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Very early lineage-specific chimerism after reduced intensity stem cell transplantation is highly predictive of clinical outcome for patients with myeloid disease

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Running head: Early chimerism predicts outcome after HSCT

Highlights

- PBMC and T cell chimerism 50 days after allogeneic-HSCT identifies three patient groups.
- Day 50 mixed chimerism in PBMC and T cells predicts early disease relapse in myeloid disease.

Abstract

Background: The importance of chimerism status in the very early period after hematopoietic stem cell transplantation is unclear. We determined PBMC and T-cell donor chimerism 50 days after transplantation and related this to disease relapse and overall survival.

Methods: 144 sequential patients underwent transplantation of which 90 had AML/MDS and 54 had lymphoma. ‘Full donor chimerism’ was defined as $\geq 99\%$ donor cells and three patient groups were defined: 40% with full donor chimerism (FC) in both PBMC and T-cells; 25% with mixed chimerism (MC) within both compartments and 35% with ‘split’ chimerism (SC) characterised by full donor chimerism within PBMC and mixed chimerism within T-cells.

Results: In patients with myeloid disease a pattern of mixed chimerism (MC) was associated with a one year relapse rate of 45% and a five year overall survival of 40% compared to values of 8% and 75%, and 17% and 60%, for those with SC or FC respectively. The pattern of chimerism had no impact on clinical outcome for lymphoma.

Conclusion: The pattern of lineage-specific chimerism at 50 days after transplantation is highly predictive of clinical outcome for patients with myeloid malignancy and may help to guide subsequent clinical management.

Keywords: chimerism, AML, allogeneic-HSCT, relapse

Abbreviations: FC-full donor chimerism, SC- split donor chimerism, MC- mixed donor chimerism.

Introduction

Reduced intensity conditioned (RIC) allogeneic haematopoietic stem cell transplantation (HSCT) is an established curative strategy for high risk haematological malignancies¹⁻³. However, disease relapse remains the major cause of treatment failure and occurs in approximately 35% of patients³. The establishment of the graft versus leukaemia (GvL) effect and maintenance of long term disease control are both thought to depend upon development of a donor T cell-mediated allogeneic immune response but this also underlies the mechanism of graft-versus-host disease (GvHD)⁴. Whilst T-cell depletion of the graft reduces the risk of both acute and chronic GvHD⁵⁻⁷ this is also associated with an increased risk of disease relapse and a reduction in relapse-free survival⁸.

Anti-thymocyte globulin (ATG) and alemtuzumab are alternative options for T cell depletion during transplant conditioning. Alemtuzumab typically leads to more intensive T cell depletion but both agents are associated with an increased incidence of mixed donor chimerism. Importantly, the presence of mixed chimerism at day 100 after transplant is strongly prognostic for subsequent clinical outcome⁹⁻¹¹. Studies with ATG-conditioned RIC HSCT have shown that mixed T-cell donor chimerism at day 100 or a decline in donor chimerism between day 30 and day 180 are associated with an increased rate of disease relapse^{12,13}. Stable mixed chimerism following alemtuzumab-conditioned RIC HSCT has also been associated with an improvement in survival¹⁴ or protection from GvHD¹⁵⁻¹⁷. However it is currently unclear if the pattern of chimerism at an earlier time-point could also be predictive as this would have substantial value in guiding subsequent clinical management.

We determined the degree of chimerism within both the PBMC and T-cell fractions at day 50 after alemtuzumab-conditioned RIC transplantation and related this to clinical outcome in patients with myeloid disease (AML/MDS) and lymphoma. We demonstrate that this information has strong prognostic value for the subsequent risk of disease relapse and overall survival in patients with myeloid disease.

Methods

HSCT regimens

144 consecutive patients undergoing a T-cell depleted and reduced intensity conditioned HSCT between 2009-2014 were studied. No patients relapsed prior to day 100 post transplant. In 139 patients (96.5%) alemtuzumab was administered at 10mg/day from day -5 pre-HSCT for 5 days and a further 5 patients (3.5%) received the same dose from day-14 to day -10. 121 patients received fludarabine (30mg/m² for 5 days) plus melphalan (140mg/m² for 1 day). 18 patients received carmustine (300mg/m² for 1 day), etoposide (200mg/m² for 4 days), cytarabine (200mg/m² for 4 days), and melphalan (140mg/m² for 1 day) and 3 of this group also received fludarabine (30mg/m² for 3 days). 5 patients received fludarabine (30mg/m² for 6 days), cytarabine (2g/m² for 4 days), amsacrine (100mg/m² for 4 days) and busulphan (3.2mg/kg for 4 days). The conditioning regime was not found to be an independent predictor of clinical outcome and so all patients were included in the study. All patients received post transplant ciclosporin A for GvHD prophylaxis. Anti-microbial prophylaxis and viral monitoring were carried out according to standard institutional policies. Escalating doses of DLI were administered to patients who displayed sustained mixed donor T-cell chimerism <95% at 6 months post-transplant.

Clinical Outcomes

The primary outcomes of interest were overall survival and time to disease relapse. Grades 2-4 acute GvHD and chronic GvHD were also recorded according to consensus conference criteria and national institutes of health criteria respectively^{18,19}. Bone marrow aspirate and trephines were performed on all patients at 100 days after transplant in order to assess engraftment and confirm remission.

Chimerism analysis

Analyses of PBMC and T-cell chimerism were performed at 50, 100, and 180 days post-transplant. As the conditioning regimen included T-cell depletion, day 50 was chosen as opposed to day 30 to ensure sufficient T-cell yields to perform microsatellite analysis for chimerism analysis. Analyses

were undertaken on whole blood derived PBMC and on purified CD3⁺ T-cell fractions, which were separated from PBMCs using magnetic bead selection (Miltenyi Biotec). The degree of donor/host chimerism was determined by multiplex PCR of microsatellite markers by applying 5 fluorescently labelled primer pairs for the loci MBP (A and B), FGA, D18S391, D18S386 and D13S634. 2µl PCR product was loaded onto a 6% polyacrylamide gel on an ABI-373 gene scanner. Relative heights of donor and host cells in the sample were calculated based on the peak heights and areas of informative alleles and had a detection limit of 1%¹⁷.

Statistical methods

Differences in mean values of donor chimerism were assessed using 2-way ANOVA. The effect of all baseline variables (Table 1) upon overall survival was estimated using the Kaplan-Meier method. Log-rank tests were used to compare survival curves. Cox proportional hazard regression multivariate analysis was used to identify independent predictive factors of overall survival. Forward stepwise models were constructed using 10% entry and exit criteria. The effects of all baseline variables on time to event outcomes with competing risks (relapse, non-relapse mortality and GvHD) were estimated by cumulative incidence analyses. Outcomes were tested with the Gray method²⁰ where ‘death from any other cause’ was the respective competing risk. Non-relapse mortality was defined as the number of days from the date of transplantation until death from causes other than disease relapse. Multivariate adjustment for predictive variables was performed using this competing risks framework²¹. Day 50 PBMC and T-cell chimerism were treated as baseline variables using a landmark analysis which measured the time from chimerism measurement to the event.

All multivariate analyses included baseline variables significantly associated with outcome according to univariate analysis (Supplementary table 2) along with day 50 chimeric status. All P values are 2-sided and with significance levels of <0.05. All calculations were performed with R 2.14.1 (the R project for Statistical computing) or GraphPad Prism. Survival graphs were generated using R 2.14.1 (the R project for Statistical computing).

Results

Patient characteristics

All 144 patients received PBMC allografts mobilised with GCSF and incorporated *in vivo* T-cell depletion with alemtuzumab prior to the transplant. 90 patients underwent HSCT for AML/MDS whereas 54 had HL or NHL as a primary disease. A matched sibling donor graft was used in 63 transplants and 81 patients received a matched unrelated donor graft (Table 1). 109 (76%) patients were alive at a median follow up of 1006 days (range 90-3773 days). Disease relapse was observed in 37 (26%) patients. Over the study period, 37 (26%) developed acute GvHD (grades II - IV) and 17 (12%) patients developed chronic GvHD (\geq moderate severity). DLI was administered at 6 months post transplant for patients who did not achieve full donor chimerism with 32 (22%) patients eventually receiving DLI. The overall survival (OS) of all 144 patients was 71% (Supplementary table 1).

The combined pattern of PBMC and T-cell chimerism identifies three groups of patients

We first analysed the pattern of chimerism within both PBMC and T cells at day 50 following transplant. Patients with $\geq 99\%$ donor chimerism were defined as ‘full donor chimerism’ and patients with $\leq 98\%$ donor chimerism were defined as ‘mixed donor chimerism’.

When the percentage of donor chimerism within the PBMC compartment was plotted against the donor chimerism within the T-cell fraction three distinct groups of patients could be distinguished on the basis of full or mixed chimerism in either the PBMC or T cell compartments (Figure 1a). The largest group consisted of 58 patients who displayed full donor chimerism in both the PBMC and T-cell fractions (FC) whereas 36 patients represented a second group who demonstrated a pattern of mixed chimerism (MC) in both PBMC and T-cells. Interestingly, the third group of 50 patients demonstrated a pattern of full donor chimerism within the PBMC fraction but mixed donor chimerism within the T cell fraction, which we have termed ‘split donor chimerism’ (SC). We did not observe

any patients who exhibited full donor chimerism in T cells and mixed chimerism in the PBMC fraction. Similar proportions of patients with AML/MDS or lymphoma were found to exhibit FC (42% & 37% respectively), SC (33% & 35%) or MC (25% & 27%) (Figure 1b).

By univariate analysis the use of a matched unrelated donor (p 0.035*), a female donor with gender mismatch (p 0.016*) and an HSCT comorbidity index (HCT-CI) score of <1 (p 0.031*) were all associated with FC but only the use of a matched unrelated donor remained as an independent predictor following multivariate analysis (HR 4.367 p 0.043*).

The pattern of lineage-specific chimerism is predictive of the risk of disease relapse in patients with myeloid disease

In order to assess whether the classification of patients into FC, MC and SC groups was predictive of clinical outcome we next compared the cumulative incidence of relapse and overall survival in each group. In patients with myeloid disease the relapse rate, at median follow up of 1006 days, was increased in the MC group (56%; C.I 28%-70%) compared to patients in the FC group (46%; C.I 24%-63%, p 0.075) or SC group (31%; C.I 3%-52%, p 0.0046; Figure 2a). The SC group demonstrated the lowest rate of relapse, with just 8% of patients with myeloid disease relapsing in the first year, but this did not reach statistical significance compared to the FC group. In contrast, chimerism was not predictive of relapse in patients with lymphoma (Figure 2b). Univariate analysis found that the only baseline variables to associate with relapse were; chimerism pattern at day 50, disease type (myeloid or lymphoid), and use of a gender mismatched donor (Supplementary table 2). However on multivariate analysis, which adjusted for these factors in a competing risk analysis against 'death without relapse', there was a strong and independent impact of the pattern of day 50 chimerism on the subsequent risk of disease relapse (MC v SC, HR 6.42, p 0.004**; MC v FC, HR 2.32 p 0.057; FC v SC, HR 0.36 p 0.11) (Table 2). Whilst there was no evidence that patients within the MC cohort had an increased prevalence of intermediate or high risk disease as defined by the disease risk index²², subgroup analysis of the myeloid patients revealed that adverse risk cytogenetics²³ also predicted for

relapse (Supplementary figure 1). However, there was no observed correlation between cytogenetic risk and chimerism status at day 50 (pearson's R 0.226, p 0.06 ns).

Current clinical practice generally recommends the delivery of prophylactic donor lymphocyte infusions to those patients who demonstrate sustained mixed chimerism at day 180 after transplant in order to promote full donor chimerism and reduce risk of relapse. However, we observed that 41% of myeloid patients with MC at day 50 had already relapsed by day 180, compared to only 3% and 8% of those in the SC and FC groups respectively (data not shown). Determination of the pattern of PBMC and T cell chimerism at day 50 could therefore of considerable value in identifying patients with myeloid disease at risk of early relapse and may have significant value in guiding their subsequent clinical management.

For those patients with mixed chimerism who do not demonstrate relapse at 6 months post transplant, the median time to DLI was 264 days (n=17), compared to the 362 days for patients with split chimerism (n=20, Supplementary figure 2a). Importantly, this demonstrates that a delay to DLI within the MC group does not contribute to their inferior outcome. In terms of response to DLI, all patients, irrespective of MC or SC status, demonstrated sequential increments to donor T-cell chimerism levels following DLI (Supplementary figure 2b). However, patients with MC maintained a significantly lower level of donor chimerism throughout follow up (p 0.02*), failing on average to increment > 80% donor. This group also failed to increment donor PBMC chimerism over the course of 1 year (Supplementary figure 2c) which remained lower than donor PBMC chimerism in SC patients (p<0.0001****).

The pattern of lineage-specific chimerism at day 50 is predictive of overall survival in patients with myeloid disease

We next investigated if the pattern of chimerism at day 50 was also predictive for overall survival. Of note, mixed chimerism (MC) at day 50 was associated with a markedly inferior survival of only 40% at 5 years in patients with myeloid disease. This compared to values of 75% in SC patients (HR

0.83, C.I 0.48 - 4.16; p 0.01* figure 2c) and 60% in patients with FC (HR 0.52, C.I 0.23 - 1.136; p 0.1). In contrast, chimerism was not predictive of survival in patients with lymphoma although the 20 patients who achieved FC by day 50 had a very favourable outcome with 96% survival at 9 year follow up (figure 2d).

To further examine the nature of the association between MC status at day 50 and reduced survival in patients with myeloid disease we next assessed the incidence of relapse mortality, GvHD and non-relapse mortality. A 4.7 fold increased rate of relapse mortality was seen in MC patients compared to SC patients (p 0.001**) with a 2 fold increase compared to FC patients (p 0.0355* data not shown). In contrast, no differences were observed in the rates of acute GvHD (grade 2 or above), moderate or severe chronic GvHD or non-relapse mortality (data not shown). This indicates that the reduced survival in myeloid patients with MC status at day 50 is due primarily to death from disease relapse.

Univariate analyses of baseline variables showed that disease and the use of a matched unrelated donor were also individually associated with overall survival (Supplementary table 2) but incorporation of these factors into a cox regression model of survival confirmed the independent and strong adverse impact of mixed chimerism status at day 50 on overall survival (MC v SC, HR 2.865, p 0.004**; MC v FC, HR 2.532 p 0.009**; FC v SC, HR 1.28 p 0.533) (Table 2).

Discussion

It has generally been assumed that successful allogeneic stem cell transplantation requires the complete replacement of the host hemopoietic and immune compartments by donor cells. However, T-cell depletion often leads to a state of mixed chimerism and the impact of this on the potency of the donor alloreactive immune response and ultimate clinical outcome remains unclear. As the median time to relapse in RIC transplant is less than 12 months after transplant³ it is crucially important to identify such patients at an early stage in order to deliver potential therapeutic interventions.

Previous studies have found that a decline in donor T cell chimerism is a risk factor for relapse in T replete RIC HSCT²⁴⁻²⁷ and ATG-conditioned RIC HSCT^{12,13}. In contrast, mixed T cell chimerism in

alemtuzumab-conditioned T cell-depleted RIC HSCT has not been associated with an increased risk of disease relapse^{14,15,17,28}. A major finding of our study was that the combined pattern of PBMC and T-cell chimerism at day 50 revealed three distinct groups of patients with different clinical outcomes in alemtuzumab-conditioned RIC HSCT (Figure 2a). Importantly, in patients with myeloid disease (AML/MDS) mixed chimerism in both the PBMC and T-cell fractions was associated with a very high rate of early disease relapse which reached 41% at only 6 months, rising to 45% by 12 months (Figure 3a). This translated into a five year overall survival of only 40% (Figure 3c). MC is therefore strongly predictive of early disease relapse although this pattern then stabilises after 12 months. In contrast, patients with FC or SC showed a pattern of less frequent and later disease relapse with improved overall survival. It was notable that the pattern of chimerism was only predictive of clinical outcome in patients with myeloid disease. Similar findings have been observed previously and may reflect differential mechanisms of relapse prevention according to disease subtype^{14,29}. Relapse rates were substantially lower in patients with lymphoid disease, at 0%, 22% and 14% at two years in those with FC, SC and MC respectively.

Assessment of PBMC chimerism in patients with myeloid disease therefore appears to be of significant prognostic value for relapse, whilst monitoring chimerism kinetics may remain important for lymphoma patients following alemtuzumab-conditioned RIC HSCT³⁰.

It might be expected that very early detection of mixed chimerism within the PBMC compartment might indicate early evidence of disease relapse. However we did not observe any correlation between the chimerism status at day 50 and the presence of tumour populations in samples of bone marrow at day 100 (data not shown) and it may therefore be the case that the detrimental impact of very early mixed chimerism is related to immunological priming of the graft versus leukaemia effect rather than overt tumour cell proliferation. Whilst it would be informative to correlate early chimerism patterns with measurable residual disease, it is notable that dendritic cells established from monocytes of patients with mixed chimerism demonstrate a reduced capacity to stimulate donor alloreactive T-cells³¹. Similarly, we have recently observed an increased expression of PD-L1 exclusively on donor derived

dendritic cells in the context of mixed chimerism³², suggesting that this donor population may have a key role in priming the T-cell driven allogeneic immune response. Alternatively, given the profound T-cell depletion achieved with *in vivo* alemtuzumab, NK cells may have a role in mediating the GvL effect following alemtuzumab-conditioned RIC HSCT³³, and their activity in different chimeric states remains unclear. Importantly, these biological variables are likely to contribute differentially to the clinical importance of T-cell chimerism in the setting of T-replete, ATG-conditioned or alemtuzumab-conditioned RIC HSCT.

Given the prognostic value of the pattern of chimerism at such an early stage after transplant it will be important to identify factors that may determine this profile³⁴. Within our cohort there was no significant correlation between chimerism status and ELN cytogenetic stratification, but a trend was observed towards a higher proportion of patients with mixed chimerism having ‘adverse’ cytogenetic profiles (supplementary figure 1b. pearson’s R 0.226, p 0.06 ns, ref 23). Recent insights into the mutational status of AML at relapse post transplant highlight that these can facilitate evasion of the allogeneic immune response³⁵, but it is not clear whether initial adverse cytogenetics also increases this risk. There was no difference in the total dose of ciclosporin given to each group within the first 50 days (data not shown) and chimerism was also not related to the nature of transplant conditioning. Only the use of a matched unrelated was found to be an independent predictor of full donor status at day 50. Recipient / donor CMV sero-status has also been shown to be an important determinant of mixed T-cell chimerism when the recipient is CMV positive but the donor CMV negative³⁶ and there may be other intrinsic factors related to either the patient or donor, or the nature of prior chemotherapy that determine the level of donor engraftment at this early time-point²⁴.

DLI is generally highly effective in this cohort²⁸ and our center employs unselected T cell DLI as standard of care for patients with T-cell donor chimerism <95% donor at 6 months following transplant. Escalating doses are employed until donor chimerism > 98%. Importantly, our study shows how patients with mixed chimerism (mixed in both purified T-cell and whole PBMC fractions) exhibit a significantly increased rate of relapse even prior to the application of DLI, constituting a group

of patients with an unmet need. Overall, our findings show that assessment of the pattern of chimerism at a very early time-point after transplant can identify patients with myeloid disease who are at very high risk of early relapse following alemtuzumab-conditioned RIC HSCT. Indeed, 41% of patients with mixed chimerism had already suffered disease relapse by the time that donor lymphocyte infusions (DLI) become available at 6 months. As such, novel clinical approaches should be investigated in this group to prevent early disease relapse. Early administration of DLI is one potential option although associated with a significant risk of GvHD³⁷. Alternative approaches might include the use of agents such as azacitidine^{38,39} or T cell checkpoint blockade⁴⁰⁻⁴².

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Authorship

Contributions. FK designed the study and prepared the manuscript. FK and AG collected, and analysed the data. FK, CI, TC, and DM performed statistical analyses and reviewed the manuscript. KW and MG performed chimerism analysis for patients. GMcI, SN, JN, and KH collected data and reviewed the manuscript. JZ, MG, CC, EN, PM, and RM reviewed the manuscript.

Conflict of Interest

None of the authors have interests that conflict with the work herein.

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Authorship

Contributions. FK designed the study and prepared the manuscript. FK and AG collected, and analysed the data. FK, CI, TC, and DM performed statistical analyses and reviewed the manuscript. KW and MG performed chimerism analysis for patients. GMcI, SN, JN, and KH collected data and reviewed the manuscript. JZ, MG, CC, EN, PM, and RM reviewed the manuscript.

Conflict of Interest

None of the authors have interests that conflict with the work herein.

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Figure 1. Pattern of PBMC and T-cell donor chimerism at day 50 .

- a. Bubble chart demonstrating PBMC and T-cell donor chimerism at day 50. 3 groups were identified. The first group (n=58, shown in black), displayed ‘full donor’ chimerism (FC) where both PBMC and T-cell donor chimerism $\geq 99\%$. The second group (n=50, shown in clear circles) were mixed only in the T-cell fraction, and termed ‘split’ donor chimeras (SC). They exhibited PBMC chimerism $\geq 99\%$, but T-cell chimerism $\leq 98\%$. The final group (MC, n=36, shown in grey) demonstrated mixed donor chimerism in both PBMC and T-cell fractions, i.e. where both PBMC and T-cell chimerism $\leq 98\%$. The area of each bubble represents the number of events (patients). To allow visual distinction of the data at axes limits (i.e. 1.00) donor chimerism levels were expressed as fractions and transformed using an asinh square root function. Dashed lines indicate the assay detection limit of 1% (i.e. 99% donor chimerism on both axes).
- b. Patterns of chimerism at day 50 in patients with myeloid or lymphoid disease .

Figure 2. Clinical outcome based on pattern of PBMC and T-cell donor chimerism at day 50

a. Disease relapse in patients with AML or MDS **b.** Disease Relapse in patients with lymphoma

c. Overall survival patients with AML or MDS **d.** Overall Survival in patients with lymphoma.

p values of cumulative incidence or survival are shown for significant associations only.

Table 1. Baseline patient characteristics.

Variable	Category	N	%
All patients		144	100
Age	Median & range	52.8 years (22-71)	
Gender	Male	92	63.9
	Female	52	36.1
Gender mismatch	Yes	68	47.2
	No	76	52.8
HLA match	10/10	132	91.7
	9/10	12	8.3
Donor Type	Sibling	63	43.8
	Matched unrelated	81	56.2
Diagnoses	AML & MDS	90	62.5
	HL & NHL	54	37.5
Disease status	CR	95	66
	PR	37	25.7
	Untreated	12	8.3
Disease risk index ¹⁹	Low	40	27.8
	Intermediate	28	19.4
	High	57	39.6
	Unknown	19	13.2
HCT-CI Score	0	101	70.1
	1	18	12.5
	2	18	12.5
	>3	7	4.9
CMV serostatus (recipient/donor)	+/+	51	
	+/-	43	
	-/+	8	
	-/-	42	
Conditioning Regimens	Flu/Mel	121	84
	BEAM	15	12.5
	Flu/BEAM	3	2.1
	FLAMSA/Bu	3	2.1
	FLAMSA	2	1.4
GvHD prophylaxis	CsA	134	93
	CsA + MTX	8	5.6
	CsA + MMF	2	1.4

Table 2. Chimerism status at day 50 remains an independent predictor of relapse and overall survival in multivariate analyses

Outcome	variable	HR	95% C.I lower	95% C.I. upper	p value
Relapse incidence					
	SC v FC	0.36	0.10	1.28	0.11
	MC v FC	2.32	0.98	5.54	0.057
	MC v SC	6.42	1.84	22.40	0.004**
	Disease	3.03	1.43	6.40	0.02*
	Gender mismatch	1.87	0.97	3.59	0.07
Overall Survival					
	FC v SC	1.28	0.567	2.89	0.533
	MC v FC	2.53	1.30	6.33	0.009**
	MC v SC	2.87	1.34	4.79	0.004**
	Disease	5.23	1.78	15.3	0.001**
	Donor type	4.02	1.50	10.75	0.002**

Table 2. Baseline characteristics or chimerism status at Day 50 (with a univariate $p < 0.1$ association with a relapse or overall survival were included in multivariate models to assess for independent prediction $p < 0.05$ in table, in bold.).

Fine and Grey regression method was used for relapse incidence, to take competing risk into account. Cox proportional hazard regression analysis was used to identify independent associations with overall survival. FC, full donor chimerism. SC, split donor chimerism. MC, mixed donor chimerism. Disease, (myeloid v lymphoid). HR, hazard ratio. CI, confidence interval.