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Short Lung Transplant Donor Telomere Length is Associated with Decreased CLAD-Free Survival

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ABSTRACT:
Telomere length (TL) decreases with cellular aging and biologic stressors. As advanced donor and recipient age are risk factors for chronic lung allograft dysfunction (CLAD), we hypothesized that decreased age-adjusted donor TL would predict earlier onset of CLAD. Shorter donor TL was associated with increased risk of CLAD or death (HR 1.26 per 1-kb TL decrease, 95%CI 1.03-1.54), particularly for young donors. Recipient TL was associated with cytopenias but not CLAD. Shorter TL was also seen in airway epithelium for subjects progressing to CLAD (P = 0.02). Allograft telomere length may contribute to CLAD pathogenesis and facilitate risk stratification.

INTRODUCTION:

Chronic lung allograft dysfunction (CLAD) reflects progressive fibrosis and constitutes the most common cause of death after the first year following transplant[1]. However, time to development of CLAD varies substantially across lung transplant recipients. While donor and recipient age are risk factors for decreased CLAD-free survival, they do not fully capture the extent of molecular aging.[2]

Telomeres are nucleoprotein caps on the terminal region of chromosomes that provide protection from chromosomal shortening during cell replication. TLs vary inversely with age but are widely distributed within cells and cell populations. TL shortening past a critical length triggers cellular senescence. Accelerated telomere attrition is associated with increased cell turnover and stress, while inherited or sporadic mutations in telomerase-associated genes cause syndromes mimicking early aging.[3]

Short allograft TL has been associated with delayed graft function and chronic allograft dysfunction following renal transplantation[4] and poor graft survival following stem-cell transplant for aplastic anemia.[5] Case series of recipients with known telomerase mutations following lung transplantation suggest an increased risk of leukopenia but did not evaluate CLAD.[6] A small retrospective lung transplantation cohort study found no association between donor or recipient peripheral blood TL and survival.[7]

Telomere dysfunction in lung disease has been increasingly appreciated, as telomere-related mutations are implicated in familial pulmonary fibrosis and chronic obstructive pulmonary disease.[8] Experimentally, telomere dysfunction induced by selective deletion of the shelterin complex proteins in alveolar type II cells results in epithelial cell failure, remodeling, and fibrosis.[9]

These observations motivated the hypothesis that shortened donor peripheral blood

telomeres would identify lung allografts at increased risk for early CLAD or death following transplantation.

METHODS:
See supplement for detailed methods. Briefly, lung allograft recipients at UCSF were included if they provided informed consent, donor and recipient DNA samples were available, and had at least 18 months of follow up data (Supplemental Table 1). We measured TL by quantitative PCR on DNA isolated from peripheral blood mononuclear cells (PBMC) or spleen. For the sub-cohort described in Supplemental Table 4, TL in endobronchial biopsies collected within 90 days following transplant was measured by quantitative Fluorescence in Situ Hybridization (Q-FISH).

RESULTS:
Demographic features of the 175 included subjects are summarized in Supplemental Table 2. Mean donor TL exceeded that of recipients by 1.0 kb (95% CI 0.8-1.2, P <0.001) and by 0.5 kb (95% CI 0.2-0.7, P <0.001) after adjusting for age. Forty-five percent of recipients developed CLAD with a median time to CLAD of 3.8 years and 28% of subjects died. Median follow up time was 4.9 years (IQR 4.0 years). TL was inversely correlated to age for the entire cohort (Figure 1A).

Shorter donor TL was associated with an increased risk of CLAD or death with an adjusted HR of 1.25 per 1-kb decrease in TL (95% CI 1.03-1.52, 88 events, P = 0.02, Figures 1B-C). Recipient TL and donor age were not associated with CLAD-free survival (Table 1). Interaction modeling suggested that short donor telomeres are most hazardous in younger donors (P = 0.01). In a competing risks analysis adjusted for subject characteristics, decreasing donor TL was associated with both CLAD censored on death (subdistribution HR 1.25, 95% CI 1.00-1.56, 72 events, P = 0.045) and death alone (subdistribution HR 1.43, 95% CI 1.06-1.93, 40 events, P = 0.02).

An increased odds ratio of mild (OR 2.5, 95% CI 1.1-5.5) and severe leukopenia (OR 4.8, 95% CI 1.8-13.0) was found amongst recipients in the lowest 20th percentile of PBMC TL (Supplemental Table 5).

Endobronchial TL was measured in subjects with extremes of time to CLAD to determine the

Table 1: Cox proportional hazards models for CLAD-free survival as a function of donor and recipient telomere length.

Multivariable risk of CLAD or death model[†] [‡]				
	Hazard ratio	**95% CI**		**P-value**
Telomere length (per 1 kb decrease)				
Donor	1.25	1.03	- 1.52	0.02
Recipient	1.02	0.87	- 1.2	0.79
Age (per decade)				
Donor	1.03	0.86	- 1.22	0.76
Recipient	1.06	0.8	- 1.41	0.67
Multivariable risk of CLAD or death interaction model[†]				
	Hazard ratio	**95% CI**		**P-value**
Telomere length (per 1 kb decrease)				
Donor	2.39	1.4	- 4.07	0.001
Age (per decade)				
Donor	0.29	0.11	- 0.79	0.02
Donor Age * Donor Telomere length				
Interaction	0.83	0.71	- 0.96	0.01
Donor-age stratified, multivariable-adjusted risk of CLAD or death models[†]				
	Hazard ratio	**95% CI**		**P-value**
Donor telomere length (per 1 kb decrease)				
Donor Age <30	1.65	1.18	- 2.31	0.004
Donor Age ≥30	1	0.79	- 1.27	0.97

[†]Includes donor and recipient telomere length, age, diagnosis group, lung allocation score, donor gender, recipient gender, non-Hispanic white donor, non-Hispanic white recipient, transplant type, and donor "ever smoker" status.
[‡]Univariable and multivariable models including all covariates are included as Supplemental Table 3.

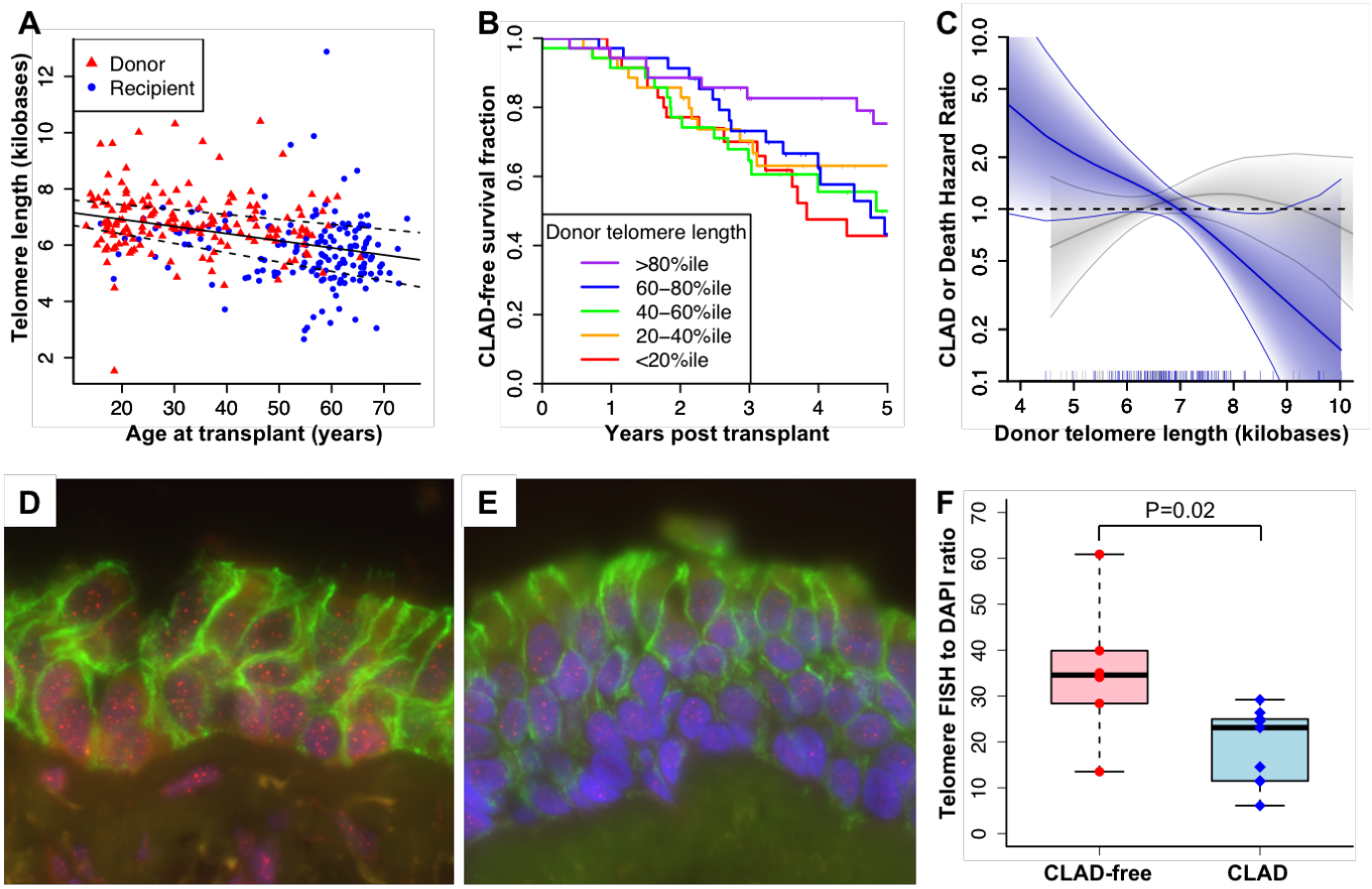

Figure 1: Short donor telomere length in peripheral blood and airway biopsies is associated with decreased CLAD-free survival. (A) Telomere length in kilobases versus age in years for donors (red triangles) and recipients (blue circles). Linear regression of telomere length versus age across both cohorts resulted in a slope of -25 (95% CI -17 to -32) base pairs per year. (B) Kaplan-Meier plot showing CLAD-free survival stratified by quintiles of donor telomere length. Improved CLAD-free survival was seen across increasing quintiles of donor telomere length (P = 0.04), with improved survival in the >80%ile group compared with the <20%ile group (P = 0.02). Increasing quintile of recipient telomere length was not associated with CLAD-free survival (P = 0.59). (C) Hazard ratios for CLAD or death per 1 KB decrease in telomere length stratified by donor age under 30 (blue) or over 30 (grey) and adjusted for subject characteristics in Supplemental Table 2 are plotted against donor telomere length. Solid line shows spline fit of data with shaded area indicating 95% confidence intervals. (D-E) Quantification of telomere length in airway epithelial cells from lung transplant recipients who would either develop CLAD or remain CLAD-free. Endobronchial biopsies collected during surveillance bronchoscopy within the first 90 days post-transplant (median 31 days) were stained for telomeric DNA (red), E-cadherin (green), and total DNA (DAPI, blue). Shown are representative images from (D) a CLAD-free subject with a telomere to DAPI ratio of 40 and (E) a CLAD subject with a telomere to DAPI ratio of 15. As shown in (F), telomere length was higher in CLAD-free subjects than in subjects with CLAD (P = 0.02 by Mann Whitney test).

association with allograft TL. The populations are described in Supplemental Table 4. Endobronchial TL was significantly greater in the >8 year CLAD-free group, relative to those that developed CLAD at a median of 2.9 years (P = 0.02, Figure 1F).

DISCUSSION:

In summary, short donor PBMC TL was associated with worse CLAD-free survival, independent of donor age, in univariable and multivariable models, while recipient TL was associated with increased incidence of post-transplant leukopenia. TL was also shorter in airway epithelial cells from lung allografts that went on to develop CLAD, suggesting that decreased TL within the lung may directly contribute to the development of CLAD.

Genetic predisposition or environmental insults affecting the lung may contribute to short telomeres. Telomere shortening beyond what is expected with aging might signify systemic or inherited telomere dysfunction, which may explain the observed interaction with donor age. Cell turnover that is either homeostatic or in response to injury is impaired in the context of critically short telomeres. Allografts with short telomeres may have a higher frequency of senescent cells and thus be at increased risk for the airway-centric or parenchymal lung fibrosis that are pathologic correlates of CLAD.[1] Lung allografts may be particularly susceptible to telomere attrition because of repeated environmental insults or high rates of cell turnover relative to other solid organs.[10] Recipient PBMC TL was associated with clinically significant leukopenia, consistent with previous reports of increased leukopenia in lung allograft recipients with telomerase mutations.[6] Short PBMC TL may reflect a larger population of senescent bone marrow cells unable to increase leukocyte production, particularly under the influence of immunosuppressive medications. Assessment of recipient TL has the potential to guide post-transplantation immunosuppressive and antiviral dosing.

Strengths of this study include the large sample size relative to other investigations of TL in lung transplantation,[7] length of follow up, uniform assessment of TL with quality controls, and confirmation with measurement of TL in airway tissue. The study is from a single center, limiting differences in clinical practice; however, external validation would strengthen the generalizability of the findings. In particular, relative to ISHLT registry reports, this cohort included more recipients transplanted for pulmonary fibrosis.[2] Also, we did not evaluate TL relevance for subjects with <18 months follow up.

Our findings, if replicated, may assist with improved stratification of donor risk, and facilitate the use of lungs from donors of advanced age if TL is found to be adequate. Telomerase activation may be of interest in allografts with short TL.[3]

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Author Contributions: H.E.F., J.R.G., J.A.G., and P.J.W. designed the experiments, J.A.G., R.R, S.R.H., J.K., and J.P.S. recruited subjects and contributed to sample collection and maintenance. H.E.F., G.G., and A.S.W. performed the experiments, H.E.F. and J.R.G. analysed data and wrote the manuscript. All authors read and approved the manuscript.

Competing Interests: The authors have no competing financial interests.

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Supplemental Data

Supplemental Table 1: Subject inclusion and exclusion table

683	Lung transplants at UCSF between 4/15/2000 and 3/2/2015
82	Survived < 18 months
285	Not approached
316	Approached for study
6	Refused
310	Consented
114	Missing donor DNA sample
81	Missing recipient DNA sample
31	<18mo follow up data available
182	DNA samples evaluated
7	Donor or recipient DNA sample degraded
175	Subjects included

Supplemental Table 1 (cont): Subject characteristics

	DNA missing	DNA available	P-value
Subjects	**135**	**175**	
Age, mean (sd)			
Recipient	54 (11)	54 (13)	0.95
Donor	32 (14)	32 (14)	0.67
Diagnosis group, N (%)			
A	18 (13%)	43 (25%)	0.03
B	6 (4%)	11 (6%)	
C	12 (9%)	21 (12%)	
D	99 (73%)	100 (57%)	
Female, N (%)			
Recipient	60 (44%)	83 (47%)	0.61
Donor	64 (47%)	62 (35%)	0.07
Transplant type, N (%)			
Double lung	124 (92%)	157 (90%)	0.80
Single lung	9 (7%)	15 (9%)	
Heart-lung	2 (1%)	3 (2%)	
White Non-Hispanic, N (%)			
Recipient	98 (73%)	136 (78%)	0.36
Donor	63 (47%)	82 (47%)	0.83
Lung allocation score, mean (sd)	59 (22)	54 (21)	0.07
Donor ever smoker, N (%)	7 (5%)	18 (10%)	0.24
CLAD-free survival, median years (95% CI)	6.1 (4.5-7.3)	5.5 (4.5-7.0)	0.62

Supplemental Table 2: Subject characteristics stratified by quintile of donor telomere length.

	Total	Quintile of donor telomere length					P-value
		<20%	20-40%	40-60%	60-80%	>80%	
Subjects	175	35	35	35	35	35	
Maximum telomere length, kilobases	11.16	6.0	6.6	6.9	7.5	11.16	
Age, mean (SD)							
Recipient	54 (12)	56 (12)	56 (10)	54 (14)	54 (13)	51 (14)	0.05
Donor	33 (14)	36 (16)	35 (14)	31 (12)	32 (12)	31 (14)	0.04
Diagnosis group, N (%)							
Group A (COPD)	43 (25%)	8 (23%)	8 (23%)	9 (26%)	8 (23%)	10 (29%)	0.12
Group B (PAH)	11 (6%)	3 (9%)	0 (0%)	1 (3%)	1 (3%)	6 (17%)	
Group C (CF)	21 (12%)	4 (11%)	2 (6%)	7 (20%)	3 (9%)	5 (14%)	
Group D (PF)	100 (57%)	20 (57%)	25 (71%)	18 (51%)	23 (66%)	14 (40%)	
Female, N (%)							
Recipient	83 (47%)	15 (43%)	16 (46%)	16 (46%)	15 (43%)	21 (60%)	0.24
Donor	62 (35%)	10 (29%)	12 (34%)	12 (34%)	12 (34%)	16 (46%)	0.18
Transplant type, N (%)							
Double lung	157 (90%)	31 (89%)	33 (94%)	32 (91%)	32 (91%)	29 (83%)	0.83
Single lung	15 (9%)	3 (9%)	2 (6%)	2 (6%)	3 (9%)	5 (14%)	
Heart-lung	3 (2%)	1 (3%)	0 (0%)	1 (3%)	0 (0%)	1 (3%)	
White Non-Hispanic, N (%)							
Recipient	136 (78%)	28 (80%)	27 (77%)	26 (74%)	27 (77%)	28 (80%)	1.00
Donor	82 (47%)	18 (51%)	15 (43%)	14 (40%)	15 (43%)	20 (57%)	0.59
Lung allocation score, mean (SD)	53 (21)	55 (21)	52 (21)	53 (21)	55 (21)	51 (21)	0.41
Donor ever smoker, N (%)	17 (10%)	0 (0%)	2 (6%)	1 (3%)	7 (20%)	7 (20%)	0.01

Supplemental Table 3: Univariable and multivariable CLAD-free survival Cox proportional hazards models.

	Univariable			Multivariable		
	Hazard ratio	**95% CI**	**P-value**	**Hazard ratio**	**95% CI**	**P-value**
Telomere length (per 1kb decrease)						
Recipient	1.02	0.88 - 1.20	0.76	1.02	0.87 - 1.20	0.79
Donor	1.25	1.04 - 1.50	0.02	1.25	1.03 - 1.52	0.02
Age (decade)						
Recipient	1.22	1.02 - 1.47	0.03	1.06	0.80 - 1.41	0.67
Donor	1.05	0.9 - 1.22	0.55	1.03	0.86 - 1.22	0.76
Diagnosis group						
Group A (COPD)	1.28	0.79 - 2.06	0.74	1.34	0.74 - 2.43	0.34
Group B (PAH)	0.74	0.26 - 2.06	0.74	0.62	0.16 - 2.39	0.48
Group C (CF)	0.81	0.42 - 1.59	0.74	0.82	0.33 - 2.07	0.68
Group D (PF)	1		-			-
Female						
Recipient	1	0.66 - 1.52	1.00	1.26	0.78 - 2.02	0.35
Donor	0.57	0.36 - 0.91	0.02	0.55	0.32 - 0.95	0.03
Transplant type						
Double lung	1		-	1		-
Single lung	1.06	0.53 - 2.12	0.72	1.20	0.52 - 2.77	0.66
Heart-lung	2.08	0.51 - 8.53	0.72	2.53	0.49 - 13.0	0.27
White Non-Hispanic						
Recipient	1.23	0.74 - 2.05	0.43	1.24	0.66 - 2.32	0.51
Donor	0.88	0.58 - 1.35	0.57	0.87	0.55 - 1.36	0.54
Lung allocation score	1	0.99 - 1.01	1.00	1.00	0.99 - 1.02	0.76
Donor ever smoker	0.92	0.49 - 1.73	0.79	1.01	0.48 - 2.10	0.99

Supplemental Table 4: Telomere-FISH nested sub-cohort subject characteristics

	Total		CLAD-free		CLAD		P-value
Subjects	175		6		9		
CLAD-free years, median	3.5		> 8		2.9		
Endobronchial biopsy days post-transplant, median (IQR)			30	(34)	37	(10)	0.32
Telomere length, kb median (IQR)							
Recipient	5.9	(1.4)	5.4	(2.0)	6.2	(1.0)	0.18
Donor	6.7	(1.1)	9.2	(1.0)	5.7	(2.7)	0.05
Age, mean (SD)							
Recipient	54	(12)	50	(11)	61	(9)	0.06
Donor	33	(14)	28	(14)	37	(18)	0.34
Diagnosis group, N (%)							
Group A (COPD)	43	(25%)	5	(83%)	5	(56%)	0.10
Group B (PAH)	11	(6%)	0	(0%)	0	(0%)	
Group C (CF)	21	(12%)	1	(17%)	0	(0%)	
Group D (PF)	100	(57%)	0	(0%)	4	(44%)	
Female, N (%)							
Recipient	83	(47%)	2	(33%)	4	(44%)	1.00
Donor	62	(35%)	3	(50%)	2	(22%)	0.58
Transplant type, N (%)							
Double lung	157	(90%)	5	(83%)	7	(78%)	1.00
Single lung	15	(9%)	1	(17%)	2	(22%)	
Heart-lung	3	(2%)	0	(0%)	0	(0%)	
White Non-Hispanic, N (%)							
Recipient	136	(78%)	4	(67%)	8	(89%)	0.69
Donor	82	(47%)	5	(83%)	6	(67%)	0.91
Lung allocation score, mean	53		33		46		NA
Donor ever smoker, N (%)	17	(10%)	1	(17%)	1	(11%)	1.00

Supplemental Table 5: Incidence of cytopenia by recipient telomere length

	Recipient telomere length				
	Shortest quintile (N=33)		**Remaining quintiles** (N=120)		**P-value**
Leukopenia, N (%)					
Mild (WBC <4,000/μL)	17	(52%)	34	(28%)	0.02
Severe (WBC <2,500/μL)	10	(30%)	10	(8%)	0.002
Anemia, N (%)					
Hb < 7 g/dL	6	(18%)	14	(12%)	0.49
Thrombocytopenia, N (%)					
Mild (Plt <100,000/μL)	14	(42%)	38	(32%)	0.34
Severe (Plt <50,000/μL)	5	(15%)	10	(8%)	0.40
Any cytopenia, N (%)					
Mild	21	(64%)	65	(54%)	0.44
Severe	14	(42%)	25	(21%)	0.02

Incidence of hematopoetic dysfunction in the first 90 days post-transplant for recipients above and below the lower 20th percentile of recipient PBMC telomere length (4.98 kb).

Supplemental Methods

Subject samples and clinical data: This study was approved under University of California, San Francisco (UCSF) Institutional Review Board protocols 13-10738 and 10-00721. Recipients of lung transplantation at UCSF were included if they provided informed consent, donor and recipient DNA samples were available, and had at least 18 months of follow up data. Donor DNA was isolated from peripheral blood mononuclear cells (PBMC) or spleen, in order of preference. Quality of DNA samples was determined by gel electrophoresis and samples excluded if there was evidence of DNA degradation. DNA degradation was detected in 2.0% of donor samples and 2.1% of recipient samples.
Serial spirometry was collected on subjects dating from the time of transplant per clinical protocols. Time to CLAD was determined from date of FEV1 or FVC decline by 20% of the post-transplant baseline as previously described.[1 2] Data on white blood cell count, hemoglobin, and platelet counts were collected from electronic medical records. Survival data was determined from UCSF electronic medical records and from the United Network for Organ Sharing (UNOS) database. Telomere shortening may even be accelerated in lung tissue relative to shortening of PBMC telomeres.[3] Therefore, in a nested case-control study, telomere length was also measured on formalin-fixed plasma-embedded endobronchial biopsies that were collected within the first 90 days following transplant. Subjects were selected based on extremes of time to development of CLAD, with the 6 CLAD-free patients having the longest CLAD-free intervals of our subjects and the CLAD patients having the shortest CLAD-free intervals.

Peripheral blood telomere length measurements: We measured telomere lengths in a blinded manner, using quantitative PCR (qPRC) on donor and recipient PBMC or splenic DNA. DNA was visualized on agarose gel to determine quality, and degraded DNA samples were excluded from the analysis. We performed uniplex qPCR in triplicate with the 36B4 gene as a reference housekeeping gene. Assays were repeated until the sample standard deviation was less than 0.5. Relative telomere lengths were determined by subtracting telomere and reference median cycle threshold (CT) values. To facilitate comparison with other studies, absolute telomere lengths were interpolated from study sample delta CT values using linear regression with delta CT values for reference samples. Telomere length for these reference samples was determined by mean terminal restriction fragment length.[4]

Telomere Q-FISH assay: In a nested case-control group of 6 subjects who developed CLAD and 12 subjects who did not, telomere lengths were measured on paraffin-embedded sections of endobronchial biopsy tissue by Quantitative Fluorescence in Situ Hybridization (Q-FISH). It should be noted that this small sample size could possibly limit power to identify associations. Briefly, after deparaffinization, tissues were suspended in 10mM sodium citrate buffer, pH 6.5, heated in a microwave, then incubated for 15 min in 0.01M HCL containing 1% pepsin (Thermo Fisher Scientific, South San Francisco, CA). The tissues were washed then treated with 10mg/ml RNase solution (Qiagen, Hilden, Germany). After washing, the tissues were incubated with 0.3 µg/ml of the TelC-Cy3 Peptide Nucleic Acid (PNA) probe (F1002, Panagene, Daejeon, Korea) suspended in formamide buffer (70% formamide, 10 mmol/L Tris, pH 7.5), heated to 78ºC for 10 min then incubated overnight at 20ºC. The tissues were then washed sequentially with formamide buffer then PBS containing 0.1% Tween, blocked with 3% BSA (Sigma, St. Louis, MO), 10% donkey serum, and incubated overnight at 4ºC with rabbit anti-human E cadherin antibody (#3195S, Cell Signaling Technology, Danvers, MA). Tissues were washed with PBS containing 0.1% Tween and incubated with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (#A11008, Thermo Fisher Scientific) at 20ºC for 1 h, washed, and mounted using prolong gold anti-fade mounting medium with DAPI (Thermo Fisher Scientific). Images were acquired using a Zeiss Axio Imager 2 microscope (Zeiss, Oberkochen, Germany) and telomere signal intensity quantified using MetaMorph imaging analysis software (Molecular Devices, Sunnyvale, CA). Telo-FISH staining and quantification were performed in a blinded manner.

Analysis: Differences in subject characteristics across donor telomere length quintile number were determined by continuous or logistic generalized linear modeling or chi-square test, as appropriate. Telomere lengths in donors and recipients as a function of age were assessed by linear regression. CLAD-free survival per one-kilobase decrease in donor and recipient telomere length was determined by Cox proportional hazards modeling.[5] All 175 available samples were analyzed, for which a post-hoc power calculation identified

a 50%, 80% and 95% power to detect a hazard ratio for CLAD or death of 1.2, 1.3, and 1.4, respectively, per 1 kb change in telomere length.[6] Quintile extremes of telomere length were compared to the remainder of the population using Kaplan-Meier analysis. In multivariable Cox models, we adjusted for donor and recipient age, gender, and race; diagnosis group; LAS score; donor smoking; and transplant procedure. Missing values were imputed using 10-fold multiple imputations by chained equations for donor smoking (N=2), donor age (N=2), donor gender (N=1), donor ethnicity (N=1), and lung allocation score (N=21).[7] Imputation using median value did not substantially alter the results. The Cox proportional hazard ratio as a function of time was computed using penalized smoothing splines.[8] We also performed competing risks analysis evaluating mortality as a competing risk for CLAD. We assessed the proportionality assumption of these Cox models through inspection of the Schoenfeld residual plot and chi-squared test of correlation between Schoenfeld residuals versus CLAD-free survival time.

Logistic regression was used to evaluate for an association between recipient telomere length in the lowest quintile and post-transplant cytopenias. Leukopenia was defined by a white blood cell count per microliter of 4,000 and 2,500, which is our clinical threshold to decrease or hold, respectively, mycophenolate mofetil and/or valgancyclovir. Tissue-specific telomere lengths between CLAD and no CLAD groups were compared by Mann Whitney test.

Statistics were calculated in R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

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