Monitoring of donor chimerism in sorted CD34+ peripheral blood cells allows the sensitive detection of imminent relapse after allogeneic stem cell transplantation

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ABSTRACT

Analysis of donor chimerism is an important diagnostic tool to assess the risk of relapse after allogeneic stem cell transplantation, especially in patients lacking a specific marker suitable for monitoring of minimal residual disease. We prospectively investigated the predictive value of donor chimerism analyses in sorted CD34+ peripheral blood cells in 90 patients with acute leukemia and myelodysplastic syndrome. The cumulative incidence of relapse after four years was significantly increased in cases with decreasing or incomplete CD34+ donor chimerism (57% *vs.* 18%, p=0.0001). Multivariate analysis confirmed decreasing CD34+ donor chimerism as an independent predictor of relapse and inferior survival. The interval between a decrease of CD34+ chimerism of less than 80% and hematologic relapse was 61 days (range 0-567). Monitoring of CD34+ donor chimerism in the peripheral blood allows prediction of imminent relapse after allogeneic stem cell transplantation even when a disease-specific marker is lacking.

Key words: donor chimerism, cell sorting, CD34+, allogeneic stem cell transplantation, relapse, leukemia.

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Introduction

In high-risk malignant diseases like acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) or acute lymphoblastic leukemia (ALL), relapse remains the major cause of treatment failure after allogeneic stem cell transplantation (SCT).[1,2] Treatment of post-transplant relapse is difficult and most strategies including donor lymphocyte infusions and second allogeneic transplantation have a limited efficacy in the majority of cases.[3-5]

Therefore, investigators have undertaken various attempts to optimize monitoring of minimal residual disease. But still, only 30-50% of cases with AML and high-risk MDS with an indication for allogeneic transplantation harbor leukemia-specific mutations which can be monitored by sensitive PCR techniques.[6,7] Thus, one could envision that a new technique allowing for serial MRD monitoring in a higher proportion of patients would potentially allow the detection of pending relapse in a higher proportion of patients after SCT. Methods to improve the sensitivity of chimerism analyses include the combination of fluorescence activated cell sorting of specific cellular subpopulations and subsequent chimerism analysis[8] or the use of fluorescence *in situ* hybridization and immunocytochemistry (FICTION).[9] Several groups have applied chimerism analysis in cells expressing the leukemic phenotype, e.g. cells expressing aberrant antigens.[10] CD34 is expressed on blast cells of more than 80% of all cases of AML and more than 50% of all ALL patients. However, sufficient numbers of CD34+ cells for chimerism analysis derived from bone marrow aspirates may be difficult to obtain at short intervals.[11] We have developed a quantitative assay for chimerism analysis requiring low cell numbers which can be used to analyze donor chimerism in CD34+ cells selected from the peripheral blood.

With this study, we confirm the prognostic impact of a decreasing CD34+ peripheral blood donor chimerism in 90 patients with AML, ALL and MDS.

Design and Methods

To establish the diagnostic algorithm, we compared CD34+ sorted peripheral blood cells and CD34+ sorted bone marrow

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cells taken at the same time-point in 36 patients with CD34+ leukemia.

The study cohort consisted of 90 patients receiving allogeneic SCT at three transplant centers between 2000 and 2004. Only patients with AML, MDS and ALL whose disease had been shown to express CD34 by the time of initial diagnosis or at relapse were eligible for the study. *Online Supplementary Table S1* summarizes the patients' characteristics. Disease categories were as follows: AML (n=67), MDS and therapy related leukemia (n=7), ALL (n=16). Patients had received peripheral blood (n=82) or bone marrow (n=8) grafts from sibling (n=35) or unrelated donors (n=55) after intensive (n=50) or reduced-intensity (n=40) conditioning. Recurrent cytogenetic abnormalities had been previously detected in 40 patients.

Disease-specific risk categories were specified according to the FAB classification

AML, ALL in first or second complete remission (CR), refractory anemia (RA) or refractory anemia with ring sideroblasts (RARS) were categorized as low-risk (n=42), AML or ALL not in remission or beyond 2nd CR and refractory anemia with excess of blasts (RAEB/RAEB-t) were defined as high-risk (n=48) with respect to probable treatment-failure and risk of relapse. Informed consent had to be obtained before day 0.

Cell sorting (FACS) and chimerism analyses

CD34+ donor chimerism was analyzed on days 28, 56, 84, 112 as well as 6, 12 and 18 months after transplantation. Pre-enrichment of CD34+ cells using magnetic beads and subsequent fluorescence activated cell sorting was performed as recently described in detail.[12] When sufficient cell numbers were available, between 1,500 and 10,000 CD34+ cells were sorted. The median purity, as measured by repeated FACS analysis, was 98% (range 88-100%). In parallel, overall WB chimerism was analyzed weekly from day 14 to day 56 and every four weeks until 18 months after transplantation out of 5-8 mL peripheral blood.

The methods for DNA isolation and chimerism analysis have been described in detail.[13] Since June 2005 the HumanType Chimera kit (Biotype, Dresden, Germany) was used which amplifies 12 STR loci and the amelogenin locus.

Clinical follow-up

In patients in whom the CD34+ or WB donor percentage fell below 80% and 95%, respectively, bone marrow aspiration and a confirmatory chimerism analysis were performed within the following 7-14 days. Whenever decreasing CD34+ or WB donor signal was confirmed and no signs of active GvHD were present, a rapid taper of immunosupression (50% every 5-7 days) or donor lymphocyte infusions (DLI) were applied according to the treating physician's decision. The median follow-up for all patients was 44 months (range 13-106) by December 15th 2008.

Statistical analysis

Median and ranges are provided for the purpose of descriptive quantitative statistics. The Wilcoxon test was used to compare paired samples obtained from 36 patients at identical time points. Estimates of overall survival (OS) and event free survival (DFS) were obtained by the Kaplan and Meier method.[14] Univariate analyses of the influence of different variables on survival were performed using the log rank statistics. The incidences of relapse and non-relapse mortality (NRM) were calculated using cumulative incidence estimates.[15] NRM and relapse were treated as competing risks. Comparisons between cumulative incidences were assessed by using the Gray test. The impact of the occurrence of decreasing CD34+ chimerism on survival and relapse was tested in a time-dependent Cox regression model.

Results and Discussion

Using the method described above in the 90 study patients, the level of CD34+ chimerism could be determined in 798 out of 863 analyses (91%) performed between day 28 and 18 months after SCT including confirmatory analyses in patients who had shown a decrease below 80%. Figure 1 provides an overview of the number of patients investigated at the pre-defined

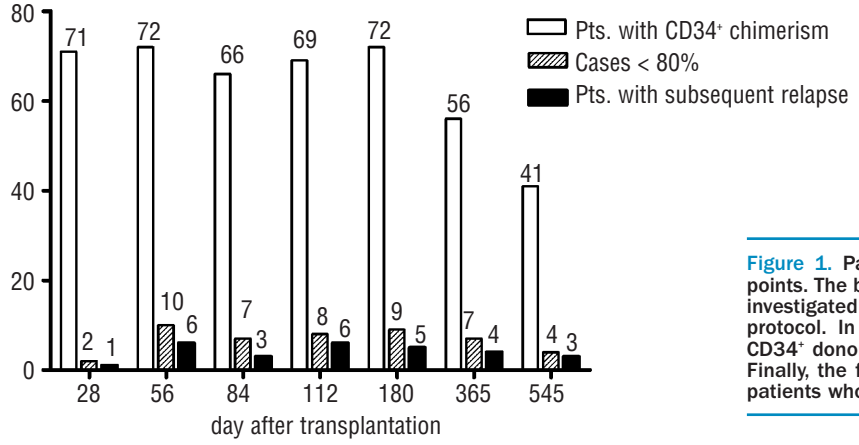

Figure 1. Patients investigated at pre-defined time points. The bar graph shows how many patients were investigated at each time point specified within the protocol. In addition, the number of cases with a CD34+ donor chimerism less than 80% is provided. Finally, the filled black bars quantify the number of patients who subsequently experienced relapse.

time points. On day 28, the blood counts of several patients were too low to be investigated. Of course, patients who had experienced hematologic relapse or transplant-related death could not be included on the respective occasion. The bar graph additionally shows the number of patients with a CD34$^+$ chimerism of less than 80% at each time point including those who subsequently experienced hematologic relapse.

An incomplete or decreasing CD34$^+$ donor chimerism was documented in 28 out of 35 patients before they experienced relapse of leukemia (positive predictive value 80%). In 6 cases, relapses occurred either very late after the last molecular analysis (day 1,152) or at least three weeks after CD34$^+$ chimerism analyses (day 211, 160, 142, 216, and 217, respectively). Only one patient experienced relapse shortly after CD34$^+$ chimerism analysis with a level of more than 80% at that time point (day 117).

The median donor chimerism in CD34$^+$ sorted peripheral blood samples was shown to be significantly lower (median 89.5%, range 1.5-99.5%) than in CD34$^+$ sorted bone marrow cells from the same patient on the same day (median 97.7%, range 0.4-100; p value= 0.0001). Only a very few (n=4) patients had higher chimerism values in the peripheral blood compared to the bone marrow arguing for a higher sensitivity of the assay performed with peripheral blood samples (Figure 1, *Online Supplementary Appendix*). This observation is difficult to explain but may be caused by an earlier release of malignant progenitors compared to donor CD34$^+$ cells and a consequent dominance of healthy donor CD34$^+$ progenitors within the marrow environment. Most impressively, the incidence of relapse after 48 months in patients with a decrease of CD34$^+$ donor chimerism of less than 80% was significantly increased compared to patients with values remaining more than 80% (57% *vs.* 18%; p=0.0001; Figure 2A). The median interval between a decrease in CD34$^+$ chimerism below 80% and hematologic relapse was 61 days (range 0 - 567). Decreasing WB chimerism could be documented only nine days (range 0-478) before hematologic relapse.

As shown in Figure 2 B and C, a decrease of CD34$^+$ donor chimerism below 80% during follow-up was associated with a significantly lower probability of overall (66% *vs.* 32%, p=0.009) and disease free survival (64% *vs.* 27%, p=0.001) at four years after SCT. Interestingly, CD34$^+$ donor chimerism was predictive for DFS in the high-risk as well as in the low-risk group. (Figure 3A and B).

In a Cox regression model including age, risk group, donor type, conditioning intensity and CD34$^+$ donor chimerism above or below 80% performed in all 90 patients only the risk group according to remission status before transplantation and CD34$^+$ donor chimerism turned out to be independent risk-factors for the probability of overall and disease free survival as well as relapse. Hazard ratios and confidence intervals are provided in the *Online Supplementary Table S2*.

One major advantage of our strategy is the use of peripheral blood which allows more frequent analyses compared to the analysis of bone marrow CD34$^+$ cells. Of course, an amount of 40 mL blood still limits the overall number of serial analyses, especially in children, and limits its current use as a routine diagnostic procedure.

The sensitivity of the method in patients with CD34$^+$

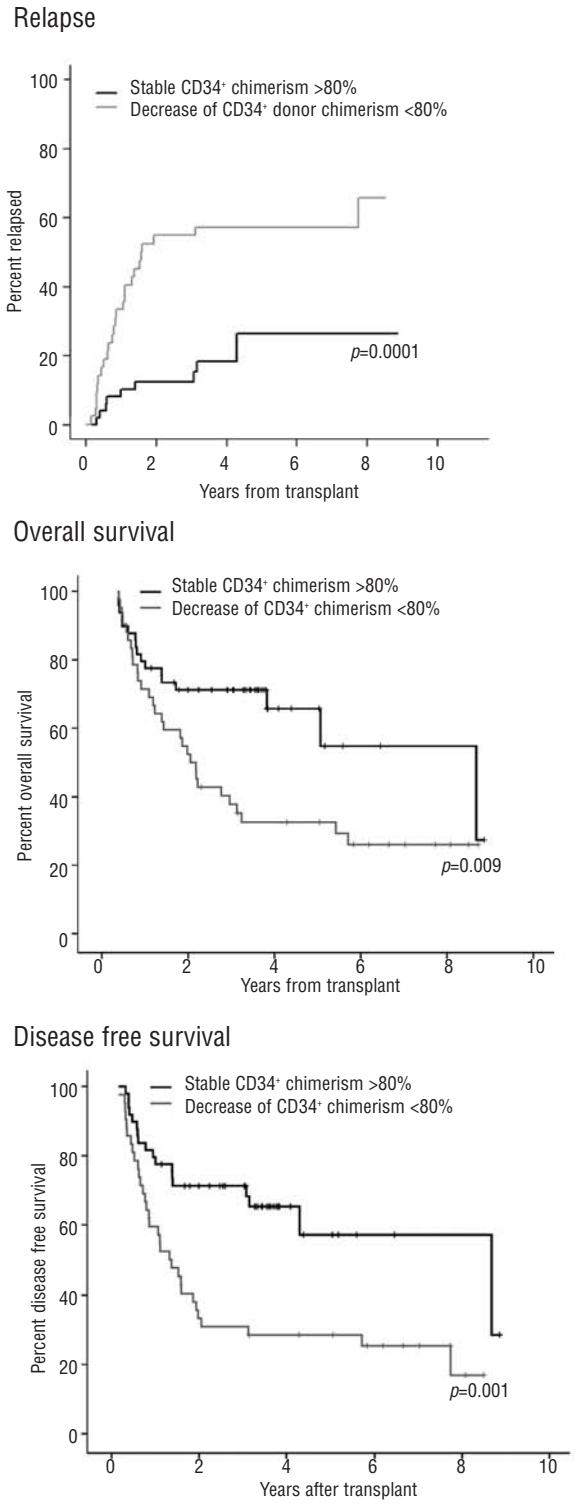

Figure 2. (A) CD34$^+$ donor chimerism and outcome. Patients in whom CD34$^+$ donor chimerism dropped below 80% had a significantly higher incidence of relapse compared to cases with a stable/complete CD34$^+$ chimerism. This was also associated with a significantly lower probability of (B) overall and (C) disease free survival.

leukemic cells achieves levels comparable to that of PCR assays amplifying leukemia-specific transcripts or mutated DNA sequences. In previous analyses we have shown that one in 40,000 leukemic cells can be detected with this technique.[12] Of course, the use of a PCR assay amplifying a leukemia-specific disease marker in cases with bcr/abl-postive ALL or AML with an altered core-binding factor subunit is clearly preferable. But still, one has to keep in mind that in case of core-binding factor positive AML bone marrow aspiration is required to achieve a comparable sensitivity. In our experience, the sensitivity of CD34+ chimerism analysis in peripheral blood is comparable to that observed with a nested PCR in bcr/abl-positive ALL or CML. In cases with recurrent cytogenetic aberrations which were found in about 40% of patients in our study, the respective metaphase analysis or interphase fluorescence *in situ* hybridization (FISH) would have a clearly lower sensitivity. Beside the use of alternative markers for the subset chimerism analyses, a considerable proportion of patients with CD34-negative AML might harbor a nucleophosmin-1 mutation which can nowadays be elegantly used for MRD studies after transplantation.[16]

Previous investigators have reported on their experience with serial analyses of CD34+ chimerism. In the first study, a decrease of CD34+ donor chimerism in the bone marrow (BM) was also associated with an increased risk of relapse.[11] Since only 20 patients could be investigated in an uncontrolled manner, no definitive conclusions on the added benefit of CD34+ chimerism in BM can be drawn. Zeiser and co-workers compared conventional and lineage-specific chimerism analysis in blood and marrow samples.[17] In contrast to our findings, imminent relapse was not detected earlier using the lineage-specific approach. The reason for this difference might in part be due to the lower purity of the CD34+ fraction enriched by immunomagnetic selection only and the consecutively lower sensitivity achieved in their studies. As demonstrated, the use of peripheral blood in our hands is even more sensitive than the analysis of CD34+ selected BM in myeloid malignancies.

In our study the analysis of CD34+ donor chimerism allowed the prediction of relapse in 80% of cases. But still, the experience of our study confirms once again that recurrence of AML or ALL is difficult to prevent by preemptive strategies in the subgroup of patients with decreasing CD34+ donor chimerism even in case of earlier detection. As previously reported, measures to harness graft-versus-leukemia reactions like rapid tapering of immunosuppression or DLI were without long-term efficacy in the majority of cases.[17] In these patients, new strategies including immunomodulatory therapies, small molecules or demethylating agents should be tested in prospective trials.[18-20]

In summary, the results of this study show for the first time that the serial chimerism analysis in CD34+ cells sorted out of peripheral blood is a sensitive technique to detect residual or reoccurring disease after allogeneic SCT. Its use allows the prediction of relapsing disease in most cases with CD34+ leukemia. The major advantage is the less frequent requirement of bone marrow aspiration and the possibility to start preemptive therapeutic interventions earlier. Since the method is time and labor-intensive, it needs further optimization before entering routine use.

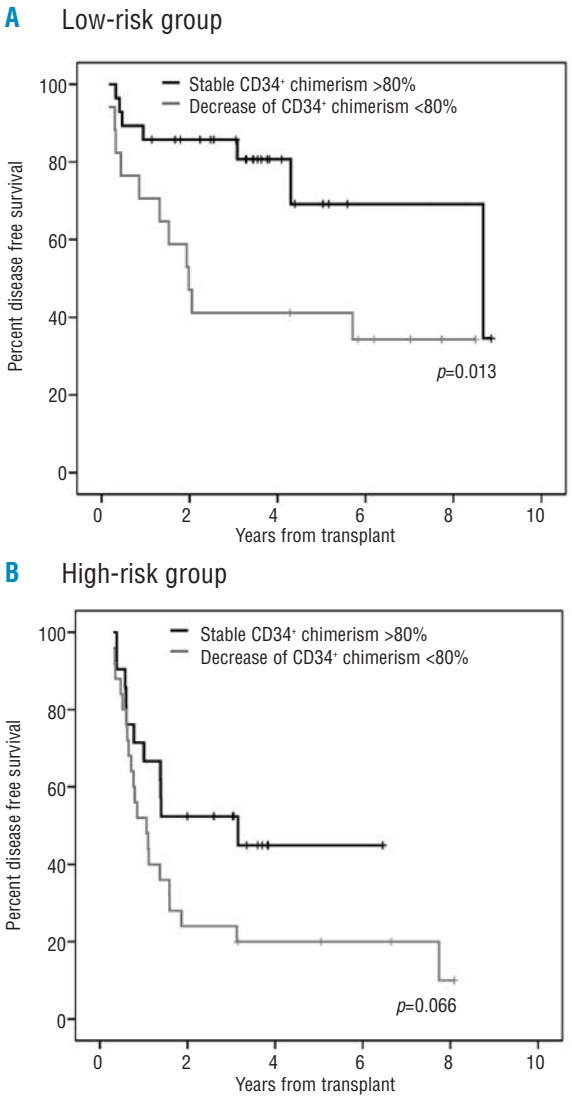

Figure 3. CD34+ donor chimerism in the low- and high-risk group
Disease free survival was influenced significantly by CD34+ donor chimerism both in (A) the low-risk and in (B) the high-risk patient cohort.

Authorship and Disclosures

MB, GE and CT designed and performed research, interpreted data and wrote the manuscript. UO, BM, KL, CT and CB performed research and developed the chimerism assay in sorted cells. CT collected data and performed statistical analyses. GB, UP, MGK, JS, AK, TI, MS, AAF, SK and HM collected data, were involved in patient care and approved the final version of the manuscript.

CT is employed at Agendix, a company performing chimerism analyses. All remaining authors reported no potential conflicts of interest.

References

1. Chang C, Storer BE, Scott BL, Bryant EM, Shulman HM, Flowers ME, et al. Hematopoietic cell transplantation in patients with myelodysplastic syndrome or acute myeloid leukemia arising from myelodysplastic syndrome: similar outcomes in patients with de novo disease and disease following prior therapy or antecedent hematologic disorders. Blood 2007;110:1379-87.
2. Tavernier E, Boiron JM, Huguet F, Bradstock K, Vey N, Kovacsovics T, et al. Outcome of treatment after first relapse in adults with acute lymphoblastic leukemia initially treated by the LALA-94 trial. Leukemia 2007;21:1907-14.
3. Platzbecker U, Thiede C, Freiberg-Richter J, Helwig A, Mohr B, Prange G, et al. Treatment of relapsing leukemia after allogeneic blood stem cell transplantation by using dose-reduced conditioning followed by donor blood stem cells and GM-CSF. Ann Hematol 2001;80:144-9.
4. Schmid C, Schleuning M, Aschan J, Ringden O, Hahn J, Holler E, et al. Low-dose ARAC, donor cells, and GM-CSF for treatment of recurrent acute myeloid leukemia after allogeneic stem cell transplantation. Leukemia 2004;18:1430-3.
5. Schmid C, Schleuning M, Ledderose G, Tischer J, Kolb HJ. Sequential regimen of chemotherapy, reduced-intensity conditioning for allogeneic stem-cell transplantation, and prophylactic donor lymphocyte transfusion in high-risk acute myeloid leukemia and myelodysplastic syndrome. J Clin Oncol 2005;23:5675-87.
6. Kern W, Haferlach C, Haferlach T, Schnittger S. Monitoring of minimal residual disease in acute myeloid leukemia. Cancer 2008;112:4-16.
7. Ogawa H, Tamaki H, Ikegame K, Soma T, Kawakami M, Tsuboi A, et al. The usefulness of monitoring WT1 gene transcripts for the prediction and management of relapse following allogeneic stem cell transplantation in acute type leukemia. Blood 2003;101:1698-704.
8. van Leeuwen JE, van Tol MJ, Bodzinga BG, Wijnen JT, van der KM, Joosten AM, et al. Detection of mixed chimaerism in flow-sorted cell subpopulations by PCR-amplified VNTR markers after allogeneic bone marrow transplantation. Br J Haematol 1991;79:218-25.
9. Baurmann H, Nagel S, Binder T, Neubauer A, Siegert W, Huhn D. Kinetics of the graft-versus-leukemia response after donor leukocyte infusions for relapsed chronic myeloid leukemia after allogeneic bone marrow transplantation. Blood 1998;92:3582-90.
10. Mattsson J, Uzunel M, Tammik L, Aschan J, Ringden O. Leukemia lineage-specific chimerism analysis is a sensitive predictor of relapse in patients with acute myeloid leukemia and myelodysplastic syndrome after allogeneic stem cell transplantation. Leukemia 2001;15:1976-85.
11. Scheffold C, Kroeger M, Zuehlsdorf M, Tchinda J, Silling G, Bisping G, et al. Prediction of relapse of acute myeloid leukemia in allogeneic transplant recipients by marrow CD34+ donor cell chimerism analysis. Leukemia 2004;18:2048-50.
12. Thiede C, Bornhauser M, Oelschlagel U, Brendel C, Leo R, Daxberger H, et al. Sequential monitoring of chimerism and detection of minimal residual disease after allogeneic blood stem cell transplantation (BSCT) using multiplex PCR amplification of short tandem repeat-markers. Leukemia 2001;15:293-302.
13. Thiede C, Florek M, Bornhauser M, Ritter M, Mohr B, Brendel C, et al. Rapid quantification of mixed chimerism using multiplex amplification of short tandem repeat markers and fluorescence detection. Bone Marrow Transplant 1999;23:1055-60.
14. Kaplan E, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457-62.
15. Lin DY. Non-parametric inference for cumulative incidence functions in competing risks studies. Stat Med 1997;16:901-10.
16. Bacher U, Badbaran A, Fehse B, Zabelina T, Zander AR, Kroger N. Quantitative monitoring of NPM1 mutations provides a valid minimal residual disease parameter following allogeneic stem cell transplantation. Exp Hematol 2009;37:135-42.
17. Zeiser R, Spyridonidis A, Wäsch R, Ihorst G, Grüllich C, Bertz H, et al. Evaluation of immunomodulatory treatment based on conventional and lineage-specific chimerism analysis in patients with myeloid malignancies after myeloablative allogeneic hematopoietic cell transplantation. Leukemia 2005;19:814-21.
18. Platzbecker U, Mohr B, von BM, Binder M, Schetelig J, Ehninger G, et al. Lenalidomide as induction therapy before allogeneic stem cell transplantation in a patient with proliferative CMML-2 and del(5q) not involving the EGR1 locus. Leukemia 2007;21:2384-5.
19. Graef T, Kuendgen A, Fenk R, Zohren F, Haas R, Kobbe G. Successful treatment of relapsed AML after allogeneic stem cell transplantation with azacitidine. Leuk Res 2007;31:257-9.
20. Stone RM, Deangelo DJ, Klimek V, Galinsky I, Estey E, Nimer SD, et al. Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. Blood 2005;105:54-60.