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ORIGINAL ARTICLE

Transforming growth factor-β1 functional polymorphisms in myeloablative sibling hematopoietic stem cell transplantation

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Hematopoietic stem cell transplantation (HSCT) with sibling donors (s.d.) is a life-saving intervention for patients with hematological malignancies. Numerous genetic factors have a role in transplant outcome. Several functional polymorphisms have been identified in *TGF-β1* gene, such as single-nucleotide polymorphism (SNP) at +29C > T within exon 1. Two hundred and forty five patient/donor pairs who underwent a s.d. HSCT in our centers were genotyped for this SNP. In the myeloablative cohort, +29CC donors were associated with an increase in severe chronic GvHD (32% vs 16%, hazard ratio (HR) 9.0, $P = 0.02$). Regarding survival outcomes, +29CC patients developed higher non relapse mortality (NRM) (1–5 years CC 28–32% vs TC/TT 7–10%; HR 5.1, $P = 0.01$). Recipients of +29TT donors experienced a higher relapse rate (1–5 years TT 37–51% vs TC 19–25% vs CC 13%–19%; HR 2.4, $P = 0.01$) with a decreased overall survival (OS) (1–5 years TT 69–50% vs TC/CC 77–69%; HR 1.9, $P = 0.05$). Similar to previous myeloablative unrelated donors HSCT results, we confirmed that +29CC patients had higher NRM. In addition we found that +29TT donors might be associated with a higher relapse rate and lower OS. These results should be confirmed in larger series. Identification of these SNPs will allow personalizing transplant conditioning and immunosuppressant regimens, as well as assisting in the choice of the most appropriate donor.

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) offers a unique opportunity for long-term disease control to many patients with severe malignant or non-malignant hematopoietic disease otherwise incurable with standard chemotherapy. The outcome has improved significantly over the last decade and HSCT is currently the standard of care for several high-risk diseases.[1–2] This ongoing improvement is being achieved through better supportive care and changes in conditioning and immunosuppressant regimes.[3]

Although HLA is the most important barrier for this procedure,[4] other highly polymorphic genes have been associated with significant impact on transplant outcome.[5–8]

Transforming growth factor β1 (TGF-β1) is involved in the regulation of numerous immunomodulatory processes. It is secreted by T cells and platelets, endothelial cells and other cell types.[9] Apart from pro-fibrotic properties[10] TGF-β1 functions mainly as an immunomodulatory cytokine. It inhibits proliferation and activation of T-effector cells,[11,12] induces proliferation of regulatory T cells (Tregs)[13,14] by an upregulation of FOXP3[15] and is part of an important pathway used by Tregs to inhibit the immune system through secretion or membrane bound expression of the cytokine.[13,14,16,17]

There have been identified many functional polymorphisms and single-nucleotide polymorphisms (SNPs) in the *TGF-β1* gene. Probably the most studied are SNP present at codon 10 (coding (c).29C > T, protein(p).P10L, rs1800470) and codon 25 (c.74G > C, p.A25P, rs1800471) within exon 1. Both SNPs are located in the signal peptide in the center of a core consisting of a sequence normally made of 8–15 hydrophobic amino acids.[18,19] This peptide, that is cleaved from the COOH terminal in the trans Golgi, is crucial for the secretion process.[19–21] This change between Proline (an indifferent or less hydrophobic amino acid) to Leucine (highly hydrophobic) may modify the tertiary structure of the protein and therefore affect the secretion process. Although conflicting data have been published regarding the impact of these SNPs and TGF-β1 plasma levels,[22–28] +29C allele has been mainly described as high producer. This allele has been associated with an increased risk of several solid organ cancer[26,29] as well as a better outcome following renal transplantation.[30]

We have previously published an association of +29C > T SNP and HSCT from unrelated donors (UD).[31] Patients with +29CC genotype experienced a higher non relapse mortality (NRM) and reduced overall survival (OS) compared with the TC and TT genotypes. The impact of this polymorphism on the outcome of sibling donor HSCT is on debate as several studies have given conflicting results, especially in aGvHD.[32–34] To date there is no published evidence in large s.d. HSCT cohorts that evaluates the possible role of these SNPs and survival transplant outcome including their relationship with NRM, OS and relapse. We hypothesized that these SNP may have a significant impact

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on s.d. HSCT outcome like our previous observation in HSCT UD.[31]

MATERIALS AND METHODS

Patients selection

A cohort of 245 patient/donor pairs who underwent a s.d. HSCT in four Argentinean centers were genotyped for the presence of the SNP at +29 and +74 of *TGF-β1* gene. Transplants took place between January 2000 and December 2014 and the median follow up time was 4.4 years.

Ethical approval

The present study was conducted according the declaration of Helsinki, and approved by local institutional ethics committee. After anonymisation of the subjects, the IRB allowed the investigators not to take written informed consent for DNA analysis. DNA was obtained from pre-transplant HLA stored samples.

Genotyping methods

Sequence-specific-primer as described by Perrey *et al.*[35] was the PCR genotyping method. The internal control for the PCR was human ß-globin (*HBB*). PCR reaction consisted of: 200 ng genomic DNA, +29 primers 0.6 μM, +74 primers 0.5 μM, *HBB* primers 0.8–0.9 μM, 100 μM dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl pH 8.3 and 0.45 U DNA Taq polymerase (Promega, Madison, WI, USA) in a final volume of 25 μl. The thermocycler consisted of initial denaturation at 95 °C for 3', followed by 10 cycles of 95 °C for 30'', 65 °C for 50', 72 °C for 45'', 25 cycles of 95 °C for 30'', 59 °C for 50'' and 72 °C for 45'' followed by a final extension of 72 ° C for 3'. The products were analyzed by a 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The target bands size was 240 bp for +29, 233 bp for +74 and 630 bp for *HBB*.

Statistical analysis

For the statistical analysis we used SPSS version 23 (SPSS Inc., Chicago, IL, USA) and R version 3.3.0 (http://r-project.org). In univariate analysis, aGvHD, chronic GvHD (cGvHD), cause of mortality, 100-day mortality and 1-year transplant-related mortality we used the X^2-test. OS and disease-free survival (DFS) were compared using log-rank (Kaplan–Meier), relapse and NRM with Gray's test using the cumulative incidence (CI) (competing event for relapse was death without relapse and for NRM was relapse). Regarding multivariate analysis (MA), logistic regression was used for dichotomic variables and Cox regression for survival including all the factors with a *P*-value < 0.2 after the univariate analysis. Outcomes were considered to be significant with a two-sided *P*-value of < 0.05, and a trend between 0.05 and 0.1. We calculated a sample size for the entire cohort of 200 transplants, assuming 66% myeloablative conditioning and a difference of 20% in the main outcome incidences.

Table 1. Cohort characteristics ($N = 245$)

	N *(%)*
Patients, age (mean 32 years)	
$<$ 40 years	161 (66)
$\geqslant$ 40 years	84 (34)
Donors, age (mean 34 years)	
$<$ 30 years	65 (37)
$\geqslant$ 30 years	112 (63)
Missing	68
Patients, sex	
Male	146 (60)
Female	99 (40)
Donors, sex	
Male	144 (59)
Female	101 (41)
Diseases	
AML	69 (28)
ALL	57 (23)
MDS	31 (13)
Lymphoproliferative	31 (13)
MPD	22 (9)
Other	35 (14)
Stage	
Early	94 (44)
Late	119 (56)
Missing	32
Conditioning	
Myeloablative	157 (64)
RIC	88 (36)
Conditioning II	
BuCy	79 (32)
TBI-Cy (myeloablative)	55 (22)
Other myeloablative Bu based	20 (8)
Fludarabine based (RIC)	64 (26)
TimoCy	11 (5)
Other	15 (6)
Missing	1
Immunosuppressant	
Tacrolimus + Mtx	133 (57)
Cyclosporine + Mtx	42 (18)
Other	49 (25)
None	8 (3)
Missing	13
Source	
PBSC	212 (88)
Bone marrow	28 (12)
Missing	5

Abbreviations: MDS = myelodysplastic syndrome; MPD = myeloproliferative disorders; RIC = reduced intensity conditioning.

RESULTS

Patient and donor characteristics are listed in Table 1. Main diagnoses were AML (28%), acute lymphoid leukemia (ALL) (23%), myelodysplastic syndrome (13%) and lymphoproliferative disease (13%). Ninety-four patients (44%) were at early stage (defined as acute leukemia and myelodysplastic syndrome in first CR, CML in chronic phase and severe aplastic anemia as first-line therapy), 64% of the patients received myeloablative conditioning regimens. Ninety-eight percent of the donors were HLA full-matched; the source was peripheral blood (PBSC) in 88% of patients, 97% received post-transplant immunosuppression, 57% of these received tacrolimus plus methotrexate and 18% cyclosporine A plus methotrexate (Table 1).

Patients' +29C>T genotype observed frequencies were CC 22.5%, TC 55% and TT 22.5% (C allele frequency 0.50 and T 0.50) and for the donors were 20%, 56% and 24%, respectively (C allele frequency 0.47 and T 0.53). For +74G>C genotype, patients frequencies were GG 89% and GC/CC 11% and for the donor were 91% and 9%, respectively. These frequencies were similar to healthy volunteer donor (130 healthy volunteers, CC 37, 29%, TC 53, 41% and TT 40, 31% (C allele frequency 0.49 and T 0.51). There was no deviation according to Hardy-Weinberg equilibrium.

GvHD

Acute GvHD was graded as none (grade 0), clinically significant (grades II–IV) and severe (grades III–IV). The overall incidence of aGvHD was 44%, GII–IV 31% and GIII–IV 10%. Chronic GvHD incidence was 35%, limited in 18% and extensive in 17%. No significant impact of the studied SNPs was observed within the entire cohort as well as in the reduced intensity conditioning subgroup.

Within the myeloablative setting aGvHD incidence was 49%, GII–IV 38% and GIII-IV 10%. +29TT recipients had a significant increase in aGvHD (67% vs 45%, hazard ratio (HR) 5.6, 95% CI 1.1–30.1, $P = 0.04$) and aGvHD GII–IV (48% vs 35%, HR 5.5, 95% CI 1.1–27.3, $P = 0.03$). Interestingly, +29CC recipients had more aGvHD GIII–IV (22% vs 7.2%, $P = 0.01$) although this was NS in

Table 2. Acute GvHD incidence depending patients and donors TGF-β1 +29 genotype

	aGvHD any grade (%)	P *(univ)*	*MVA HR (95% CI)*	*aGvHD II–IV (%)*	P *(univ)*	*MVA HR (95% CI)*	*aGvHD III–IV (%)*	P *(univ)*	*MVA HR (95% CI)*
Patient's genotype									
p CC	53	0.04	NS	47	0.10	NS	22	0.03	NS
p TC	41			30			5.1		
p TT	67			48			12.5		
p CC	53	0.65	NS	47	0.24	NS	22	0.01	NS
p TC-TT	49			35			7.2		
p TT	67	**0.02**	**5.6 (1.1–30)**	48	**0.16**	**5.5 (1.1–27)**	12.5	0.74	NS
P TC-CC	45			35			10		
Donor's genotype									
d CC	43	0.21	NS	35	0.25	NS	13	0.31	NS
d TC	45			33			12		
d TT	62			50			3		
d CC	43	0.51	NS	33	0.55	NS	13	0.51	NS
d TC-TT	50			39			9		
d TT	62	0.07	NS	50	0.09	NS	3	0.16	NS
d TC-CC	44			34			12		

Abbreviations: CI = cumulative incidence; d = donor; HR = hazard ratio; MVA = multivariate analysis; NS = not significant; p = patient. The bold entries indicate the significant *P* values.

Table 3. Chronic GvHD incidence depending on patient and donor TGF-β1 +29 genotype

	cGvHD (%)	P *(univ.)*	*MVA HR (95% CI)*	*Severe cGvHD (%)*	P *(univ.)*	*MVA HR (95% CI)*
Patient's genotype						
p CC	44	0.71	NS	22	0.44	NS
p TC	38			16		
p TT	45			26		
p CC	44	0.69	NS	22	0.67	NS
p TC-TT	40			18		
p TT	45	0.56	NS	26	0.30	NS
P TC-CC	39			17		
Donor's genotype						
d CC	55	0.18	NS	32	0.01	NS
d TC	36			12		
d TT	39			29		
d CC	55	0.07	NS	32	**0.05**	**9.0 (1.3–62)**
d TC-TT	37			16		
d TT	39	0.79		29	0.15	
d TC-CC	41			17		

Abbreviations: CI = cumulative incidence; d = donor; HR = hazard ratio; MVA = multivariate analysis; NS = not significant; p = patient. The bold entries indicate the significant *P* values.

MA (HR 2.2, 95% CI 0.7–7.5, $P = 0.18$). Patients receiving cells from +29TT donors had a trend to a higher aGvHD incidence (62% vs 44%, $P = 0.08$) and aGvHD GII–IV (50% vs 34%, $P = 0.09$) (Table 2).

The incidence of cGvHD was 40%, limited in 21% and extensive in 19%. Patient's genotype showed no differences, however recipients from a +29CC donors had a significant increase in extensive cGvHD (32% vs 16%, MA HR 9.0, 95% CI 1.3–62, $P = 0.02$) (Table 3).

Survival analysis

The OS and NRM in the entire cohort at 5 years were 51% and 17%, respectively, relapse incidence was 36% and DFS 46%. No significant impacts on these parameters were observed when the SNPs were analysed neither in the full cohort nor the reduced intensity conditioning sub-analyses.

However, patients' genotype showed a significant impact on survival outcomes in the myeloablative cohort. In this group, 5-year NRM incidence was 15%, OS 56%, relapse 33% and DFS 52%. Patients +29CC had a significant increase in 100-day mortality (19% vs 7%, HR 3.2, 95% CI 1.01–10.2, $P = 0.04$), 1-year transplant-related mortality (29% vs 7%, HR 7.1, 95% CI 1.3–37.1, $P = 0.02$) and NRM (1–5 years CC 28–32% vs TC/TT 7–10%, Gray's test $P < 0.01$; HR 5.1, 95% CI 1.36–19.2, $P = 0.01$) (Figure 1) with no significant difference in OS. When we analyzed the different causes of mortality, we found that +29CC patients had a higher probability of dying because of GvHD (18% vs 4%, $P = 0.07$) and sinusoidal obstruction syndrome (18% vs 0%, $P = 0.01$) but not because of infections (12% vs 17%, $P = 0.48$).

Probably the most interesting results were related to donor +29C > T genotype. Recipients from +29TT donors had a significant increase in relapse rate (1–5 years TT 37–51% vs TC 19–25% vs CC 13–19%, Gray's test $P = 0.02$, HR 2.4, 95% CI 1.2–4.9, $P = 0.01$) (Figure 2). Without differences observed in NRM, these recipients had a trend to a lower DFS (1–5 years 57–36% vs 73–63%, log-rank $P = 0.05$, HR 2.5, 95% CI 0.97–6.5, $P = 0.05$) (Figure 3) and a significant decrease in OS compared with other genotypes (1–5 years TT 69–50% vs TC/CC 77–69%, log-rank $P = 0.04$, HR 1.9, 95% CI 0.99–3.8, $P = 0.05$) (Figure 4).

No significant effect of patients or donors +74 genotype was found on any transplant outcome.

DISCUSSION

We have shown that *TGF-β1* +29 SNP significantly impaired the outcomes following s.d. HSCT and its impact was restricted to

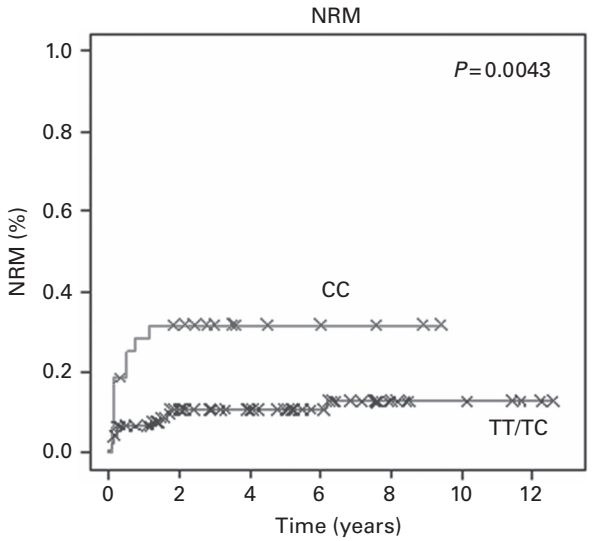

Figure 1. Survival analysis depending on patients' TGF-β1 +29 genotype. Patients +29CC vs others (TT-TC) had higher NRM. A full color version of this figure is available at the *Bone Marrow Transplant* journal online.

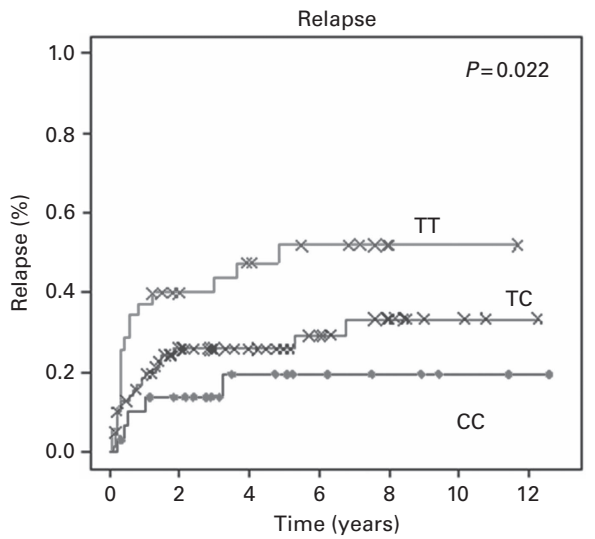

Figure 2. Survival analysis depending on donors' TGF-β1 +29 genotype. Donors +29TT vs others had higher relapse rate. A full color version of this figure is available at the *Bone Marrow Transplant* journal online.

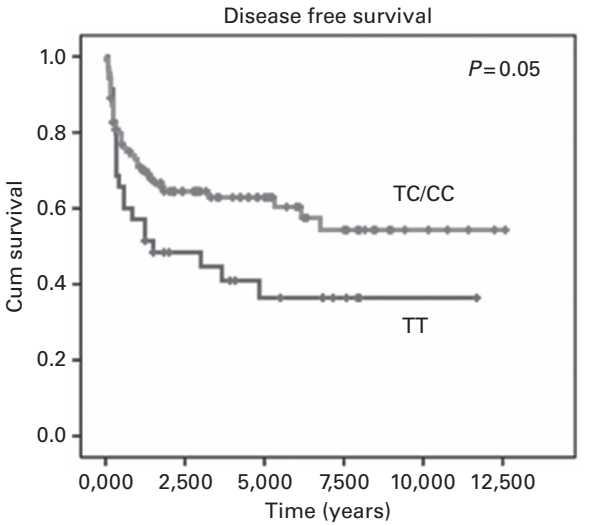

Figure 3. Survival analysis depending on donors' TGF-β1 +29 genotype. Donors +29TT vs others had decreased DFS. A full color version of this figure is available at the *Bone Marrow Transplant* journal online.

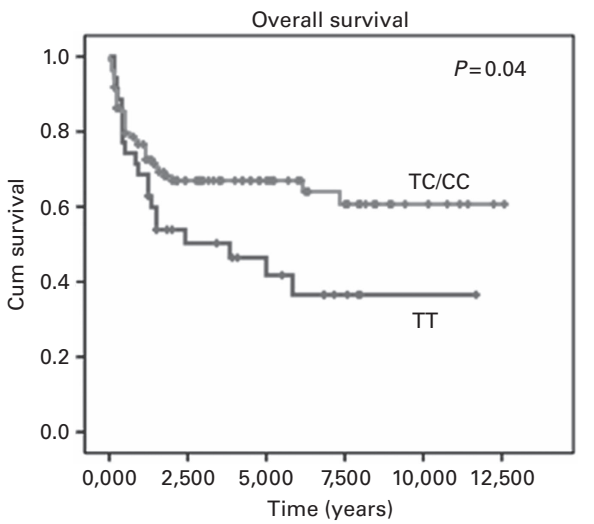

Figure 4. Survival analysis depending on donors' TGF-β1 +29 genotype. Donors +29TT vs others had OS. A full color version of this figure is available at the *Bone Marrow Transplant* journal online.

the myeloablative cohort, as we have previously observed in UD HSCT.[31] It is well recognized that this highly toxic conditioning produces an inflammatory scenario known as 'cytokine storm'.[36,37] In a murine model, the conditioning regimen did alter the contribution of CD8+ or CD4+ subsets to the total FOXP3+ pool with functional importance in defining transplant outcome and are important for protection from GvHD. These studies confirmed that TGF-β, but not IL-10, was required for the conversion of the highly suppressive CD8+FOXP3+ Treg.[38] The above mentioned hypothesis of the impact of these SNPs on the secretion process may explain the effect of the polymorphism in this type of transplant.

We observed that +29CC patients had a significant increase in NRM, similar to what we previously observed in UD HSCT, nevertheless with no significant impact on the OS. NRM is increased in UD HSCTs compared with s.d.,[39] which may explain why we found no significant impact in OS after s.d. However, we found that patients homozygous for this SNP had a higher probability of death due to GvHD and sinusoidal obstruction syndrome. GvHD is an exacerbated inflammatory response that leads to the destruction of healthy host tissues by donor immune cells. The effect of TGF-β1 might depend on the timing, the target tissue and the origin of the producing cells (donor/host). Probably, the presence of this SNP in recipient endothelial or dendritic cells may influence the immunosuppressive environment.

The novel finding from the current paper is the impact of donor +29 genotype on transplant outcomes. Although +29TT donors showed a trend to an increased aGvHD incidence, this was not associated with a higher Graft-vs-Tumor effect but the opposite with a significant increase in the relapse rate with a suggestion of dosage effect of the SNP. This effect resulted in a trend to a reduction in DFS and a significant reduction in OS for the

recipients of +29TT donors. On the other hand CC donors were associated with a significant increase in cGvHD extensive forms.

This 'dual' behavior of the SNP where the recipients of +29TT donors (lower TGF-β1 producers) had more aGvHD but more relapse and on the other side the recipients of +29CC donors (higher TGF-β1 producers) had more extensive cGvHD was described in a similar way by Banovic *et al.*[40] in a murine model. They observed that in post-myeloablative transplant early phases TGF-β1 was secreted by donor lymphocytes and the inhibition of TGF-β1 was associated with more aGvHD but also with more relapse, and in later phases was secreted by donors monocytes and its inhibition was associated with less cGvHD. Consistent evidence has been published regarding this dual effect of TGF-β1 and GvHD in *in vivo* post-transplant patients. Higher levels of TGF-β1 at early post-transplant phases were associated with a reduction in aGvHD, probably related to Tregs-mediated immunosupression,[41–43] whereas higher levels of the cytokine at later phases were associated with an increased incidence of cGvHD, probably related to the pro-fibrotic properties.[44–46]

Some conflicting results have been published regarding the influence of *TGF-β1* on sibling and UD HSCT outcome.[42,47,48] We thought that this is mainly due to the reduced population-based analyses, the fact that most of the publications do not make focus on myeloablative cohort sub-analysis, and that the donor effect might overcome with T-cell depletion protocols. Nevertheless, some new data confirmed the impact of *TGF-β1* genotype on UD HSCT. A recent publication from Anthony Nolan showed that the p001 haplotype including the C allele at +29 position, so called 'high producers' patients, had a significant reduction in OS due to an increase in NRM.[49]

Our study has strengths. It is one of the largest cohorts reported in this topic. We used a comprehensive adjusted model with the principal characteristics in the MA. A clear limitation of our study, due to the retrospective nature, is that some missing data might had been useful for the MA. An example of this is the HCT-CI score, described by Sorror *et al.*[50] a validated tool that can predict the risk for NRM which was not available to us. In addition, loss of follow-up can interfere with long-term outcomes like OS.

In conclusion, we have confirmed that as in UD HSCT, +29CC patients had an increased NRM after myeloablative conditioning s.d. HSCT. In addition, we found that +29TT donors might be a worst choice compared with TC/CC due to an increase in relapse rate and a decreased OS. These data emphasize the importance of TGF-β1 in this setting and pursuing further analysis is granted by our group. Identification of these SNPs pre-transplant may allow for transplant conditioning and immunosuppression regimens to be tailored to the individual patient, as well as assisting in the most appropriate choice of donor.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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