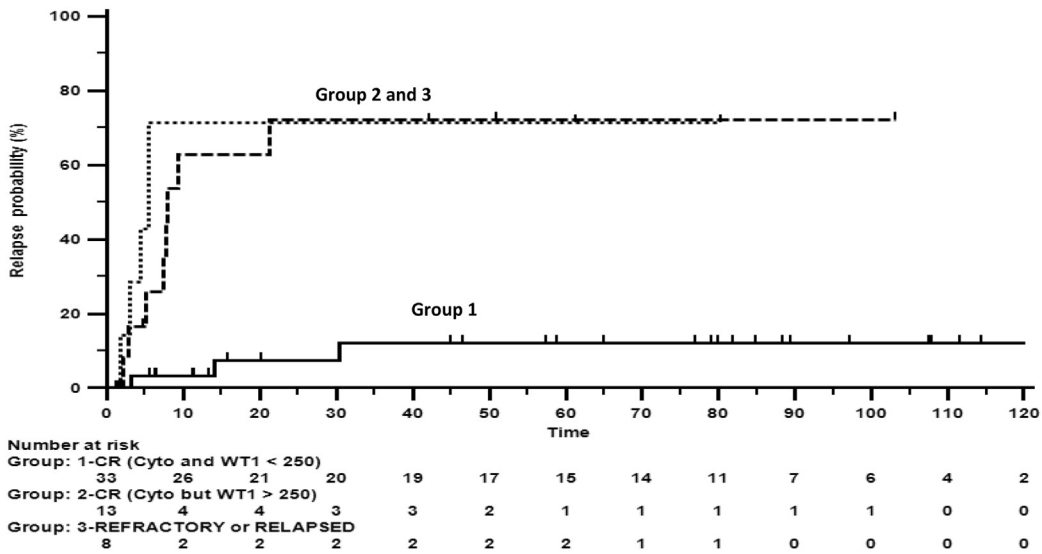


**Figure 4.** Probability of relapse after allo-SCT according to disease status at transplantation (cCR and molecular remission, cCR but not molecular remission, refractory/relapsed AML; log–rank test between group 1 and groups 2–3, $p < 0.0001$).

**Table 5.** Cox regression analysis of variables affecting DFS and OS after allo-SCT in FLT3-positive AML

| Covariate | OS | | | DFS | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p* | HR | 95% CI | *p* |
| Molecular-Cytogenetic Risk (High vs Intermediate) | 2.41 | 0.462–12.564 | 0.29 | 1.28 | 0.324–5.053 | 0.72 |
| FLT3 Mutation (ITD vs D835) | 0.39 | 0.067–2.24 | 0.29 | 0.63 | 0.148–2.695 | 0.53 |
| Cytologic + Molecular CR (WT1 <250 Copies) | 3.82 | 1.430–10.182 | 0.0078 | 6.21 | 2.465–15.667 | 0.0001 |
| EBMT Risk Score (<4 vs >4) | 0.83 | 0.237–2.949 | 0.78 | 0.48 | 0.139–1.712 | 0.26 |
| Conditioning Regimen (Myeloablative vs Not Myeloablative) | 1.02 | 0.384–2.730 | 0.96 | 0.1 | 0.407–2.433 | 0.99 |
| Stem Cell Source (BM vs PB) | 1.02 | 0.369–2.730 | 0.96 | 1.57 | 0.639–3.869 | 0.32 |
| aGVHD (Grade <2) | 3.05 | 1.134–8.213 | 0.028 | 3.35 | 1.317–8.529 | 0.011 |
| cGVHD (Present vs Absent) | 0.50 | 0.161–1.598 | 0.24 | 0.39 | 0.126–1.205 | 0.1 |

aGVHD = acute graft versus host disease; allo-SCT = allogeneic stem cell transplantation; AML = acute myeloid leukemia; BM = bone marrow; cGVHD = chronic graft versus host disease; CR = complete remission; DFS = disease-free survival; EBMT = European Society for Blood and Marrow Transplantation; FLT3 = FMS-like tyrosine kinase 3; ITD = internal tandem duplication; OS = overall survival; PB = peripheral blood; SD = standard deviation; WT1 = Wilms' tumor.

chemotherapy and after transplantation with this specific marker [8].

Regarding the role of allo-SCT in FLT3-positive AML patients, several studies, mostly retrospective and/or from registries, showed a clear benefit of allo-SCT in FLT3-mutated patients, especially if the transplantation is performed in first CR [8,11,12,14,26,29,30]. A recent meta-analysis, published in 2015, confirmed the efficacy of allo-SCT in terms of OS, DFS, and relapse rate in FLT3-positive patients compared with patients consolidated with chemotherapy or undergoing autologous transplantation (OR = 1.55, 95% CI = 1.33–1.82, $p < 0.00001$) [16]. In addition, a recent study from the M.D. Anderson Cancer Center group pointed out that allo-SCT is effective in FLT3-mutated AML patients regardless of the FLT3 allelic ratio at diagnosis; however, in this study, no outcome according to MRD stratification at SCT was reported [31]. In summary, the more recent literature data regarding allo-SCT in FLT3-mutated AML patients confirm its efficacy (regardless of FLT3 allelic ratio at diagnosis) and the expected survival of these patients, especially of those receiving transplantation in first cCR, is comparable to that of FLT3-negative patients undergoing allo-SCT [8,31,32]. However, it should be underscored that actual specific data on the prognostic impact of molecular MRD at the time of transplantation in FLT3-positive patients are not yet available.

In our case series consisting of 62 FLT3-positive AML patients, mostly (84%) in cCR at the time of allo-SCT, we found a median OS of 79 months (with a 12- and 36-month OS of 70% and 62%, respectively) and a median DFS of 58 months (with a 12- and 36-month DFS of 63% and 57%, respectively); these data are in agreement with those reported by a recent review and by the National Marrow Donor Program in 2016 (OS 60% at 12 months and 49% at 36 months) and support the role of allo-SCT in overcoming the unfavorable prognostic FLT3 effect [33,34].

Furthermore, in our population, we have verified the predictive value of molecular MRD, checked at the time point of transplantation, for prediction of post-allo-SCT outcome. Due to the fact that FLT3 is not currently considered an optimal molecular MRD marker, we evaluated the post-allo-SCT outcomes and the risk of relapse related to the pre-allo-SCT levels of expression (not mutation) of the pan leukemic MRD marker WT1. It is well known that the WT1 gene is expressed in >90% of AML cases at diagnosis and it is stable over time; in addition, standardized quantitative methods for its expression detection are available [18]. Therefore, expression of WT1 is currently considered an adequate and reliable marker for monitoring molecular MRD in the postchemotherapy and post-SCT phase [18,35,36]. However, it is documented that, in the post-allo-SCT phase, the time to recurrence of this MRD marker relative to the subsequent cytological relapse can be variable and, if overexpression of WT1 is too close to cytological relapse, an early and effective preemptive therapy may be unworkable [36]. In this respect, the WT1 pre-SCT expression evaluation could be more helpful. However, no data are available regarding the predictive value of WT1 levels of expression in bone marrow samples collected at the pre-allo-SCT time point.

In our series, 54 patients (87%) had a valuable of WT1 level both at diagnosis and at the time of transplantation (preconditioning). All of these 54 cases overexpressed WT1 at diagnosis (mean 9747 ± 8064 copies). Comparing post-allo-SCT OS and DFS of the patients overexpressing and not overexpressing WT1 at the pre-SCT time point (WT1-positive and WT1-negative cases), we noted a statistically significant difference in favor of the WT1-negative patients, both for OS (log–rank $p = 0.0005$, HR = 3.7, 95% CI = 1.5–9) and DFS (log–rank $p = 0.0001$, HR = 4.38, 95% CI = 1.9–10) (Fig. 2A and B). Further, stratifying the 54 WT1-monitored patients into three classes according to pre-SCT status (group 1 = patients with cCR + molecular remission-WT1-negative; group 2 = patients with cCR but not molecular remission-WT1-positive;

and group 3 = patients with cytological evidence of disease at transplantation-refractory or relapsed), the excellent outcome of patients in group 1 was still more evident (5-year OS and DFS of 70% and 67%, respectively), showing that, in FLT3-positive patients, it is possible to discriminate, through pre-SCT quantitative analysis of WT1, a subset (both in cCR and WT1-negative) with a post-allo-SCT excellent outcome and low probability of relapse (9% of relapse rate in group 1 of our series; Fig. 3A and B and Table 4).

Furthermore, it was possible to identify a second subset of FLT3-positive AML patients at allo-SCT (cCR but still WT1-positive at transplantation, group 2) for whom the post-allo-SCT outcome was extremely unfavorable (median DFS of 8 months) and comparable to that of patients with cytological active disease at the time of transplantation (median OS and DFS of 7 and 5 months, respectively). For group 2 patients with positive molecular WT1-MRD, in which most relapses occurred within 12 months from allo-SCT, it could be possible to consider a more comprehensive transplantation strategy, including a relapse prophylaxis with targeted treatments such as FLT3 inhibitors ± donor lymphocyte infusion or demethylating agents, to be started before allo-SCT in order to obtain MRD negativity (WT1 ± FLT3) or as soon as possible after the allo-SCT procedure. This important predictive role of the WT1 expression at time of transplantation in FLT3-positive AML was also confirmed by the multivariate analysis, in which only the expression amount of WT1 at transplantation and the post-SCT development of mild aGVHD were the significant factors (Table 5).

In summary, the data obtained from the present population (62 FLT3-positive AML patients undergoing allo-SCT at our department of hematology in the last 11 years) confirmed the ability of allo-SCT to overcome the unfavorable prognostic impact of FLT3 mutations. Moreover, our data emphasize the importance of molecular MRD status at the time of transplantation in predicting the outcome and risk of post-allo-SCT relapse and support the recently published data by the Fred Hutchinson Cancer Research Center group, which used a phenotypic analysis (LAIP) as MRD to stratify the patient risk in the pre-SCT setting [37]. In our case, the use of WT1 gene expression as a molecular marker of MRD before transplantation allowed us to optimally stratify FLT3-positive AML patients, showing how the post-allo-SCT outcome is very favorable in patients with cytologic and molecular remission (both in cCR and WT1-negative) in the pre-SCT workup; conversely, patients with cCR who were not in molecular remission (WT1-positive and MRD-positive at the time of allo-SCT) have an extremely unfavorable post-allo-SCT outcome. For these patients, considering the increasing availability of FLT3 inhibitors (sorafenib, midostaurin, quizartinib, lestaurinib, etc), a more comprehensive strategy involving a prophylactic use of these drugs also in the post-allo-SCT phase should be considered in order to reduce the high but predictable incidence of relapses [32,38].

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