Project Title: The impact of donor and recipient age-related factors on long-term haematopoietic potential and telomere length following allogeneic haematopoietic progenitor cell transplant

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Summary:

OBJECTIVE: Advanced donor and recipient age-related factors negatively impact outcomes of haematopoietic progenitor cell (HPC) transplant. Telomere shortening, which occurs due to rapid HPC proliferation during the transplant process, is a potential mechanism for age-related haematopoietic dysfunction due to the induction of HPC senescence. This study aims to determine whether age-related factors impair long-term haematopoiesis (manifested by decreased peripheral blood counts) following allogeneic HPC transplant and assess the potential role of telomeres. Significant age-related differences could help guide graft selection in cases where both old and young HLA-identical grafts are available for transplant.

METHODS: The Manitoba Blood and Marrow Transplant database was reviewed to identify allogeneic transplant patients with donors or recipients of young (<22) or old (>40) age. Age- and sex-normalized peripheral blood counts and engraftment times were compared between groups at one year post-transplant. Telomere length was assessed in subset of these patients using real-time PCR and quantitative 3D fluorescent *in situ* hybridization.

RESULTS: Young donor and recipient age significantly correlate to improved erythroid and megakaryocyte indices at one year post-transplant. Telomere length was inversely correlated with donor age and was reduced after transplantation. Telomere length was not correlated to erythroid and megakaryocyte indices in the late post-transplant period.

CONCLUSION: Reduced erythroid and megakaryocyte indices in the late, but not early, post-transplant period may be due to age-related changes in HPCs. It is unlikely that telomere dysfunction is the underlying cause of these changes. Multivariate analysis of a larger cohort is warranted to confirm the findings.

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Introduction

Haematopoietic progenitor cell (HPC) transplant is an option when treating a variety of non-malignant and malignant haematological diseases, such as aplastic anaemia, immunodeficiency haemoglobinopathies, leukemia and lymphoma (1-4). Appropriate graft selection is multi-factorial and depends on the patient's disease and the characteristics of the available grafts. Allogeneic transplantation is preferred in the setting of inherited conditions, such as haemoglobinopathies and immunodeficiency as transplantation of the haematopoietic system from another individual lacking the genetic lesion is necessary to restore functional haematopoiesis in the recipient (2,3). For malignant diseases, the graft-versus-leukemia effect of an allogeneic graft may help resolve residual disease and prevent relapse (4-7).

In situations where allogeneic transplantation is desired, there are few guidelines for graft selection. Human leukocyte antigen (HLA) compatibility between donor and host is considered paramount, as the degree of mismatch at HLA loci correlates with the incidence and severity of graft-versus-host disease (GVHD) (8,9). Progenitor cell content of the graft, as determined by the number of $CD34^+$ cells or nucleated cells, is another consideration (10). However, sufficient progenitor cells are usually available in grafts obtained by bone marrow harvest or aphaeresis of mobilized peripheral blood stem cells (PBSCs) and low $CD34^+$ counts usually only become an issue in umbilical cord blood transplant (11). Other criteria for graft selection include the relationship between the donor and the recipient and cytomegalovirus (CMV) serology. Related donors show a decreased severity of GVHD and the graft-versus-leukemia effect, which may or may not be desirable in certain situations (4,7). CMV serology is only considered in a seronegative recipient, in which case, use of a seropositive graft would be undesirable. Situations often arise in which there are several grafts available that are equivalent in terms of HLA match and contain adequate numbers of progenitor cells. Additional criteria are needed in order for clinicians to rationally select the best graft for a particular patient in these situations.

Recipient and donor age has been shown to influencing the outcome of HPC transplant and may provide additional criteria for graft selection. In human HPC transplantation, outcome with advanced recipient age is traditionally thought to be due to treatment related morbidity, chronic GVHD and other medical co-morbidities (12). This idea has recently been challenged as mortality, GVHD and disease-free survival have been shown not to vary with age (13). Studies on the quality of haematopoiesis and HPC function in older transplant recipients are lacking. In animal studies, HPC function of young progenitor cells has been shown to be negatively affected by transplantation into an older recipient. These changes were shown to be mediated by paracrine and endocrine factors present in the aged haematopoietic microenvironment, which may modulate a many aspects of cell physiology and lineage differentiation (14).

The relationship between improved transplant outcome and donor age is more firmly established. The use of younger grafts increases the overall and disease-free survival rates as well as providing a modest reduction in the incidence of acute and chronic GVHD (15-17). However, this observation is not consistent across all studies. (18). Long term graft failure and delayed immunologic recovery following transplants are associated with transplantation from older donors (19,20). Animal studies demonstrate a decreased repopulating capacity of HPCs with age (21), which is also accompanied by a loss in granulocyte-monocyte colony forming units within the graft (22). The mechanisms underlying impaired graft function with donor age is unknown and warrants further study. Telomere shortening, which is strongly associated with aging and cell senescence, may play a role in these age-related differences in graft function.

Telomeres are polynucleotide $(TTAGGG)_n$ repeats located on the distal ends of chromosome arms (23). Among other functions, telomeres serve as a solution to the so called "end replication problem" (24). This problem arises because DNA polymerase requires a RNA primer in order to bind to DNA during replication. Following replication of a segment of DNA, the primer dissociates, leaving a small, unreplicated region. This causes no problem when replication is initiated at the internal regions of the chromosome, as another primer simply binds

upstream of the initial primer and fills in the unreplicated gaps (24). However, at the extreme ends of chromosome arms, there is no upstream sequence for subsequent primers to bind to. This results in small segments of DNA going unreplicated, causing loss of genomic material equivalent to 30-150 bp with each cell division (25). Telomeres protect genomic integrity by providing a repetitive, non-coding sequence to be lost with every cycle of cell division (25). As the organism ages and cells continue to divide, telomeres become shorter and their functionality is compromised. Telomere shortening is irreversible in terminally differentiated cells. However, some stem and progenitor cells, including HPCs, express the enzyme telomerase (hTERT), which catalyzes the elongation of telomere sequences (26). In HPCs, hTERT activity is sufficient to slow the rate of telomere attrition but not to halt it entirely. As a result, telomeres in HPCs exhibit gradual shortening of approximately 9bp/year throughout the life of the individual (27).

Telomeric sequences complex with proteins to form the shelterin complex. The shelterin complex's most important functions are preventing telomeric regions from being recognized by the cell's double strand break repair machinery and preventing the end-end fusion of telomere sequences of different chromosomes (25). Telomeric sequences contain a 3' overhang that would normally be recognized as a double strand break, triggering cell senescence. If the shelterin complex is intact, this does not occur and the cell survives. This function is impaired if the telomeres become shortened and proteins in the shelterin complex dissociate (27). Telomeres lacking proteins of the shelterin complex will undergo p53- and p21-mediated cell senescence (25,29). This mechanism prevents the persistence of cells with critically shortened telomeres. This is important as short, uncapped telomeres increase the likelihood of non-homologoous end joining with other chromosomes. This in turn may predispose the chromosomes to break-fusion-bridge cycles, resulting in non-reciprocal translocations and an increased risk of malignant transformation (30,31). Short telomeres have been shown to increase the risk and severity of several hematologic malignancies and other solid tumors (32-34).

Telomere dynamics in the context of HPC transplantation are important both in terms of donor and recipient age. Numerous studies have demonstrated telomere shortening in precursor across several haematological cell lineages following transplantation (35,36). The majority of telomere shortening occurs in the first year post-transplant, in which the small number of HPCs within the graft must rapidly proliferate to regenerate the entire haematopoietic system in the recipient (37). Most studies estimate this to be equivalent to 10-20 years of normal aging, although estimates are as high as 45 years of normal aging (36,38,39). Whether this impairs the biological function of the graft is controversial (40). Late graft failure with marrow aplasia due to telomere exhaustion and progenitor cell senescence has been noted in case reports, but its actual prevalence is unknown (19). Several congenital and acquired marrow failure syndromes such as dyskeratosis congenita, Fanconi anaemia and aplastic anaemia are associated with telomere abnormalities (41,42). The incidence of secondary malignancies following HPC transplant is also high and could potentially be explained by telomere dysfunction (43). One could expect that the recipients of grafts from older donors, who's HPCs have already experienced a lifetime of cell division, may be at increased risk for impaired haematopoiesis, graft failure or secondary malignancy.

The link between the age of the haematopoietic microenvironment telomere length has not been well studied. The haematopoietic microenvironment consists of adipocytes, fibroblasts and stromal cells in the recipient's marrow. These cells have the ability to modulate the activity of HPCs through paracrine signalling and direct cell-cell contact. Animal studies have demonstrated that the cytokine milieu in the aged haematopoietic microenvironment can alter the physiology of young HPCs and cause them to behave like aged HPCs (14). The effect that these physiological alterations may have on telomere dynamics are unknown. However, hTERT activity can be modulated by several factors present in the microenvironment. These included interferons, androgens and TGF-β (inhibitors) as well as IGF-1, VEGF and IL-2,4,6,7 and 13 (activators) (44). Expression of genes for IGF-1, VEGF, IL-2 and IL-4, by bone marrow mesenchymal cells decreases with age (45,46). TGF-β levels increase with age of the microenvironment (47). Therefore, it is possible that alterations in hTERT activity precipitated by age-related changes in paracrine signalling networks could predispose older transplant recipients to telomere attrition.

This pilot study proposes to evaluate the effects of donor and recipient age on haematopoietic function in the late post-transplant period. Cases in which donor and recipient are of the same age (either old or young) or of disparate ages (old donor/young recipient and vice versa) will be studied to help understand the contribution of aging in HPCs and aging in the haematopoietic microenvironment to graft function. Peripheral blood counts at one year-post transplant will be used to indicate the quality of haematopoiesis in the recipient.

Telomere lengths will be measured on peripheral blood lymphocytes, which are highly correlated to telomere lengths of HPCs (48). The feasibility of using real-time PCR and quantitative three dimensional fluorescent *in situ* hybridization (3D qFISH) to measure telomere length will be assessed. Both methods will help indicate whether changes in telomere length may underlie changes in haematopoietic quality. We predict that haematopoietic function and telomere length will decline with the age of the donor and the recipient. If present, age-related differences in haematopoietic function or telomere characteristics may change the practice of graft selection. This is particularly true in the paediatric setting, where the graft must resist telomere shortening and progenitor cell exhaustion and retain functionality for the considerable remaining life of the child.

Methods

Participant selection

The Manitoba Blood and Marrow Transplant database was reviewed to identify all living recipients of allogeneic HPC transplant since June 1, 2005. Patients receiving follow-up care at institutions outside of Manitoba were excluded due to unavailability of medical records. Eligible patients were grouped according to donor and recipient age. Those under the age of twenty two were considered "young" and those over the age of forty were considered "old". Age cut-offs were determined arbitrarily in an attempt maximize age-related differences between groups while excluding a minimal number of patients. Patients were grouped as followed: young donor/young recipient (Y/Y), young donor/old recipient (Y/O), old donor/old recipient (O/O), old donor/young recipient (O/Y) and cord blood donor/young recipient (CB).

Effect of age on long- and short-term haematopoietic function

The Manitoba Blood and Marrow Transplant database and the patients' electronic charts were reviewed to obtain demographic and clinical transplant data, including age, sex, transplant date, indication for transplant, source of haematopoietic stem cells (peripheral blood, bone marrow or umbilical cord blood), relationship between the donor and recipient (related or unrelated) and ongoing use of immunosuppression at one year post-transplant. Peripheral blood counts at one year post-transplant were taken as a primary endpoint and used assess the quality of haematopoiesis using parameters associated with the erythroid (RBCs, haemoglobin, haematocrit), megakaryocyte (platelets), granulocyte/monocyte (WBCs, neutrophils, monocytes) and lymphoid (lymphocytes) cell lineages. All haematological indices were analyzed, and haemoglobin, platelet count and neutrophil count were selected as representative measures of erythropoiesis, megakaryopoiesis and granulopoiesis, respectively. Haematology parameters were standardized by computing z-scores using North American age- and sex-specific population norms (49). Patients were classified as cytopenic if their scores were two standard deviations below the normal age-and sex-specific population mean. Haematopoietic function in the acute post-transplant period was assessed by measuring time to platelet recovery to $20x10^9$/L and neutrophil recovery to $0.5x10^6$/L. Differences in blood counts and engraftment time were analyzed using one-way ANOVA with Tukey's test for post-hoc comparisons (Kruskall-Wallis with Dunn's test was used for non-normally distributed data) (α=0.05). Differences in proportion were compared using Fischer's exact test. All statistical tests were performed using SigmaPlot 11.0 software.

Sample processing

Donor and recipient peripheral blood lymphocytes were obtained in accordance with the University of Manitoba Research Ethics Board policy. Pre-transplant donor lymphocytes and DNA are routinely collected and archived during the process histocompatibility testing and were obtained to give a baseline measure of telomere length.

Recipient lymphocytes were obtained via peripheral blood draw at least one year post-transplant. Approximately 20mL of blood was drawn into tubes anti-coagulated with 100 units of preservative-free heparin (Organon). Samples were stored 4°C for a maximum of one day before lymphocytes were isolated.

Recipient lymphocytes were isolated using ficoll density separation. Patient samples were diluted 1:1 with Plasma-lyte A (Baxter) and layered onto ficoll solution (MJS BioLynx) in approximately a 1:1 ratio diluted sample to ficoll. Cells were centrifuged at 400g for 30 minutes. Following centrifugation, the lymphocyte layer was aspirated and washed twice in Plasma-lyte A, with centrifugation at 300g for 10 minutes between washes. Lymphocytes were cryopreserved in a 3:1:1 solution of Plasma-lyte A:human serum AB (Invitrogen):dimethyl sulphoxide (Bioniche Pharma) and stored at -80°C.

Genomic DNA was extracted from isolated lymphocytes using an EZ1 BioRobot extraction system whole blood protocol (Qiagen) according to the manufacturer's instructions.

Lymphocytes to be analyzed by 3D qFiSH were subject to short term culture for 96 hours at 37°C. Optimal culture conditions were established experimentally by culturing cells in various volumes of culture media supplemented with different growth factors and observing cell proliferation and viability. Cell number was assessed using a Beckman-Coulter AcT Diff automated cell counter and viability was assessed using trypan blue staining. Patient lymphocytes were cultured in RPMI 1640 medium (Gibco) with 10% human serum supplemented 1:400 phytohemagglutinin (Invitrogen) and 100U/mL interleukin-2 (Stem Cell Technologies).

Real-time PCR determination of telomere length

Real-time monochrome multiplex PCR was conducted as previously described (50). Briefly, all reactions were conducted in 96 well microplates in a CFX96 Touch real-time PCR detection system (BioRad) using with 25µL reaction volumes. Final reagent concentrations were: 1x SYBR Green reagent (Qiagen), 720nM each of sense and antisense telomere primer (Sigma), 400 nM each of sense and antisense β_2-microglobulin primer (Sigma), 1.6ng/µL genomic DNA. β_2-microglobulin was used as a single copy control gene. Primer sequences and thermocycling conditions were as described by Cawthon, except that 37 cycles of amplification were performed instead of 32 (50). Ct values were computed by non-linear regression using CFX Manager software. Telomere length is expressed as the ratio of telomere to β_2-microglobulin signal (T/S ratio) and calculated using the standard curve method (50). For analysis, patients were grouped according to donor age (<20 years vs. >33 years). Differences between old and young donors and between pre- and post-transplant samples were compared using the student's t-test (Mann-Whitney test for non-normal data; α=0.05). Post-transplant telomere measurements were correlated to peripheral blood counts by linear regression.

3D qFiSH determination of telomere length

Protocol was conducted as previously described (51). Briefly, lymphocytes were fixed in a 3:1 methanol:acetic acid solution then spread on slides. Slides were incubated in 3.7% formaldehyde/1xPBS solution (20 minutes), then washed thrice in 1xPBS (5 minutes) then once in 0.5% triton X-100 (10 minutes). Slides were then incubated in 20% glycerol before being subject to four freeze-thaw cycles in liquid nitrogen. Slides were then washed twice in 1xPBS (5 minutes) and once in 0.1M HCl (5 minutes) before being incubated in 70% formamide/1xSCC buffer. Slides were denatured at 80°C for three minutes then hybridized for two hours at 30°C using a Vysis/Abbott hybrite using a telomere-specific Cy3 conjugate PNA probe (DAKO). Slides washed twice in 70% formamide/2xSCC (10 minutes), then 1xPBS (1 minute), then 0.1xSCC (5 minutes, 55°C), then twice in 0.05% Tween 20/2xSCC. Nuclei were stained with DAPI then dehydrated 70%, 90% then 100% ethanol (2 minutes each). Slides were mounted in VectaShield anti-fade (Vector Laboratories) and stored at 4°C overnight.

Imaging was performed using an AxioImager ZI microscope (Zeiss) and AxioCamHRm charge-coupled device (Zeiss) using a 63x oil objective. Exposure times were kept constat at 187 ms for Cy3 and 2 ms for DAPI. Thirty

cells were analyzed for each sample. Eighty z-stacks of image were taken with a 200 nm slice thickness and 107 nm lateral sampling distance. Images were processed using constrained iterative deconvolution and analyzed using TeloView software to determine integrated intensities of Cy 3 signals, which are proportional to telomere lengths (51). Sample size did not permit statistical analysis.

Results

Effect of age on long term haematopoiesis

As of June 1, 2010, there were 108 survivors of allogeneic HPC transplant in the province of Manitoba who were transplanted after January 1, 2005. Of these patients, 31 (28.7%) received bone marrow, 66 (61.1%) received mobilized peripheral blood and 9 (8.3%) received cord blood. Related donors were available for 64 (59.2%) of patients. More males (60.2%) received transplants than females. The median recipient age was 42 (interquartile range: 17-52) and the median donor age was 36 (interquartile range: 23-48). Seventy-one patients met the criteria for extreme donor and recipient age; thirteen of which were excluded because relevant blood counts were unavailable. Group characteristics are summarized in Table 1. In addition to age, groups differed significantly by primary diagnosis ($p<0.001$), cell source ($p<0.001$) and donor-recipient relatedness ($p=0.009$) but not sex or immunosuppression use. Numbers were too small to support multivariate analysis.

Considered collectively, allogeneic transplant patients showed lower peripheral blood counts than age- and sex-matched populations with haemoglobin, platelets and neutrophils z-scores of -1.58, -1.31 and -0.53, respectively at one year post-transplant. A high percentage of patients met our criteria for cytopenia, with 16% of patients having z-scores less than -2.0 for haemoglobin, 16% for platelets, 14% for both platelets and haemoglobin and 0% for neutrophils. When patients were grouped according to donor and recipient age, patients in the Y/Y and CB group had higher haemoglobin and platelet, but not neutrophil, z-scores, than the Y/O, O/O and O/Y (median haemoglobin: 0.29, -0.30, -1.82, -1.66, -1.46; median platelets: -0.04, -0.95, -2.46, -1.75, -1.04; median neutrophils: -0.88, -0.52, -1.04, -1.11, -0.82 for Y/Y, CB, Y/O, O/O and O/Y, respectively.) (Figure 1A-C). Fewer patients in the Y/Y and CB group met the criteria for cytopenia (Figure 1D). Short-term granulopoiesis was not affected by age as all groups showed similar median time to engraftment (range of 14.5-20 days). Median time to platelet engraftment was delayed in the CB group relative to all other groups (37 vs. 16.5-27 days).

Real Time PCR determination of telomere length

Telomere length was determined on eleven patients. For all patients, pre-transplant donor samples had longer telomeres than samples collected at least one year post-transplant (Figure 2). Patients with donors younger than twenty years of age had a mean T/S ratio of 3.00 ± 0.53 before transplant and 1.36 ± 0.26 after transplant. This differed significantly from patients with old donors, which had T/S ratios of 1.63 ± 0.22 and 0.80 ± 0.08 in the pre- and post-transplant samples, respectively ($p<0.005$) (Figure 3A). Recipients of young grafts showed a 2.23 ± 0.25 fold change in T/S ratio between pre-and post-transplant samples, which was not significantly different from recipients of old grafts, who experience a 2.03 ± 0.22 fold reduction ($p=0.21$) (Figure 3B). The fold reduction in T/S ratio was not related to the dose of nucleated cells/kg, $CD34^+$ cells/kg or colony forming units in the graft at time of transplant (data not shown). Post-transplant T/S ratios did not correlate to age- and sex-specific haemoglobin ($r^2 = 0.001$), platelet ($r^2=0.083$) or neutrophil ($r^2=0.099$) counts at one year post-transplant (Figure 4).

Effect of short term culture on lymphocytes

The optimal primary culture conditions for lymphocytes cryopreserved according to cell therapy lab standards as outlined in methods was determined to be RPMI/10% human serum supplemented with 100U/mL IL-2 and 1:400 phytohemmoglutin. If plates were seeded with 10^6 lymphocytes/mL in at least 2-4mL of medium and cultured for 4-5 days, 2-3 fold expansion of the lymphocyte population was routinely achieved while

understand the contribution of the age of HPCs and the recipient's microenvironment to long term HPC function. Further analysis on a patient cohort large enough to support multivariate analysis is warranted.

Telomere length measurement by real-time PCR are consistent with other observations that telomere length decreases with age, as pre-transplant samples from young donors had greater T/S ratios than old donors (Figure 3A). We have also confirmed that significant telomere shortening occurs in the post-transplant period as post-transplant T/S ratios are significantly less than pre-transplant T/S ratios in both age groups (Figure 3A). The amount of telomere shortening is likely significant as many patients showed a greater than two-fold change in T/S ratio between pre- and post-transplant samples. Unfortunately, we do not know how much of this change in T/S ratio is due to the transplant process itself and how much is due to normal aging as changes in T/S ratio in healthy age-matched controls per year of aging was not determined. Also, comparison of changes in telomere length to those found by other investigators is not easily performed. Most other studies use restriction enzyme or fluorescent intensity based methods (such as 2D FiSH and flow FiSH), which give telomere length in base pairs and fluorescent intensity units, respectively (37,54). Although the T/S ratio can be converted to a base-pair measure of telomere length through linear correlation with telomere restriction fragment length, this work has yet to been done with this data (55).

Fold change in T/S ratio did not differ between patients with different donor or recipient ages (Figure 3B). This seems to indicate that HPCs in patients of any age undergo similar replicative stress during the transplant process. Also, hTERT activity in HPCs has the potential to attenuate telomere shortening during times of replicative stress (26). Since no differences in the change in T/S ratio were observed between groups, it is possible that no difference also exists in their hTERT activity. This could indicate that hTERT activity in young and old HPCs does not differ in response to the transplant process and/or that the old and young haematopoietic microenvironments do not differ in their ability to induce hTERT activity. A direct assay of hTERT activity in HPCs from these patients is needed to confirm this speculation.

Although telomere length is reduced during the transplant process, it is unlikely that these changes underlie the impaired long-term haematopoiesis noted in transplant recipients. Post-transplant telomere length was not significantly correlated with haemoglobin, platelet or neutrophil counts (Figure 4). Had telomere length been reduced enough to induce widespread progenitor cell senescence, deficiencies in multiple blood cell lineages should have been noted and have been proportional to telomere length. It is possible that telomere attrition due to HPC transplant may cause impaired haematopoiesis in the very late post-transplant period as already shortened telomeres continue to shorten as the patient ages. Analysis of patient blood counts several years post-transplant rather than at only the one year post-transplant time point will help clarify this question. However, this data is consistent with the work of Den Elzen *et al*, who have showed that telomere length does not correlate with the incidence of anaemia or other abnormalities in haematological parameters in the elderly (56). Our results should also be interpreted with caution as participant numbers were not adequate to obtain sufficient statistical power.

Post-transplant samples analyzed by 3D qFiSH are somewhat in agreement with results from the real-time PCR assay. Little difference was observed between the post-transplant samples from patients PB003, VJ004, DJ005 and CS006, who also had very similar T/S ratios (Figure 2). When the pre-and post-transplant sample from patient CS006 are compared, a reduction in median fluorescent intensity is observed in the post-transplant sample (Figure 2,5). The post-transplant sample also had fewer telomeres with fluorescent intensities ranging from $2.0x10^4$-$4.0x10^4$ and more telomeres with fluorescent intensities ranging from $3.0x10^3$-$1.5x10^4$. This could be due to shortening of intermediate length telomeres from the $2.0x10^4$-$4.0x10^4$ range to the $3.0x10^3$-$1.5x10^4$ range during the transplant process. However, the differences between the pre- and post-transplant samples from CS006 are subtle and statistical analysis is not readily performed on this single sample. Also, the fact that only subtle differences exist between the two samples calls the validity of the measurements into question, as more substantial differences in telomere length were observed using real-time PCR. Considering the consistency of

the PCR data across all samples, it seems more likely that problems were encountered with the 3D qFiSH method.

Several aspects of this study highlight the difficulties in using 3D qFiSH for the retrospective analysis of archived material. The advantage of 3D qFiSH is that it provides information on not only mean/median telomere length, but the variation in telomere lengths of individual cells, the spatial organization of telomeres in the nucleus and the presence of telomere aggregates (57) (Figure 6). However, in order for these measurements to be valid, the three dimensional architecture of the nucleus must be preserved in the sample. It was previously noted that DMSO found in cryopreservatives distorts the 3D nuclear architecture (Mai, unpublished), altering the number of telomeres, number of aggregates and length of telomeres detected. Although we have demonstrated that cells placed in short term culture following cryopreservation are similar to non-cryopreserved cells (Table 2), difficulties still remain in using manipulated archived material. The quantity and quality of cells stored at the time of histocompatibility typing was not as rigorously controlled as cryopreservation of post-transplant samples collected explicitly for this study. Poor growth of pre-transplant samples may not reverse DMSO-induced changes in nuclear architecture or may not yield sufficient cells for analysis, as was the case with the majority of our samples. It is also possible that some of our sample processing techniques have induced changes in telomere architecture as a large percentage of cells, even control cells that were not subject to culture, demonstrated aggregates. Typically only 3% of lymphocytes show aggregates (Ishdorj; unpublished), while approximately 50% of our control samples showed aggregates. The reason for this discrepancy is still under investigation. A potential cause may be heterogeneity in the population of lymphocytes, as nuclear size and telomere staining does show considerable variation within a sample (Figure 6A), and selection of a different sub-population of lymphocytes for imaging (i.e. all large nuclei or all small nuclei) account for some of these differences.

The major drawback with 3D qFiSH is that it is a multi-step, labour intensive process. Care must be taken at all steps of the procedure to ensure consistency between samples. Differences in fixation efficacy, hybridization efficiency and photobleaching by ambient light, among other things, can affect the reproducibility of fluorescent intensity measurements (55,58). Therefore, this technique is best used by experienced researchers in studies with small cohorts of patients.

Low patient enrolment, low statistical power and difficulties with methodology prevent the true impact of changes in telomere length on long term haematopoiesis from being assessed. This question would be best answered by a prospective multi-center study in which haematopoietic progenitor cells could be obtained from a large number of donors at time of transplant, and from recipients at one or more years follow-up. This would allow telomere measurements to be made directly on HPCs instead of using lymphocyte telomere length as a surrogate marker. It would also allow samples to be processed immediately without cryopreservation, removing some of the difficulties encountered with the 3D qFiSH method. 3D qFiSH remains an attractive method for examining telomeres, as it is able to detect critically short telomeres and telomere aggregates (57). If a predisposition towards forming aggregates exists, it could potentially explain the increased risk of second malignancies in transplant patients (43). For simple measures of average telomere length, real-time PCR has proven to be a simple and effective method and is suitable for use in large scale, high enrolment studies.

Based on data from the present study, other causes of age-related HPC dysfunction should be investigated to explain impaired haematopoiesis in the late post-transplant period. These may include the analysis of the surface phenotype and gene expression profiles of different progenitor cell populations from old and young donors. Mouse models have demonstrated that certain subpopulations of HPCs have a greater capacity to restore long-term haematopoiesis in certain blood cell lineages (59). Recipient factors should also be considered as we demonstrated that advanced recipient age was also associated with poor haematopoiesis. These may include the expression of surface receptors such as SDF-1 on bone marrow stromal cells, which influence the homing and engraftment of HPCs in the marrow, as well a production of paracrine factors in the marrow microenvironment, which may influence lineage commitment and cellular physiology of engrafted cells (14,45,60)

In closing, we have demonstrated that the recipients of allogeneic HPC transplant exhibit lower erythroid and megakaryocyte indices on their complete blood counts at one year post-transplant, but do not exhibit differences in platelet and neutrophil engraftment in the early post-transplant period. Depressed blood counts were more pronounced in older recipients and in those receiving grafts from older donors. We investigated telomere attrition, which can cause cell senescence and graft impairment, as a potential cause for impaired haematopoiesis. Although telomeres did shorten during transplant, and older donors had shorter telomeres before and after transplant, the post-transplant telomere lengths were not correlated with peripheral blood counts. Therefore, other potential causes of age-related HPC dysfunction should be sought to explain impaired haematopoiesis in the late post-transplant period.

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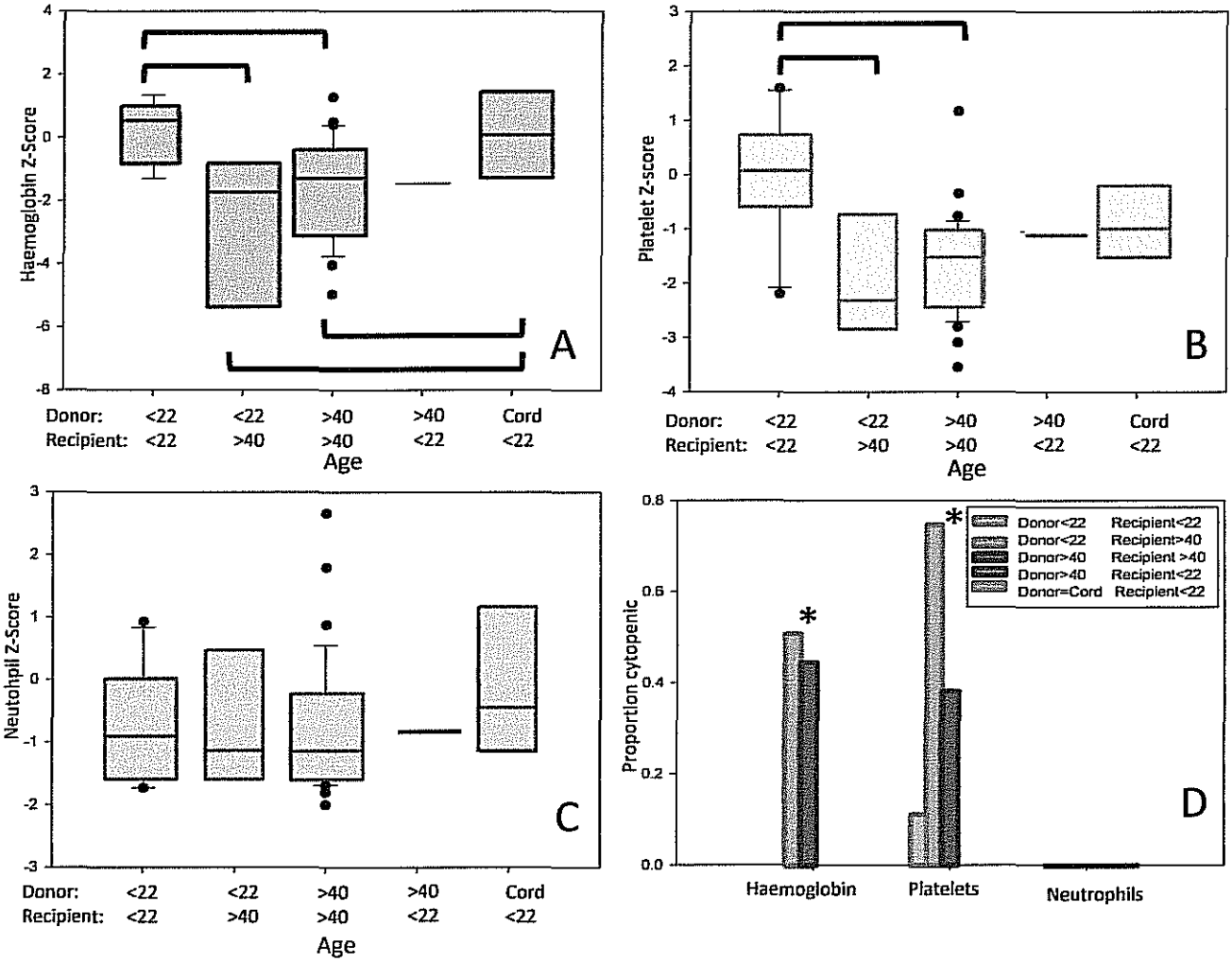

Figure 1. Z-scores of **(A)** haemoglobin, **(B)** platelet and **(C)** neutrophil levels at one year post-transplant. Significance by one-way ANOVA ($\alpha<0.05$, $\beta>0.80$) is shown with square brackets. **(D)** Proportion of patients with z-scores less than -2.0, representing marked blood count depression. Asterix indicates significant differences between study groups by Fisher's exact test ($\alpha<0.05$, $\beta>0.80$).

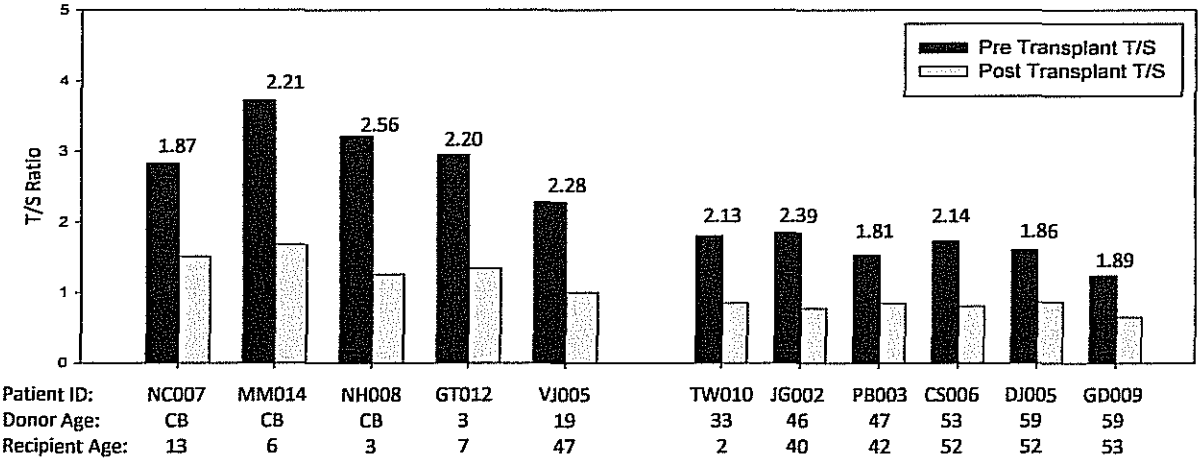

Figure 2. T/S ratios as a representation of telomere length in pre- and post-transplant patient samples. Donor and recipient ages for individual patients are given below. Fold change between pre- and post-transplant T/S ratio are given above the bars.

Jeffrey Tompkins

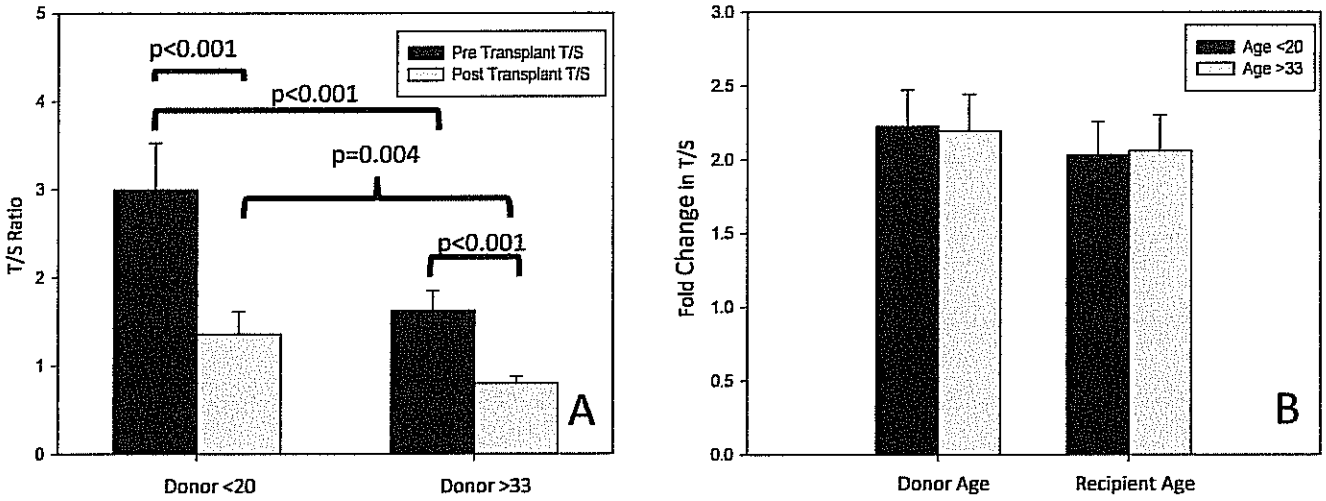

Figure 3. **(A)** Mean T/S ratio of pre- and post- transplant samples with patients grouped according to donor age. **(B)** Fold change in T/S ratio with patients grouped according to both donor age and recipient age. Error bars in both graphs represent one standard deviation. Squre brackets show significance by the student's t-test, pointed brackets show significance by the Mann-Whitney test ($\beta>0.80$).

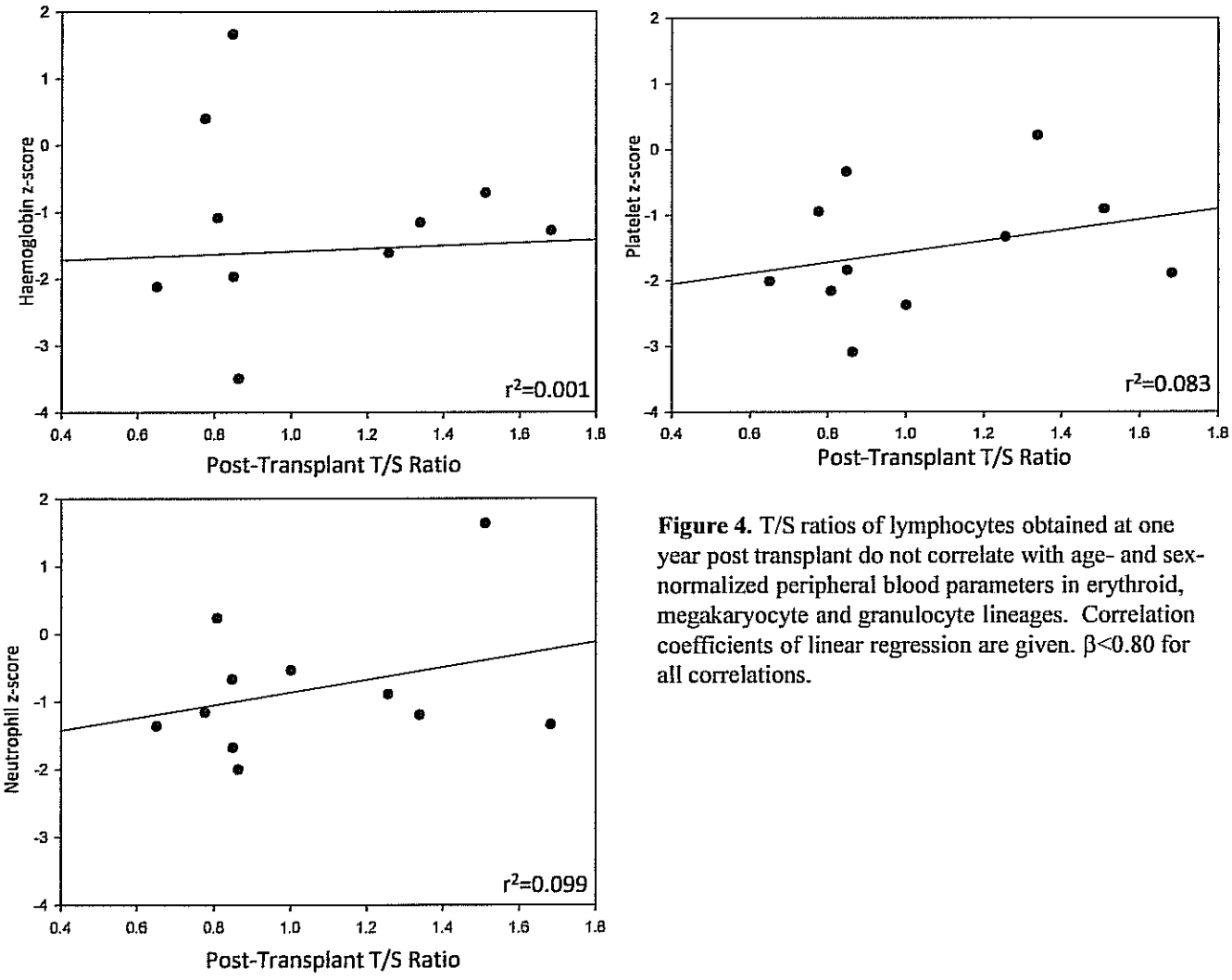

Figure 4. T/S ratios of lymphocytes obtained at one year post transplant do not correlate with age- and sex-normalized peripheral blood parameters in erythroid, megakaryocyte and granulocyte lineages. Correlation coefficients of linear regression are given. $\beta<0.80$ for all correlations.

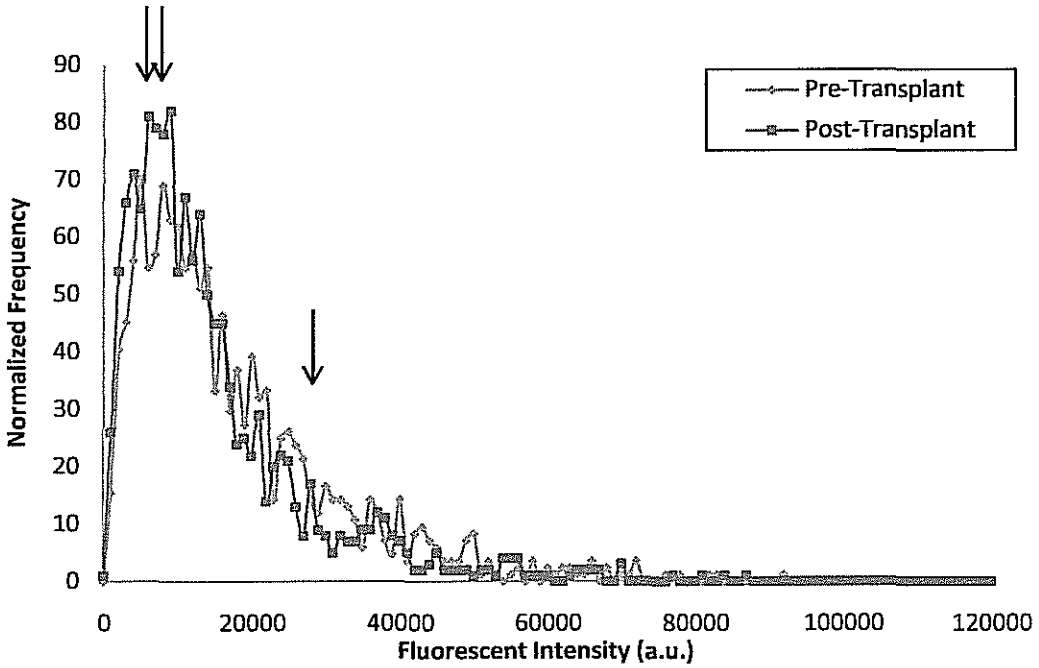

Figure 5. Frequency distribution of telomere intensities pre- and post-transplant samples for patient CS006. Frequencies are normalized to the post-transplant sample to account for differences in total number of telomeres detected. The pre-transplant sample had a greater frequency of telomeres at high fluorescent intensity (single arrow) and the post-transplant sample had a greater frequency of telomeres at low intensity (double arrow)

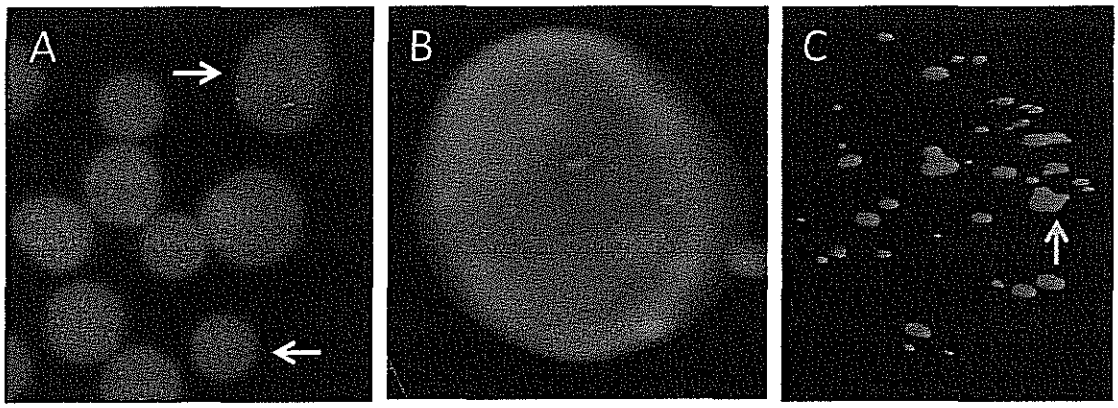

Figure 6. (A) Two dimensional fluorescent micrograph showing cell nuclei (blue; DAPI) and telomere (red; Cy3) signals. Heterogeneity is seen in terms of nuclear diameter, with both large (white arrow) and small (yellow arrow) nuclei visible. **(B)** Three dimensional reconstruction following deconvolution showing the distribution of telomeres within the interphase nucleus. **(C)** Three dimensional reconstruction showing clustering of some telomere signals (green arrow).

Table 1. Demographic and clinical characteristics of all participants. Significant differences between groups were determined using Fisher's exact test for all categories except for donor age (Kruskal-Wallis test) and recipient age (one way ANOVA).

	Y/Y	Y/O	O/O	O/Y	CB	Total	p
n	10	4	34	2	8	58	-
Age							
Donor Age: Median	16	19	49	46	0	-	<0.001
Donor Age: IQR	8-20	18.5-20	45-55	-	0	-	
Recipient Age: Median	7	51	52	11	3	-	<0.001
Recipient Age: IQR	5-12	48-56.5	46-55	-	1.5-7.5	-	
Sex							
Male	8	2	21	1	3	35	0.413
Female	2	2	13	1	5	23	
Donor-Recipient Relatedness							
Related	8	0	26	1	0	35	0.009
Unrelated	2	4	8	1	8	23	
Graft Cell Source							
Bone Marrow	10	1	5	1	0	17	<0.001
Mobilized Peripheral Blood	0	3	29	1	0	33	
Cord Blood	0	0	0	0	0	8	
Immunosuppression One Year Post Transplant							
Yes	3	2	18	1	5	29	0.712
No	7	2	16	1	3	29	
Primary Diagnosis							
Aplastic Anemia	4	0	0	1	0	5	<0.001
Immunodeficiency	3	0	0	0	0	3	
Myeloid Leukemia/Myelodysplasia	2	2	12	0	5	21	
Lymphoid Leukemia	1	1	5	0	2	9	
Lymphoma	0	1	15	1	0	17	
Other	0	0	2	0	1	3	

Table 2. Comparison of telomere characteristics (± standard deviation where relevant) of cells subject to cryopreservation followed by 96 hour culture prior to fixation to cells fixed immediately after phlebotomy and ficoll isolation of lymphocytes (fresh cells).

	Fresh Cells	Cultured Cells
Average telomere signals per cell	35.5 ± 5.5	38.3 ± 9.1
Percentage of cells with aggregates	57.6	45.5
Median fluorescent Intensity	13 072	11 162
Average Nuclear diameter (μm)	10.78 ± 0.76	10.39 ± 2.15