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Mononuclear Cell Telomere Attrition Is Associated with Overall Survival after Nonmyeloablative Allogeneic Hematopoietic Cell Transplantation for Hematologic Malignancies

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After allogeneic hematopoietic cell transplantation (allo-HCT), transplanted cells rapidly undergo multiple rounds of division. This may cause extensive telomere attrition, which could potentially prohibit further cell division and lead to increased mortality. We therefore characterized the development in telomere length after nonmyeloablative allo-HCT in 240 consecutive patients transplanted because of hematologic malignancies and tested the hypothesis that extensive telomere attrition post-transplant is associated with low overall survival. Telomere length was measured using quantitative PCR in mononuclear cells obtained from donors and recipients pretransplant and in follow-up samples from recipients post-transplant. Telomere attrition at 9 to 15 months post-transplant was calculated as the difference between recipient telomere length at 9 to 15 months post-transplant and donor pretransplant telomere length, divided by donor pretransplant telomere length. Although allo-HCT led to shorter mean telomere length in recipients when compared with donors, recipients had longer mean telomere length 9 to 15 months post-transplant than they had pretransplant. When compared with donor telomeres, recipients with extensive telomere attrition at 9 to 15 months post-transplant had low overall survival (10-year survival from 9 to 15 months post-transplant and onward: 68% in the tertile with least telomere attrition, 57% in the middle tertile, and 39% in the tertile with most attrition; log-rank $P = .01$). Similarly, after adjusting for potential confounders, recipients with extensive telomere attrition had high all-cause mortality (multivariable adjusted hazard ratio, 1.84 per standard deviation of telomere attrition at 9 to 15 months post-transplant; 95% confidence interval, 1.25 to 2.72; $P = .002$) and high relapse-related mortality (subhazard ratio, 2.07; 95% confidence interval, 1.14 to 3.76; $P = .02$). Taken together, telomere attrition may be a clinically relevant marker for identifying patients at high risk of mortality.

INTRODUCTION

Telomeres form protective caps at the chromosome tips and consist of a protein complex and hundreds to thousands of tandem repeats of the nucleotide sequence TTAGGG [1]. In most somatic cells the telomeric DNA shortens with each cell division due to the end replication problem [2,3]. After allogeneic hematopoietic cell transplantation (allo-HCT), the transplanted cells rapidly go through multiple rounds of division, which may lead to extensive telomere attrition that can prohibit further cell divisions by cells becoming senescent or undergoing apoptosis [3-6].

When compared with donor telomeres, recipient telomeres attrite rapidly in the immediate post-transplant period in patients treated with myeloablative allo-HCT [5-20]. This accelerated telomere attrition seem to slow down approximately 1 year post-transplant, after which the rate of telomere attrition resembles what is seen in the general population. It is currently unknown whether the extent of telomere attrition after allo-HCT is associated with overall survival. However, the hypothesis is clinically important because studies on patients undergoing autologous HCT have found that extensive

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post-transplant leukocyte telomere attrition may be a risk factor for therapy-related myelodysplastic syndrome, acute myeloid leukemia, and/or bone marrow failure [21-24].

Similarly, pretransplant donor and/or pretransplant recipient telomere length could also potentially influence the outcome after allo-HCT, but studies on survival in allo-HCT patients according to pretransplant donor and/or pretransplant recipient telomere length have produced conflicting results [25-30]. In a study on 330 patients treated with allo-HCT due to severe aplastic anemia, long donor leukocyte telomeres were associated with high overall survival [25]. In contrast, a study on 178 consecutive patients undergoing myeloablative allo-HCT mainly due to hematologic malignancies found that short pretransplant recipient telomeres were associated with high treatment-related mortality, whereas donor telomere length was not associated with overall survival or treatment-related mortality [26]. However, although nonmyeloablative conditioning is used in more than 40% of allo-HCT procedures performed in Europe [31], no previous studies have specifically examined whether short donor and/or recipient telomere length are associated with survival in consecutive patients undergoing nonmyeloablative allo-HCT for hematologic malignancies. Likewise, there is limited knowledge on the development in telomere length in consecutive patients undergoing nonmyeloablative allo-HCT, because only a single cross-sectional study with 23 patients has compared donor and recipient post-transplant telomere length in consecutive patients treated with nonmyeloablative allo-HCT [32].

Therefore, our objective was to characterize the development in telomere length in mononuclear cells (MNCs) after nonmyeloablative allo-HCT and to test the following 4 hypotheses: (1) short pretransplant donor telomeres are associated with low overall survival, (2) short pretransplant recipient telomeres are associated with low overall survival, (3) short recipient telomeres at 9 to 15 months post-transplant are associated with low overall survival thereafter, and (4) when compared with donor telomeres, extensive telomere attrition in recipients at 9 to 15 months post-transplant is associated with low overall survival thereafter. For this purpose we prospectively studied 240 consecutive patients undergoing nonmyeloablative allo-HCT for hematologic malignancies. Telomere length was measured in samples of MNCs obtained from donors and recipients pretransplant as well as in sequential follow-up samples obtained from the recipients post-transplant, and information on deaths and complications was collected prospectively for all patients during follow-up.

METHODS

Patients and Treatment Regimens

We studied 240 consecutive adult patients treated with nonmyeloablative allo-HCT at Rigshospitalet, Copenhagen University Hospital, from 2000 to 2011. All adult patients with available samples of MNCs after treatment with nonmyeloablative allo-HCT at Copenhagen University Hospital from 2000 to 2011 were included irrespective of disease. All patients were transplanted because of hematologic malignancies, and 239 patients received a peripheral blood stem cell graft, whereas 1 patient received a bone marrow graft.

Conditioning was fludarabine 30 mg/m^2 on days −4, −3, and −2 plus total body irradiation with 2 Gy in 210 patients, 3 Gy in 1 patient, and 4 Gy in 28 patients; 1 patient did not receive total body irradiation. In patients with a sibling donor, mycophenolate mofetil was stopped at day +28, whereas patients with an unrelated donor had mycophenolate mofetil tapered from day +100 to day +180. All patients were planned to stop calcineurin inhibitors 6 months after transplantation, unless graft-versus-host disease (GVHD) was present. Procedures for infection prophylaxis and cytomegalovirus (CMV) surveillance are described in Supplementary Methods.

After the transplantation patients were followed with regular outpatient visits, and no patients were lost to follow-up. As follow-up for this study ended on April 4, 2017, patients were followed for a median of 6 years (range, 0 to 17). The study was approved by the Regional Ethics Committee for the Capital Region of Denmark, and all patients provided written, informed consent.

Collection of Blood Samples

One blood sample was collected from each donor pretransplant. For 108 donors, samples of peripheral blood were acquired directly by venipuncture before administration of granulocyte colony-stimulating factor, whereas 132 of the donor samples were taken from peripheral blood stem cell grafts obtained by leukapheresis. From each recipient up to 4 blood samples were collected at the following 4 time points: pretransplant (immediately before conditioning), approximately 1 month post-transplant (range, 3 to 5 weeks), approximately 3 months post-transplant (range, 9 to 15 weeks), and approximately 1 year post-transplant (range, 9 to 15 months). Pretransplant samples were available for all recipients and their respective donors, whereas the number of recipients alive without relapse and with samples available became gradually lower for each of the post-transplant time points, as seen from Supplementary Figure S1. Shortly after sample collection MNCs were isolated using the Ficoll method and frozen at −80°C overnight after adding DMSO and human serum to a final concentration of 10% DMSO and 40% human serum [33].

Telomere Length Measurements

All samples were thawed in 2017, and DNA was extracted from MNCs using the QIAsymphony DSP DNA kit (Qiagen, Hilden, Germany) [34]. Telomere length was measured on a CFX384 real-time PCR detection system (Bio-Rad, Hercules, California) using a modified monochrome multiplex quantitative PCR method [35]. In summary, the telomeric DNA sequence and the single-copy gene for albumin were amplified concurrently in the same well to adjust for different amounts of DNA in the samples. Telomere length was expressed as the telomere (T) to single-copy gene (S) ratio (T/S ratio).

Samples were distributed randomly across PCR plates. To monitor precision of the measurements, a set of 3 control samples was included in all plates: 1 control sample consisted of NTERA-2 cell line DNA, whereas the other 2 control samples were from 2 patients treated with allo-HCT and consisted of DNA extracted from MNCs using the exact same procedures for sample storage and handling as used for the experimental patient samples. Coefficients of variation for the T/S ratio across plates were 8.9% for NTERA-2 cell line DNA (mean T/S ratio, .40), 4.9% for patient control sample A (mean T/S ratio, .51), and 5.5% for patient control sample B (mean T/S ratio, .55). As recently recommended in a series of commentaries in *International Journal of Epidemiology*, precision was further monitored by performing repeat measurements of the T/S ratio on a subset of samples and calculating the intraclass correlation coefficient [36-38]. Based on 275 samples measured twice on separate days, we found an intraclass correlation coefficient of .90 (95% confidence interval [CI], .88 to .92) using a 1-way random effects model [39]. More details on experimental procedures and quality control are described in Supplementary Methods.

Statistical Analysis

Statistical analyses were performed using Stata (StataCorp, College Station, Texas) version 13.1. All *P* values are 2-sided. Univariate linear regression was used when modeling telomere length as a function of age. Likewise, univariate linear regression was used to model telomere attrition as a function of age and to model telomere attrition as a function of days elapsed since transplantation. We used *t*-tests to compare telomere length between donors and recipients and when comparing recipient telomere length at different time points.

Telomere attrition at 9 to 15 months post-transplant ($\text{telomere}_{\text{attrition}}$) was calculated as the difference between recipient telomere length at 9 to 15 months post-transplant ($\text{recipientTL}_{\text{9 to 15 months post}}$) and donor-pretransplant telomere length ($\text{donorTL}_{\text{pretransplant}}$) divided by donor pretransplant telomere length ($\text{donorTL}_{\text{pretransplant}}$) by using the following formula: $\text{telomere}_{\text{attrition}} = (\text{recipientTL}_{\text{9 to 15 months post}} - \text{donorTL}_{\text{pretransplant}}) / \text{donorTL}_{\text{pretransplant}}$. Hence, loss of telomere length during the first 9 to 15 months post-transplant results in negative values for telomere attrition, whereas gain of telomere length results in positive values.

When using telomere length as a categorical variable, recipient pretransplant telomere length, recipient telomere length at 9 to 15 months post-transplant, and telomere attrition at 9 to 15 months post-transplant were categorized into recipient age–specific tertiles by calculating tertiles separately for each of the following groups of recipient age: <40 years, 40 to 50 years, 50 to 60 years and >60 years. Likewise, donor pretransplant telomere length was categorized into donor age-specific tertiles by calculating tertiles separately for each of the following groups of donor age: <40 years, 40 to 50 years, 50 to 60 years and >60 years.

Overall survival was assessed using the Kaplan-Meier method and by modeling risk of all-cause mortality using Cox proportional hazards regression. Risk of cause-specific mortality and risk of complications were modeled using competing risk regression as described by Fine and Gray [40]. All multivariable models were adjusted for recipient and donor age, recipient and donor sex, recipient diagnosis, donor relation/match, recipient and donor pretransplant CMV antibody status, European Society for Blood and Marrow Transplantation risk score [41], and year of transplant. For the analysis on mortality and complications according to recipient telomere length at 9 to 15

months post-transplant and according to telomere attrition at 9 to 15 months post-transplant, multivariable models were further adjusted for the exact number of days elapsed from the transplantation date until the 9 to 15 months sample was obtained and for whether or not patients had been diagnosed with CMV reactivation, acute GVHD, and/or chronic GVHD before the 9 to 15 months sample was obtained. Selection of variables for inclusion in multivariable models was done a priori. The rationale behind also adjusting analyses on donor-derived MNCs (telomere length at 9 to 15 months post-transplant and telomere attrition at 9 to 15 months post-transplant) for recipient age was a previous study reporting that post-transplant telomere attrition is less pronounced in young than in older recipients [42] as well as our own observation that higher recipient age was associated with more telomere attrition at 9 to 15 months post-transplant. More details on statistical analyses are described in Supplementary Methods.

RESULTS

Among the 240 patients treated with nonmyeloablative allo-HCT, higher recipient age was associated with shorter recipient pretransplant telomeres (linear regression on recipient pretransplant telomere length as a function of recipient age with $P = .0002$; $R^2 = .06$). Likewise, higher donor age was associated with shorter donor pretransplant telomeres ($P = 1 \times 10^{-5}$; $R^2 = .08$). Therefore, baseline characteristics for the 240 patients are shown according to recipient age–specific tertiles of recipient pretransplant telomere length and according to donor age-specific tertiles of donor pretransplant telomere length (Table 1).

Telomere Length Before and After Transplantation

For the primary analysis on differences in telomere length, we performed a paired analysis among the 129 patients who had a complete set of follow-up samples available from all 3 post-transplant time points and who did not experience relapse before the 9 to 15 months post-transplant sample was obtained. Donors had longer telomeres than recipients before

Table 1
Baseline Characteristics of 240 Nonmyeloablative Allo-HCT Patients According to Age-Specific Tertiles of Recipient Pretransplant Telomere Length and Donor Pretransplant Telomere Length

	Recipient Pretransplant Telomere Length Tertiles				Donor Pretransplant Telomere Length Tertiles			
Characteristic	First (shortest)	Second	Third (longest)	*P* for trend	First (shortest)	Second	Third (longest)	*P* for trend
No. of patients	81	80	79		81	80	79	
Telomere length, T/S ratio	.41 (.10)	.60 (.07)	.78 (.10)		.65 (.08)	.78 (.05)	.96 (.12)	
Recipient age, yr	56 (10)	55 (10)	55 (10)	Not calculated*	54 (11)	55 (9)	56 (10)	.36
Donor age, yr	42 (13)	43 (13)	43 (14)	.96	42 (14)	43 (13)	42 (14)	Not calculated†
Age of related donors, yr	52 (10)	53 (10)	53 (10)		54 (9)	53 (9)	52 (11)	
Age of unrelated donors, yr	35 (11)	34 (9)	33 (10)		34 (10)	35 (9)	33 (11)	
Sex, recipient–donor, n (%)				.42				.55
Male–male	38 (47)	38 (48)	32 (41)		43 (53)	27 (34)	38 (48)	
Male–female	18 (22)	13 (16)	14 (18)		11 (14)	22 (28)	12 (15)	
Female–male	8 (10)	10 (13)	14 (18)		12 (15)	12 (15)	8 (10)	
Female–female	17 (21)	19 (24)	19 (24)		15 (19)	19 (24)	21 (27)	
Diagnosis, n (%)				.003				.14
Acute myeloid leukemia	15 (19)	28 (35)	26 (33)		23 (28)	24 (30)	22 (28)	
Chronic lymphocytic leukemia	26 (32)	8 (10)	10 (13)		13 (16)	9 (11)	22 (28)	
Lymphoma	20 (25)	22 (28)	22 (28)		20 (25)	26 (33)	18 (23)	
Myelodysplastic syndrome	18 (22)	12 (15)	13 (16)		15 (19)	14 (18)	14 (18)	
Multiple myeloma	2 (2)	6 (8)	2 (3)		4 (5)	3 (4)	3 (4)	
Other leukemia‡	0 (0)	4 (5)	6 (8)		6 (7)	4 (5)	0 (0)	
Donor relation/match, n (%)				.54				.73
HLA-identical sibling donor	33 (41)	38 (48)	39 (49)		36 (44)	38 (48)	36 (46)	
Matched unrelated donor (10/10 or 9/10)	45 (56)	38 (48)	38 (48)		41 (51)	39 (49)	41 (52)	
One-antigen mismatched unrelated donor	3 (4)	4 (5)	2 (3)		4 (5)	3 (4)	2 (3)	
CMV antibody status, recipient–donor, n (%)				.15				.79
Positive–positive	31 (38)	31 (39)	24 (30)		29 (36)	29 (36)	28 (35)	
Positive–negative	24 (30)	22 (28)	15 (19)		19 (23)	18 (23)	24 (30)	
Negative–positive	11 (14)	9 (11)	14 (18)		11 (14)	14 (18)	9 (11)	
Negative–negative	13 (16)	15 (19)	24 (30)		21 (26)	15 (19)	16 (20)	
Missing	2 (2)	3 (4)	2 (3)		1 (1)	4 (5)	2 (3)	
EBMT risk score, n (%)				.02				.09
0-2	36 (44)	48 (60)	52 (66)		45 (56)	49 (61)	42 (53)	
3-4	44 (54)	31 (39)	26 (33)		33 (41)	31 (39)	37 (47)	
5-6	1 (1)	1 (1)	1 (1)		3 (4)	0 (0)	0 (0)	
Year of transplant, n (%)				.57				.009
2000-2005	29 (36)	22 (28)	25 (32)		34 (42)	24 (30)	18 (23)	
2006-2011	52 (64)	58 (73)	54 (68)		47 (58)	56 (70)	61 (77)	
Source of donor sample, n (%)				.35				.62
Donor sample obtained directly by venipuncture	34 (42)	35 (44)	39 (49)		38 (47)	36 (45)	34 (43)	
Donor sample obtained from peripheral blood stem cell graft	47 (58)	45 (56)	40 (51)		43 (53)	44 (55)	45 (57)	

Mean (standard deviation) is shown for continuous variables, whereas n (%) is shown for categorical variables. Tertiles of recipient pretransplant telomere length were adjusted for recipient age by calculating recipient age-specific tertiles, whereas tertiles of donor pretransplant telomere length were adjusted for donor age by calculating donor age–specific tertiles. Calculation of age-specific tertiles was performed by calculating tertiles of telomere length separately for each of the following age groups: <40 years, 40-50 years, 50-60 years, and >60 years. For continuous variables *P* for trend is from univariate linear regression. For categorical variables with 2 levels *P* for trend is from the Mann-Whitney U test, and for categorical variables with 3 or more levels *P* for trend is from the Kruskal-Wallis test. EBMT indicates European Society for Blood and Marrow Transplantation, HLA indicates human leukocyte antigen.

* For recipient age, *P* for trend across age-specific tertiles of recipient telomere length was not calculated because recipient telomere length tertiles were already adjusted for recipient age.

† For donor age *P* for trend across age-specific tertiles of donor telomere length was not calculated because donor telomere length tertiles were already adjusted for donor age.

‡ The category "Other leukemia" include acute lymphoblastic leukemia (n = 1), chronic myeloid leukemia (n = 6), and T cell prolymphocytic leukemia (n = 3).

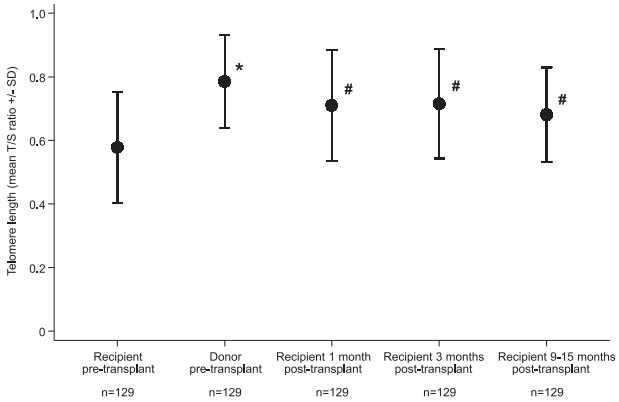

Figure 1. Paired comparison of donor and recipient pretransplant telomere length with telomere length in recipients at 1, 3, and 9 to 15 months post-transplant. The figure only include samples from the 129 patients who had a complete set of follow-up samples available from all 3 post-transplant time points and who did not experience relapse before the 9 to 15 months post transplant sample was obtained. Telomere length is shown as mean T/S ratio (*solid circles*) ± standard deviation (*whiskers*). *P* values are from a paired *t*-test.
*$P < 1 \times 10^{-5}$ for difference with recipient pretransplant telomere length.
#$P < 1 \times 10^{-5}$ for difference with recipient pretransplant telomere length and $p < 1 \times 10^{-5}$ for difference with donor pretransplant telomere length.
SD indicates standard deviation.

the transplantation, and donor pretransplant telomeres were also longer than recipient telomeres at 1, 3, and 9 to 15 months post-transplant. Recipients had longer telomeres at 1, 3, and 9 to 15 months post-transplant when compared with recipient pretransplant telomeres (Figure 1). Results were similar when telomere lengths were compared using an unpaired instead of a paired *t*-test and when including all 240 patients (Supplementary Figure S1).

Mortality and Complications According to Donor and Recipient Telomere Length

Donor pretransplant telomere length was not associated with overall survival when adjusting for donor age (5-year overall survival: 54% in the tertile with longest age-adjusted donor telomeres, 53% in the middle tertile, and 54% in the shortest; log-rank *P* = .96) or when adjusting for several potential confounders using the multivariable adjusted model (hazard ratio [HR], .88 for all-cause mortality per standard deviation shorter donor telomeres; 95% CI, .73 to 1.06) (Figure 2). Similarly, we found no association between recipient pretransplant telomere length and overall survival when adjusting for recipient age (5-year overall survival: 49% in the tertile with longest age-adjusted recipient pretransplant telomeres, 60% in the middle tertile, and 52% in the shortest; log-rank *P* = .42) or in the multivariable adjusted model (HR, .86 for all-cause mortality per standard deviation shorter pretransplant recipient telomeres; 95% CI, .71 to 1.05).

In contrast, we observed a nonsignificant trend toward short recipient telomeres at 9 to 15 months post-transplant being associated with low overall survival after adjusting for recipient age (5-year overall survival: 74% in the tertile with longest recipient age-adjusted recipient telomeres at 9 to 15 months post-transplant, 73% in the middle tertile, and 54% in the shortest; log-rank *P* = .07). Results were similar when adjusting for donor age instead of recipient age (5-year overall survival: 76% in the tertile with longest donor age-adjusted recipient telomeres at 9 to 15 months post-transplant, 70% in the middle tertile, and 56% in the shortest; log-rank *P* = .07). However, the association was attenuated when adjusting for potential confounders using the multivariable adjusted model (HR, 1.26 for all-cause mortality per standard deviation shorter recipient telomeres at 9 to 15 months post-transplant; 95% CI, .88 to 1.80; *P* = .20) (Figure 2). Results were similar when using the multivariable adjusted model and categorizing recipient telomeres at 9 to 15 months post-transplant into tertiles, showing an HR of 1.88 (95% CI, .90 to 3.94; *P* = .09) in the tertile with shortest telomeres and an HR of .73 (95% CI, .33 to 1.62; *P* = .44) in the middle tertile, when compared with the tertile with longest recipient telomeres at 9 to 15 months post-transplant (Supplementary Figure S2).

Neither donor pretransplant telomere length nor recipient pretransplant telomere length were associated with risk of relapse-related mortality, treatment-related mortality, relapse, CMV reactivation, acute GVHD, or chronic GVHD (Supplementary Figure S3). Similarly, recipient telomere length at 9 to 15 months post-transplant was not associated with risk of relapse-related mortality, treatment-related mortality, relapse, or chronic GVHD (Supplementary Figure S3).

Telomere Attrition during the First 9 to 15 Months Post-Transplant

In total, 175 patients were alive and without relapse at 1 year post-transplant, and among these 142 had samples available for measurements of recipient telomere length at 9 to 15 months post-transplant. To compare post-transplant recipient telomere length with pretransplant telomere length from the recipient's respective donors, telomere attrition at 9 to 15 months post-transplant was calculated as the difference

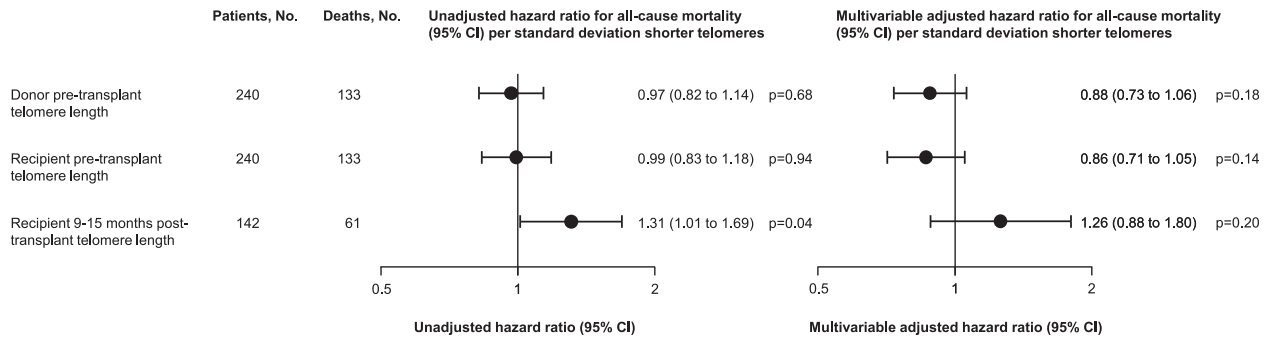

Figure 2. Risk of all-cause mortality per standard deviation shorter donor pretransplant telomere length, recipient pretransplant telomere length, and recipient telomere length at 9 to 15 months post-transplant. For the analysis on recipient telomere length at 9 to 15 months post-transplant, recipients were only included in the analysis if a sample had been obtained at approximately 1 year post-transplant (range, 9 to 15 months) and if relapse had not occurred before the sample was obtained.

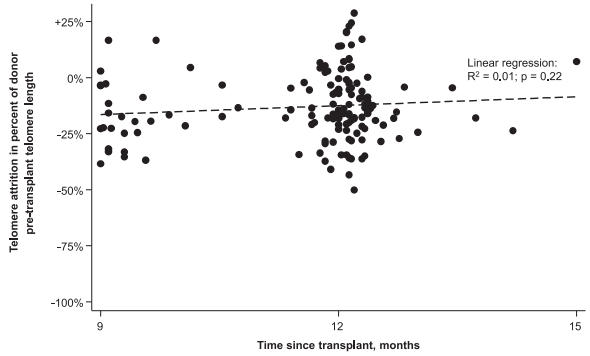

Figure 3. Telomere attrition at 9 to 15 months post-transplant as a function of the exact number of days elapsed between transplantation and obtainment of the 9 to 15 months post-transplant sample for 142 nonmyeloablative allo-HCT patients. Telomere attrition was calculated for each patient as the difference between recipient telomere length at 9 to 15 months post-transplant and donor pretransplant telomere length, divided by donor pretransplant telomere length. Hence, loss of telomere length during the first 9 to 15 months post-transplant results in negative values for telomere attrition, whereas gain of telomere length during the first 9 to 15 months post-transplant results in positive values for telomere attrition. Patients were only included in the analysis if a sample had been obtained at approximately 1 year post-transplant (range, 9 to 15 months) and if relapse had not occurred before the sample was obtained.

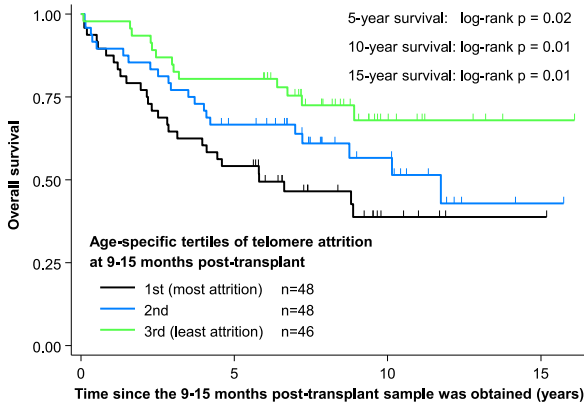

Figure 4. Kaplan-Meier curve for overall survival according to recipient age-specific tertiles of telomere attrition at 9 to 15 months post-transplant. Telomere attrition was calculated for each patient as the difference between recipient telomere length at 9 to 15 months post-transplant and donor pretransplant telomere length, divided by donor pretransplant telomere length. Tertiles of telomere attrition at 9 to 15 months post-transplant were adjusted for recipient age by calculating age-specific tertiles separately for each of the following age groups: <40 years, 40 to 50 years, 50 to 60 years, and >60 years. Patients were excluded from the analysis if the patient had been diagnosed with relapse before the 9 to 15 months post-transplant sample was obtained. *Vertical lines* mark censorings due to end of follow-up. *P* values are from the log-rank test.

between recipient telomere length at 9 to 15 months post-transplant and donor pretransplant telomere length divided by donor pretransplant telomere length. Mean telomere attrition at 9 to 15 months post-transplant was −13% (range, −50% to +29%) of donor pretransplant telomere length (Figure 3). As seen from Figure 3 the samples were obtained in the time interval 9 to 15 months after the transplantation, because it was not logistically possible to obtain 1-year post-transplant samples from all recipients at exactly 12 months after the transplantation. Within the 9 to 15 months post-transplant time interval, we found no association between number of days elapsed since transplantation and the extent of telomere attrition (univariate linear regression, $P = .22$; $R^2 = .01$).

Survival and Complications According to Extent of Telomere Attrition

When examining overall survival according to the extent of telomere attrition at 9 to 15 months post-transplant, telomere attrition was categorized into recipient age–specific tertiles (Supplementary Table S1), because each year of higher recipient age was associated with a telomere attrition at 9 to 15 months post-transplant of −.3% (95% CI, −.1% to −.5%; $P = .01$; $R^2 = .05$). In contrast, telomere attrition at 9 to 15 months post-transplant was not associated with donor age (linear regression, $P = .10$; $R^2 = .02$).

Recipients with extensive telomere attrition had low overall survival after adjusting for recipient age, as the 5-year overall survival from the 9 to 15 months sample was obtained an onward was 80% in the tertile with least age-adjusted telomere attrition at 9 to 15 months post-transplant, 67% in the middle tertile, and 54% in the tertile with most telomere attrition (log-rank $P = .02$) (Figure 4). Correspondingly, the 10-year overall survival was 68% in the tertile with least age-adjusted telomere attrition at 9 to 15 months post-transplant, 57% in the middle tertile, and 39% in the tertile with most telomere attrition (log-rank $P = .01$). Likewise, when adjusting for potential confounders using the multivariable adjusted model, extensive telomere attrition was associated with high all-cause mortality with an HR of 4.28 (95% CI, 1.88 to 9.77) in the tertile with most telomere attrition and an HR of 1.78 (95% CI, .81 to 3.90) in the middle tertile, when compared with the tertile with least telomere attrition at 9 to 15 months post-transplant (Figure 5). Similarly, when using telomere attrition at 9 to 15 months post-transplant as a continuous variable, we found a multivariable adjusted HR for all-cause mortality of 1.84 (95% CI, 1.25 to 2.72; $P = .002$) per standard deviation of telomere attrition.

When examining risk of cause-specific mortality, extensive telomere attrition at 9 to 15 months post-transplant was associated with high risk of relapse-related mortality with a sub-HR of 2.07 (95% CI, 1.14 to 3.76; $P = .02$) per standard deviation of telomere attrition (Figure 6). Likewise, sub-HRs per standard deviation of telomere attrition at 9 to 15 months post-transplant were above 1 for treatment-related mortality (sub-hazard ratio 1.21; 95% CI, .72 to 2.04), relapse (1.36; 95% CI, .75 to 2.47), and chronic GVHD (1.62; 95% CI, .91 to 2.88), but none of these findings were statistically significant at the $P < .05$ level.

Sensitivity Analyses

To further examine whether the association between extensive telomere attrition at 9 to 15 months post-transplant and high all-cause mortality could be caused by complications or prognostic factors already present when the 9 to 15 months post-transplant samples were obtained, we performed sensitivity analysis where the association was studied exclusively in each of the following subgroups: recipients with full chimerism at time of blood sampling, recipients who had not received donor lymphocyte infusions before blood sampling, recipients without CMV reactivation occurring before blood sampling, recipients without acute GVHD occurring before blood sampling, recipients without chronic GVHD occurring before blood sampling, recipients transplanted due to myeloid malignancies, and recipients transplanted due to lymphoid malignancies (Supplementary Figure S4). We found no indication of confounding due to the above-mentioned complications or prognostic factors, as risk estimates for all-cause

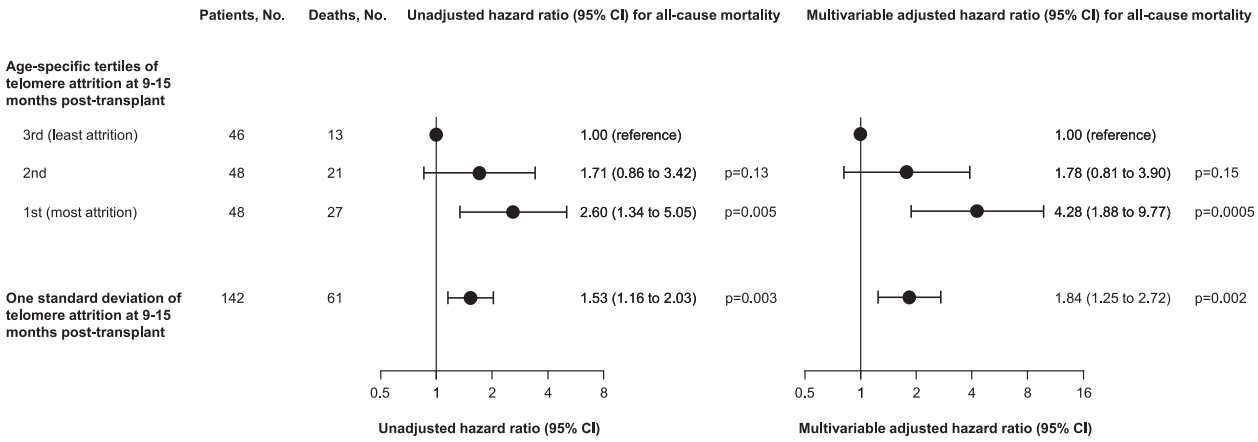

Figure 5. Risk of all-cause mortality according to telomere attrition at 9 to 15 months post-transplant. Telomere attrition was calculated for each patient as the difference between recipient telomere length at 9 to 15 months post-transplant and donor pretransplant telomere length, divided by donor pretransplant telomere length. Tertiles of telomere attrition at 9 to 15 months post-transplant were adjusted for recipient age by calculating age-specific tertiles separately for each of the following age groups: <40 years, 40 to 50 years, 50 to 60 years, and >60 years. Patients were excluded from the analysis if the patient had been diagnosed with relapse before the 9 to 15 months post-transplant sample was obtained.

mortality remained stable in each subgroup. Likewise, the association between extensive telomere attrition and high all-cause mortality remained stable when only including recipients without relapse within the first 2 years post-transplant, which indicates that the association is not likely to be caused by relapses occurring shortly after the 9 to 15 months post-transplant samples were obtained (Supplementary Figure S4).

To examine whether extensive telomere attrition at 9 to 15 months post-transplant was associated with both short- and long-term mortality, we examined risk of all-cause mortality separately in 2 time intervals by modeling time to death separately in each interval. In the first interval patients were followed from the 9 to 15 months post-transplant sample was obtained and 2 years onward, whereas in the second time interval patients were followed from 2 years after the 9 to 15 months post-transplant sample was obtained and onward (ie, from approximately 3 years post-transplant and onward) (Supplementary Figure S5). Patients with extensive telomere attrition at 9 to 15 months post-transplant had high all-cause mortality both during the first 2 years of follow-up after the 9 to 15 months blood sample was obtained (HR, 1.90 per standard deviation of telomere attrition; 95% CI, .93 to 3.92) and during the time interval beginning 2 years after the 9 to 15 months blood sample was obtained (HR, 1.74; 95% CI, 1.06 to 2.86). As described in detail in Supplementary Results, we found no indication that our observed associations were caused by confounding due to the different methods used for obtaining donor samples (Supplementary Figure S6) or due to variation in leukocyte differential counts at 9 to 15 months post-transplant (Supplementary Figure S7).

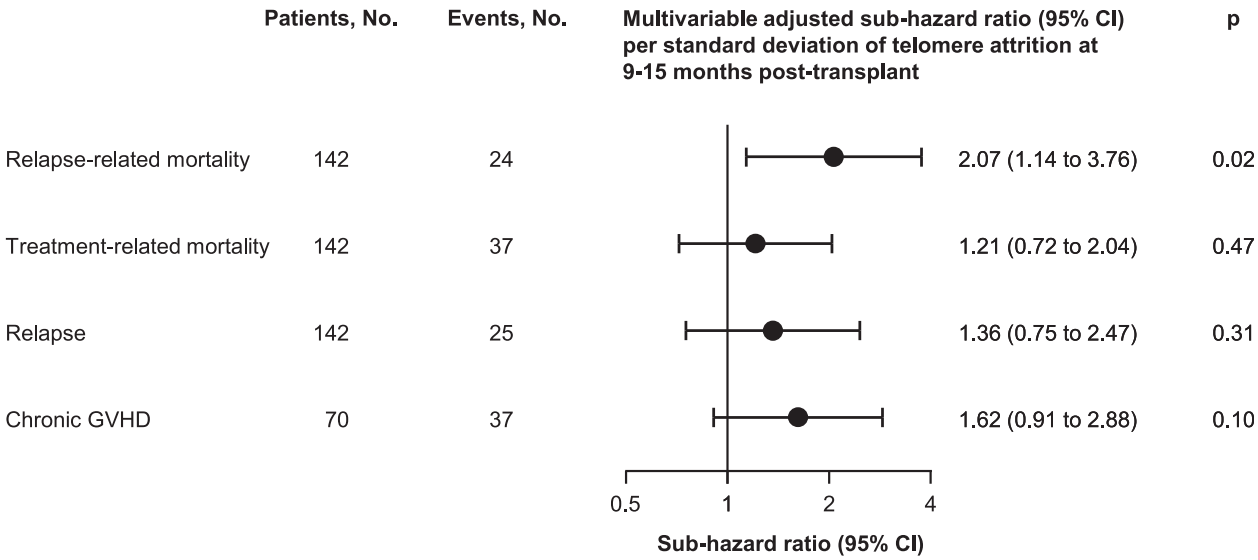

Figure 6. Risk of cause-specific mortality and complications per standard deviation of telomere attrition at 9 to 15 months post-transplant. Telomere attrition was calculated for each patient as the difference between recipient telomere length at 9 to 15 months post-transplant and donor pretransplant telomere length, divided by donor pretransplant telomere length. Patients were excluded from the analysis if they had been diagnosed with relapse before the 9 to 15 months post-transplant sample was obtained. Relapse-related mortality was defined as death after relapse or progression of disease. Risk estimates for CMV reactivation and acute GVHD could not be calculated because only 1 patient was diagnosed with a first CMV reactivation after the 9 to 15 months post-transplant sample was obtained and no patients developed acute GVHD after the 9 to 15 months post-transplant sample was obtained. The number of patients is lower in the analysis on chronic GVHD than in the other analyses because some patients had already developed chronic GVHD before the 9 to 15 months post-transplant sample was obtained.

DISCUSSION

In this study of 240 consecutive patients treated with nonmyeloablative allo-HCT for hematologic malignancies, we found that when compared with pretransplant telomere length in the recipients' respective donors, recipients with extensive telomere attrition at 9 to 15 months post-transplant had low overall survival and high relapse-related mortality. Although the allo-HCT procedure led to shorter mean telomere length in the recipients when compared with donor pretransplant telomeres, recipients on average still had longer telomeres at 9 to 15 months post-transplant when compared with recipient pretransplant telomeres. These are novel findings.

The low overall survival in recipients with extensive MNC telomere attrition at 9 to 15 months post-transplant could hypothetically be caused by impaired adaptive immune functions as a result of attrited telomeres in lymphocytes and lymphocyte precursors, limiting the cells replicative capacity. This potential mechanism is supported by previous studies on healthy volunteers and individuals from the general population, reporting that short leukocyte telomere length was associated with high risk of infections and high risk of infection-related death, which indicates that leukocyte telomere length may be a marker of immune competence [43-46]. Similarly, in a study on 22 healthy adults, longer telomeres in B lymphocytes were associated with higher antibody production after receiving an influenza vaccine in vivo, whereas telomere length in $CD8^+$ influenza-specific T lymphocytes was positively correlated with the level of replicative expansion after in vitro stimulation of the CD8 cells with a synthetic influenza peptide [47]. However, in contrast to our finding that all-cause mortality was higher in patients with extensive telomere attrition at 9 to 15 months post-transplant, the attained recipient telomere length at 9 to 15 months post-transplant was not convincingly associated with risk of all-cause mortality when adjusting for potential confounders but ignoring the extent of telomere attrition. Hence, our findings indicate that it is the rate of telomere attrition during the first 9 to 15 months post-transplant and not the level of telomere length attained at 9 to 15 months post-transplant that predicts overall survival. This is in contrast to what would be expected if short lymphocyte telomeres were a direct cause of increased mortality, suggesting that the association between extensive telomere attrition and high mortality is less likely to be due to a causal effect. However, although the association was not significant at the $P < .05$ level after multivariable adjustments, we did observe a trend toward higher overall survival in recipients with long attained telomere length at 9 to 15 months post-transplant, and our results cannot exclude the possibility of a causal association between recipient post-transplant telomere length and overall survival.

Previous studies have reported that having high numbers of B cells or naïve T cells post-transplant were associated with lower risk of treatment failure (defined as relapse or death) (48,49) and that B cells and naïve T cells have longer telomeres than most other lymphocyte subtypes [50-53]. Therefore, another potential mechanism behind the low overall survival in patients with extensive telomere attrition post-transplant could be that telomere attrition is a marker of immune reconstitution through different levels of telomere attrition reflecting variation between patients in which lymphocyte subtypes that are predominantly present in peripheral blood [42]. Importantly, however, even though the mechanisms causing our observed associations are still unknown, the more than 4-fold higher multivariable adjusted risk of all-cause mortality we observed in the tertile of patients with the most extensive telomere attrition indicates that telomere attrition may be a clinically relevant marker for identifying patients at high risk of mortality.

When analyzing telomere length in sequential samples obtained after nonmyeloablative allo-HCT, we found that the development in telomere length after nonmyeloablative allo-HCT appeared to be similar to what has previously been described for patients treated with myeloablative allo-HCT [5-20]. Hence, when compared with donor pretransplant telomere length, we found that most recipients experienced accelerated telomere attrition in the immediate post-transplant period, but the accelerated telomere attrition seemed to occur primarily in the early post-transplant period, as telomere length was stable at 1 year (range, 9 to 15 months) post-transplant. Importantly, however, we also compared pretransplant and post-transplant recipient telomere length in consecutive patients irrespective of diagnosis, which has not been done in previous studies. We found that although the allo-HCT procedure led to shorter mean telomere length in the recipients when compared with donors, recipients on average still had longer telomeres post-transplant when compared with recipient pretransplant telomeres. Although the long-term consequences of having shortened telomeres due to allo-HCT are not fully known, studies on patients undergoing autologous HCT have found that extensive leukocyte telomere attrition post-transplant may be a risk factor for later development of therapy-related myelodysplastic syndrome, acute myeloid leukemia, and/or bone marrow failure [21-24]. Therefore, it is reassuring that our results suggests that undergoing allo-HCT is not unfavorable with regards to mean telomere length in the recipients, as the quite short telomeres found in recipients before undergoing conditioning results in recipients on average having longer telomeres 9 to 15 months after undergoing allo-HCT than they had pretransplant.

Our finding that donor telomere length was not associated with overall survival or risk of complications in consecutive patients undergoing nonmyeloablative allo-HCT is similar to the findings reported from a study on 1085 acute leukemia patients undergoing myeloablative allo-HCT [29]. In contrast, our results differ from the findings from a study on 330 aplastic anemia patients undergoing allo-HCT, which found that long donor leukocyte telomere length was associated with high overall survival [25]. Because a follow-up study on 197 of the above-mentioned aplastic anemia patients indicated that a low risk of infection-related death could explain the observed high survival in patients receiving transplants from donors with long leukocyte telomeres [54], the different results between studies on aplastic anemia patients and patients with hematologic malignancies could be due to the difference in leading causes of deaths between these patient populations (infection and GVHD versus relapse) [55]. The different results might also be explained by different graft sources, as 239 of the 240 patients in our study received a peripheral blood stem cell graft. This is especially relevant because another follow-up study with a total of 706 aplastic anemia patients, including most patients from the above-mentioned original aplastic anemia study, found that the association between long donor telomeres and low all-cause mortality was primarily seen in recipients younger than 40 years of age receiving bone marrow grafts [27]. Our finding that recipient pretransplant telomere length was not associated with overall survival or risk of complications is supported by a study on 536 acute leukemia patients treated with myeloablative allo-HCT [28]. In contrast, we cannot confirm the findings of high treatment-related mortality in recipients with short pretransplant leukocyte telomeres, as reported by 2 separate studies

each including 178 consecutive patients undergoing allo-HCT, of whom most were transplanted because of hematologic malignancies [26,30].

Among the strengths of this study is the availability of both pretransplant donor and pretransplant recipient samples as well as sequential post-transplant recipient samples, which made it possible to study each patient's development in telomere length over time. Furthermore, all patients were transplanted at a single hospital and followed prospectively post-transplant with no losses to follow-up even though patients were followed for up to 17 years post-transplant. The study is limited by not being able to determine the causality of the associations because of the observational design of the study. Also, as we measured telomere length in samples of MNCs that consist of several subtypes of monocytes and lymphocytes that may differ in telomere length [50-53], telomere attrition may reflect both direct attrition in each cells telomere length as well as shifting distributions of cell types present in peripheral blood. To examine the potential clinical usefulness of extensive telomere attrition post-transplant as a marker to identify patients at high risk of mortality, further studies are needed to confirm our observations in other cohorts and to determine clinically relevant cut-offs for defining patients with extensive telomere attrition. Further studies are also needed to investigate whether our observed associations can be replicated if other laboratory methods are used for telomere length measurements, especially since telomere length measurements by other methods such as Southern blot may be more precise than measurements by quantitative PCR [36,37]. Importantly, to examine the mechanisms underlying our observed associations, further studies on overall survival according to telomere attrition in individual MNC subtypes are needed.

In conclusion, we studied 240 consecutive patients treated with nonmyeloablative allo-HCT and found that when compared with donor pretransplant telomeres, recipients with extensive telomere attrition at 9 to 15 months post-transplant had low overall survival and high relapse-related mortality. These results indicate that telomere attrition may be a clinically relevant marker for identifying patients at high risk of mortality.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2018.09.025.

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