

## ORIGINAL ARTICLE

# Chimerism analysis within 6 months of allogeneic stem cell transplantation predicts relapse in acute myeloid leukemia

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**The role of chimerism analysis as a prognostic indicator of relapse after hematopoietic stem cell transplantation (SCT) is controversial. We monitored chimerism status by short tandem repeat-based polymerase chain reaction (PCR) in T- and non-T-cell subsets and retrospectively evaluated clinical outcome in 96 patients with acute myeloid leukemia after myeloablative (MA) or reduced-intensity conditioning SCT. Fifty-six percent of 80 patients in the MA group demonstrated complete donor chimerism (CC) at all time points, whereas 6% had decreasing mixed chimerism (MC), 8% stable MC, 25% increasing MC and 3% increasing and decreasing MC. In 16 RIC patients, these percentages were 12, 50, 6, 6 and 19, respectively, together with 6% nonengraftment. Forty-three out of 96 patients experienced relapse. The last chimerism evaluation before relapse revealed increasing MC in only eight patients. In samples taken between 1 and 6 months post SCT, CC/decreasing MC was significantly related with a lower risk of relapse (31 versus 83%,  $P < 0.000$ ) and mortality (38 versus 83%,  $P < 0.000$ ) than with MC/increasing MC. However, the development of relapse was very rapid. Only very frequent monitoring of chimerism status by highly sensitive methods might identify impending relapse and allow early immunological intervention.**

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## Introduction

Chimerism analysis is an important tool to assess the origin of lymphohematopoietic cells after stem cell transplantation (SCT). Discrimination between donor- and recipient-

derived hematopoiesis allows evaluation of engraftment. However, it is controversial whether chimerism status is also a prognostic indicator of relapse.

Reliable monitoring of chimerism became possible with the development of fluorescent *in situ* hybridization in sex-mismatched transplantation in the late 1980s.<sup>1,2</sup> Since the development of DNA techniques, analysis of highly polymorphic short tandem repeats (STRs) or variable number tandem repeats (VNTRs) has increased the sensitivity for detecting small numbers of donor or recipient cells. Apart from full or complete donor chimerism (CC), a 2001 international workshop recognized split chimerism (one cell lineage complete donor and another complete host) and mixed chimerism (MC).<sup>3</sup> In the latter case, it is important to determine whether the percentage of recipient DNA is stable, increasing or spontaneously decreasing over time.

In the past decade, more than 40 studies have addressed the possible role of chimerism analysis in the detection of minimal residual leukemia after SCT. Whether relapse can be detected early enough for useful intervention in the form of immunotherapy is highly dependent on the sensitivity of the technique. STR- or VNTR-based PCR has a moderate sensitivity of 1–5%,<sup>4–7</sup> compared with the conventional morphologic definition of relapse as  $\geq 5\%$  leukemic blasts in the bone marrow. The sensitivity of STR- and VNTR-based methods can be increased by the use of lineage-specific cell populations. However, this is labor intensive and expensive. Amplification of translocation breakpoints would be the most sensitive tool, but is only possible in a minority of acute leukemia cases. It was recently reported that real-time PCR for the SRY gene on the Y chromosome allows the detection of 1:100 000 female donor cells in a male recipient.<sup>8</sup>

Apart from differences in the sensitivity of the applied methods, the majority of published papers are about chimerism status in pediatric patients or have focused on chronic myeloid leukemia. The kinetics of leukemic cells in these populations can probably not be extrapolated to those of adults with acute myeloid leukemia (AML). Moreover, the significance of MC after SCT has become even more complicated since the introduction of reduced-intensity conditioning (RIC) SCT.

In the current study, we analyzed the correlation between MC in T and non-T cells and clinical outcome

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in 96 patients with AML, after either myeloablative (MA) partially T-cell-depleted SCT or RIC unmanipulated SCT.

## Patients and methods

### Patients

Between January 1993 and October 2004, 120 consecutive AML patients received either allogeneic bone marrow transplantation or peripheral blood SCT at our institution. The conditioning MA regimen consisted of cyclophosphamide (60 mg/kg/day for 2 days) followed by total body irradiation (TBI) (600 cGy/day for 2 days) with partial shielding of the lungs (total lung dose 850 cGy). The graft was partially T-cell depleted,<sup>9</sup> consisting of  $1-2 \times 10^5$  T cells/kg and was infused after the second TBI fraction. RIC SCT patients received fludarabine (30 mg/m<sup>2</sup>) and 200 cGy TBI, followed by a non-T-cell-depleted graft. Antithymocyte globulin (Thymoglobulin, Sangstat, Amstelveen, The Netherlands) was given to matched-unrelated donor (MUD) patients before cyclophosphamide or fludarabine was infused, at a total dose of 20 mg/kg until April 1999 and 8 mg/kg thereafter.

Post-transplant immunosuppression consisted of cyclosporin monotherapy after MA conditioning or cyclosporin in combination with mycophenolate mofetil in the case of RIC. In the absence of active graft-versus-host disease (GVHD), post-transplant immunosuppression was discontinued within 3 months of MA SCT or 6 months of RIC SCT. GVHD was diagnosed according to the Seattle criteria<sup>10</sup> and treated with 1–2 mg/kg/day prednisolone and resumption of full-dose immunosuppression if applicable. Donor lymphocyte infusions (DLIs) were administered in case of relapse at a dose of  $0.01-1.0 \times 10^8$  T-cells/kg.

### PCR analysis

Peripheral blood samples were collected from the donor and the recipient before SCT and scheduled at 1, 2, 3, 6, 9, 12, 18, 24 and 48 months post SCT. T and non-T cells were separated from peripheral blood samples by Automated Magnetic Cell Sorting (Auto-MACS, Miltenyi Biotec, Utrecht, The Netherlands).

Chimerism analysis was performed by PCR-based amplification of STR sequences. Briefly, DNA was isolated from T- and non-T-cell fractions of peripheral blood samples using the salting out method.<sup>11</sup> STR chimerism analysis was performed as described previously<sup>12</sup> by using the STR markers SE33, HUMTHO, HUMVWFA, FGA, D3S1358, D19S253 and D11S554. The two most informative markers (discriminatory between donor and recipient) of these seven STR markers were used for chimerism screening after SCT using ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

### Definition of chimerism status and relapse

CC was defined as the presence of only donor cells at all time points post SCT. Recipient signals immediately post SCT, which decreased spontaneously during follow-up; were classified as decreasing MC. An increase of  $\geq 5\%$  recipient signal between sequential assessments was defined

as increasing MC. MC could not be further classified in case of a single measurement or because of fluctuations in the recipient signal of  $< 5\%$ . Relapse included the appearance of more than 5% blasts in the bone marrow or new extramedullary leukemic lesions.

### Statistical methods

Differences between patients receiving MA or RIC regimens were analyzed by the Pearson  $\chi^2$  test. Continuous variables were compared by the Student's *t*-test. Univariate and multivariate analyses were performed by logistic regression. The prevalence of chronic GVHD could be calculated for patients surviving  $> 100$  days. Overall and disease-free survival was estimated by Kaplan–Meier analysis. For all tests, a two-sided *P*-value of  $\leq 0.05$  was considered statistically significant. Calculations were performed using SPSS/PC + 12.0 (SPSS Inc., Chicago, IL, USA).

## Results

### Characterization of patients and chimerism status

Analysis of chimerism status could be performed in 96 out of 120 patients. In 18 patients, follow-up took place in another hospital. In three patients, a pre-transplant DNA sample of donor or recipient was missing. One patient who received two allogeneic transplantations was excluded. Two patients died before the first sample was taken. Median follow-up was 102 weeks after MA SCT and 93 weeks after RIC SCT. Patient and transplantation characteristics are shown in Table 1.

Follow-up of chimerism status was performed until 48 months post SCT. Samples collected after the time of documented relapse or administration of DLI were not included in the analysis. CC at all time points was documented in 56% of patients after MA conditioning and in only 12% of the RIC SCTs (see Table 2). After MA SCT, MC was observed in 44% of patients, compared with 81% of patients after RIC SCT. MC could be further classified as decreasing or increasing in 6 and 25% of the MA group, respectively. For RIC patients, the percentage of decreasing and increasing MC was 50 and 6, respectively. Clinically, it is probably most relevant to group patients with CC and decreasing MC together as well as those with stable and increasing MC. There was no difference between CC/decreasing MC after MA or RIC SCT (65 versus 62%, respectively). Nonengraftment occurred in one patient directly after RIC, whereas another patient had increasing MC, resulting in nonengraftment after 90 days.

### Cell line specificity of chimerism status

In the majority of patients, the percentage of recipient signals was similar in both T and non-T cells. In nine patients (9%), there was a discrepancy in chimerism status between T and non-T cells of  $> 5\%$ . All of these had received an MA transplantation. In these cases, we determined final chimerism status by the cell line with the highest percentage of recipient cells (T cells in four and non-T cells in five patients). This could have resulted in

**Table 1** Patient characteristics

Characteristics	MA SCT	RIC SCT
Total no. of patients	80	16
Male	36 (45%)	9 (56%)
Female	44 (55%)	7 (44%)
Age (years)		
Median	37	60
Range	18–55	38–69
AML risk		
Low	51 (64%)	11 (69%)
High	29 (36%)	5 (31%)
Type of transplantation		
Sib-BMT	26 (32%)	0
MUD-BMT	16 (20%)	0
Sib-PBSCT	24 (30%)	12 (75%)
MUD-PBSCT	14 (18%)	4 (25%)
aGVHD		
Grade 0–1	53 (66%)	9 (56%)
Grade 2–4	27 (34%)	7 (44%)
cGVHD		
No/not applicable	49 (61%)	7 (44%)
LD/ED	31 (39%)	9 (56%)
Relapse		
Yes	37 (46%)	6 (38%)
No	43 (54%)	10 (62%)
Disease-free survival (weeks)		
Median	130	102
Range	11–623	5–167
Death		
Yes	39 (49%)	6 (38%)
No	41 (51%)	10 (62%)
Overall survival (weeks)		
Median	156	133
Range	16–623	5–167

Abbreviations: AML=acute myeloid leukemia; aGVHD=acute graft-versus-host disease; BMT = bone marrow transplantation; cGVHD = chronic graft-versus-host disease; CR1 = low risk; CR2 = high risk; ED = extensive disease; LD = limited disease; MA = myeloablative; MUD = matched-unrelated donor; PBSCT = peripheral blood stem cell transplantation; RIC = reduced-intensity conditioning; SCT = stem cell transplantation; sib = sibling.

ambiguities of chimerism status in four patients. We encountered technical problems due to stutter signals in only one patient.

#### Chimerism, relapse and survival

Overall, 43 patients (45%) experienced relapse. Median time to relapse was 31 weeks after MA SCT (range 11–134 weeks) and 20 weeks after RIC SCT (range 5–102 weeks). Table 3 shows the chimerism status of MA and RIC groups at the last evaluation before relapse and at the time of relapse. Remarkably, seven patients were CC when overt relapse was established by bone marrow analysis. An increasing number of recipient cells could be detected before relapse in only eight patients. Seven of these patients

**Table 2** Overall chimerism status

Chimerism status	MA SCT	%	RIC SCT	%
Complete donor	45	56	2	12
Complete patient	0	0	1	6
Mixed chimerism				
Decreasing MC	5	6	8	50
Increasing MC	20	25	1	6
Stable MC	8	10	1	6
i- and d-MC	2	3	3	19

Abbreviations: MA = myeloablative; MC = mixed chimerism; RIC = reduced-intensity conditioning; SCT = stem cell transplantation. During 48 months post SCT and aborted after relapse and/or donor lymphocyte infusion.

**Table 3** Chimerism status and relapse

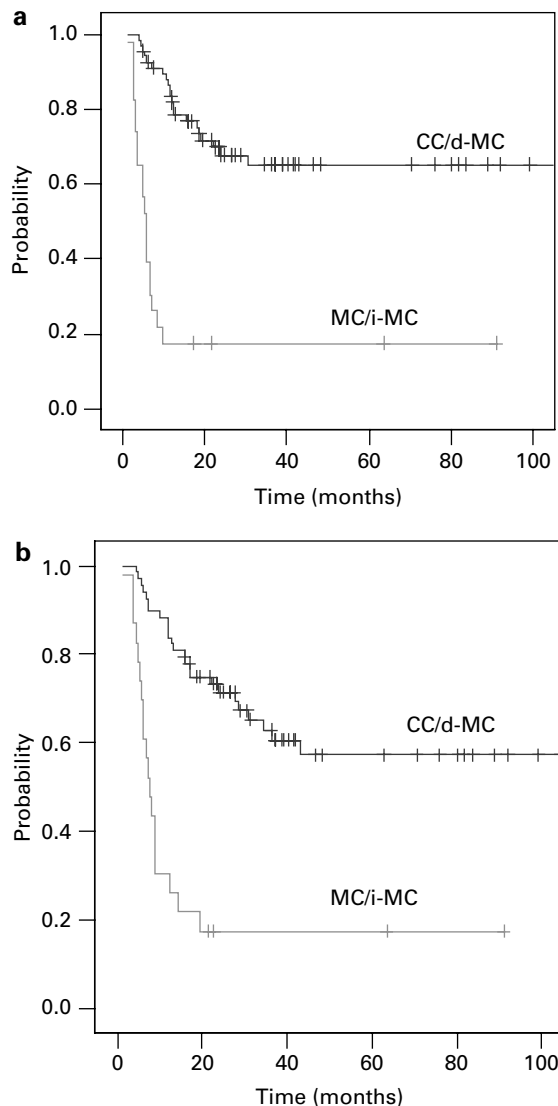
	No. of patients	Chimerism status					
		Last evaluation before relapse					
		CC	d-MC	MC	i-MC	CP	NE
Myeloablative SCT	37	20	0	7	7	0	3
RIC SCT	6	2	0	2	1	1	0
		At the time of relapse					
		CC	d-MC	MC	i-MC	CP	NE
Myeloablative SCT	37	6	0	4	16	0	11
RIC SCT	6	1	0	0	2	1	2

Abbreviations: CC = complete donor chimerism; CP = complete patient; d-MC = decreasing mixed chimerism; i-MC = increasing mixed chimerism; MC = mixed chimerism; NE = not evaluated; RIC = reduced-intensity conditioning; SCT = stem cell transplantation.

had received MA conditioning and the time interval between the detection of increasing MC and relapse was 1 week in two, 2 weeks in three, 4 weeks in one and 5 weeks in another patient. One RIC patient had spontaneously increasing and decreasing values and finally relapsed after 87 weeks.

#### Prognostic value of chimerism with respect to clinical outcome

As the last chimerism evaluation before relapse was indicative in only a very small subset of patients, we tried to establish whether serial chimerism analysis within a certain time interval would be more informative. For this model, we focused on the samples taken between 1 and 6 months post SCT as the vast majority of relapses occurred within the first year of SCT. As the percentage of patients developing CC/decreasing MC within this period was similar after both MA and RIC conditioning, we grouped these together. Patients with CC/decreasing MC in samples taken between 1 and 6 months post SCT had a higher incidence of acute graft-versus-host disease (aGVHD) grade 2–4 (40 versus 22%,  $P=0.14$ ) and chronic graft-versus-host disease (cGVHD) (47 versus 22%,  $P=0.05$ ) than patients demonstrating MC/increasing MC. Those with CC/decreasing MC between 1 and 6 months post SCT



**Figure 1** Probability of remaining free of relapse (a) and survival (b). In patients demonstrating complete chimerism (CC) or decreasing mixed chimerism (d-MC) as compared to (increasing) mixed chimerism within 6 months post stem cell transplantation.

had a significantly lower risk of relapse (31 versus 83%,  $P < 0.000$ ) and mortality (38 versus 83%,  $P = 0.000$ ) (see Figure 1). Moreover, in the (increasing) MC groups, all but one death were relapse-related.

Table 4 shows the outcome of the univariate analysis on prognostic indicators of relapse and mortality. The risk of relapse was significantly increased with age  $\leq 40$  years ( $P = 0.02$ ), MUD donor type ( $P = 0.04$ ), grade 0–1 aGVHD ( $P < 0.000$ ), the absence of cGVHD ( $P = 0.04$ ) and MC/increasing MC in samples taken between 1 and 6 months post SCT ( $P = 0.000$ ). There was also a significant relationship between mortality and no/low grade aGVHD, absence of cGVHD and MC/increasing MC between 1 and 6 months post SCT.

The uncommon finding of poor outcome with MUD donor type was attributable to poor patient characteristics

(previous autologous SCT in 12 versus 0% of sibling SCT and 65% patients in CR2+ versus 19% of sibling SCT,  $P = 0.002$  and  $P = 0.000$ , respectively). Moreover, MUD-SCT was performed in younger patients ( $\leq 40$  years in 71% versus 37% in sibling-SCT,  $P = 0.000$ ). There was no difference in the percentage of MUD transplantations between the CC/decreasing MC and the MC/increasing MC group (35 versus 30%,  $P = 0.80$ ).

In subsequent multivariate analysis, CC/decreasing MC in samples taken between 1 and 6 months post SCT ( $P = 0.000$ ), aGVHD  $\geq$  grade 2 ( $P = 0.004$ ) and age  $> 40$  years ( $P = 0.007$ ) emerged as significant factors predicting lower risk of relapse. Moreover, mortality was significantly decreased in patients demonstrating CC/decreasing MC between 1 and 6 months post SCT in this multivariate analysis ( $P = 0.000$ ).

## Discussion

Our analysis of nearly 100 AML patients focused on the association between MC and clinical outcome after partial T-cell-depleted MA or unmanipulated RIC SCT. (Increasing) MC is associated with relapse in the majority of these patients, and especially patients who do not develop CC or decreasing MC within 6 months of SCT are at significant risk of relapse and mortality. However, the development of relapse is very rapid. Even in T- or non-T-cell subsets, STR-based PCR techniques are not sensitive enough to detect minimal residual disease in AML and generally do not allow therapeutic manipulations aiming to prevent clinically overt relapse.

As has been suggested before,<sup>13</sup> the kinetics of acute leukemia in adults are too rapid to be diagnosed with a detection limit of 1%. Most studies that identified MC as a useful prognostic indicator of relapse have been reported in different patient populations, namely children and chronic myeloid leukemia (CML) patients (for recent reviews see Thiede *et al.*, 2004<sup>13</sup> and Bader *et al.*, 2005<sup>14</sup>). In a direct comparison, Guimond *et al.*<sup>15</sup> demonstrated that only in children and not in adults, MC in T and natural killer (NK) cells was found at the time of relapse. In young children, T-cell regeneration is thymus-dependent, whereas in adults, T-cell reconstitution relies primarily on expansion of peripheral T cells. As these are more alloreactive, the authors hypothesized that in adults, residual normal host cells can be eliminated more effectively, so that no host T and NK cells were detectable at relapse. CML cells differ from acute leukemic cells in their slower proliferation rate and moreover, detection of the BCR-ABL fusion gene is nowadays more informative than chimerism status in this condition. Table 5 shows an overview of previous reports on the association between MC and relapse in adult patients with AML.<sup>16–22</sup> It is important to note that the two studies that did not find a relation between MC and relapse did not distinguish between stable and increasing MC.

The current study describes a large group of AML patients treated with a partially T-cell-depleted SCT protocol. T-cell depletion results in a higher incidence of MC,<sup>23–25</sup> but the association with relapse is controversial. One group demonstrated a relation between myeloid MC

**Table 4** Univariate analysis of prognostic indicators of relapse and mortality

	Relapse (n = 43)			Mortality (n = 47)		
	OR	95% CI	P	OR	95% CI	P
<i>Age</i>						
≤40 years, n = 47	1.0			1.0		
>40 years, n = 49	0.31	0.16–0.83	0.01	0.72	0.32–1.60	0.42
<i>Risk</i>						
Low, n = 62	1.0			1.0		
High, n = 34	1.67	0.72–3.87	0.23	1.85	0.80–4.32	0.15
<i>Type of donor</i>						
Sibling, n = 62	1.0			1.0		
MUD, n = 34	2.42	1.03–5.70	0.04	1.28	0.56–2.96	0.56
<i>Type of SCT</i>						
MA, n = 80	1.0			1.0		
RIC, n = 16	0.70	0.23–2.10	0.52	0.57	0.19–1.72	0.32
<i>aGVHD</i>						
Grade 0–1, n = 62	1.0			1.0		
Grade 2–4, n = 34	0.15	0.52–0.40	0.00	0.42	0.18–0.99	0.046
<i>cGVHD</i>						
None/NA, n = 56	1.0			1.0		
LD/ED, n = 40	0.42	0.18–0.97	0.04	0.38	0.16–0.87	0.02
<i>Chimerism 2–6 months</i>						
CC/d-MC, n = 68	1.0			1.0		
MC/i-MC, n = 23	10.6	3.2–35.1	0.00	7.67	2.35–25.1	0.00

Abbreviations: aGVHD = acute graft-versus-host disease; CC = complete donor chimerism; cGVHD = chronic graft-versus-host disease; CI = confidence interval; CR1 = low risk; CR2+ = high risk; d-MC = decreasing mixed chimerism; i-MC = increasing mixed chimerism; ED = extensive disease; LD = limited disease; MA = myeloablative; NA = not applicable; MUD = matched-unrelated donor; OR = odds ratio; RIC = reduced intensity conditioning; SCT = stem cell transplantation.

**Table 5** Studies on the association between MC and relapse in adults with AML and MDS

Study	No. of AML patients	No. of AML patients with MC	Method	Outcome <sup>a</sup>
Miura (2006)	21/70	variable <sup>b</sup>	PCR microsatellite primer, lineage-specific cells	Positive
Barrios (2003)	68/133	10/26	PCR-VNTR	Positive
Schaap (2002)	53/231	3/19	PCR-STR, cell subsets	Negative
Mattson (2001)	30/30	14/14	PCR-VNTR, cell separation according to leukemia phenotype <sup>c</sup>	Positive
Wasch (2000)	39/101	NR/25	PCR-VNTR, separation of MNC and granulocytes	Positive
Choi (2000)	15/30	7/14	PCR-STR	Negative
Najfeld (1997)	7/27	NR	Interphase FISH with dual-color XY probes	Positive

Abbreviations: AML = acute myeloid leukemia; FISH = fluorescent *in situ* hybridization; MC = mixed chimerism; MDS = myelodysplastic syndrome; MNC = mononuclear cells; NR = not reported; PCR = polymerase chain reaction; STR = short tandem repeat; VNTR = variable number tandem repeat.

<sup>a</sup>Association between MC and relapse.

<sup>b</sup>Depending on time point and type of SCT (myeloablative versus RIC).

<sup>c</sup>In contrast, no correlation between T-cell MC and relapse.

and relapse in myeloid malignancies whereas T-cell MC was associated with graft rejection.<sup>26</sup> Another recent study assessed the significance of long-term MC after partially T-cell-depleted SCT in acute leukemia.<sup>21</sup> The authors found that especially in patients treated with a less intensive conditioning regimen, persistent MC after 12 months post SCT was not associated with relapse. In our study, however, many cases of relapse occurred within this period and only four out of 33 patients with (increasing) MC did not experience relapse. One of these patients had received a RIC SCT.

Initial MC is a common finding after RIC SCT.<sup>27</sup> Several groups have recently evaluated the relation between MC and clinical outcome after RIC SCT. Most studies found that in RIC patients, MC could be demonstrated at the time of acute GVHD<sup>28</sup> and regression of disease.<sup>28–31</sup> Others, however, demonstrated that acute GVHD and disease regression occurred only after CC was achieved.<sup>32</sup> None of these analyses focused on AML patients. In a multivariate analysis of more than 300 patients with hematological malignancies, achievement of CC was associated with significantly decreased risk of relapse.<sup>33</sup>

This study focused on the ultimate development of CC and did not involve the time interval post SCT, which turned out to be most informative in our evaluation. Another study in a heterogeneous patient population also demonstrated a negative impact of MC on relapse and overall survival, whereas the role of MA conditioning versus RIC did not reach statistical significance.<sup>34</sup> In our analysis, the development of CC/decreasing MC was similar after both MA and RIC regimens (65 versus 62%), in spite of an initially higher incidence of MC after RIC SCT.

Another important finding of our study is that different outcomes in T- and non-T-cell fractions were observed in 9% of patients. Separation of T and non-T cells is labor-intensive and expensive, and our results do not support its routine use in chimerism analysis of AML patients. Moreover, in a few cases, CC in peripheral blood was established at the time of clinical relapse in the bone marrow. The sensitivity of the technique may be increased by the use of lineage-specific analysis of cell subsets enriched for cells that may contain minimal residual disease, such as cells with the immunophenotype of the initial leukemia cells.<sup>3</sup>

In spite of the retrospective nature of our study, our data illustrate that the kinetics of chimerism after SCT in adult AML patients are different from those previously reported in adults or children with ALL or in CML patients. It is noteworthy that AML patients who do not develop CC or decreasing MC within 6 months of SCT have a high risk of pending relapse and mortality. Ideally, intensive monitoring of chimerism status would allow pre-emptive immunotherapy in patients with (increasing) MC, aiming to prevent disease relapse. However, relapse develops very rapidly. Early detection of increasing patient signals, therefore, requires very frequent measurements. Likewise, more sensitive methods than STR-based PCR in T- and non-T-cell subsets would have to be applied. Incorporation of these strategies would be labor-intensive and costly but deserves further study because identification of impending relapse and attempts to early immunological intervention might contribute to improved success of SCT in AML patients.

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