Epigenetic age acceleration in hematopoietic stem cell transplantation

Older recipients of allogeneic stem cell transplantation (HSCT) display a higher risk of non-relapse mortality (NRM).[1] As the number of transplant procedures in the over 65-70-year old patients increases, physicians urgently need efficient assessment of the elderly patient's fitness. Nowadays, Sorror's Comorbidity Index (HCT-CI)[2] is commonly used in clinical practice because of its extensive validation for NRM prediction as well as its easy and fast assessment. Biological age, a measure of the individual aging speed, that has been proved to be effective in predicting all-cause mortality in the general population,[3,4] might be informative of the patient's health status also in the context of HSCT. 'Epigenetic clocks', based on DNA methylation assessment, have been widely applied to estimate biological age in several conditions.[5] In the setting of HSCT, epigenetic age has been described as an intrinsic property of transplanted human hematopoietic stem cells (HSC),[6] and a potential role for cellular aging in clinical outcomes has been suggested based on the observation that donors with accelerated epigenetic aging go on to have an elevated risk of graft-*versus*-host disease (GvHD).[7] Notably, Stolzel *et al*. reported accelerated epigenetic aging of donor-derived HSC in an 8-year observation period post HSCT.[8] Moreover, a rapid shortening of telomere length in the first year post HSCT has been reported.[9]

In the present study, we aim to test whether biological age acceleration, measured through a biological clock based on targeted DNA methylation measurement (henceforth referred to as tDNAMet) can provide additional insights into the biology of transplantation, and correlate with relevant clinical outcomes. tDNAmet, like other widely applied epigenetic clocks, includes both those regions that are highly correlated with chronological age and those regions with a weaker correlation. This allows the epigenetic clocks to pick up the characteristic features of physiological aging and, at the same time, identify any deviation from healthy trajectories. tDNAMet includes 6 genomic regions (ELOVL2, NHLRC1, SIRT7/MAFG, AIM2, EDARADD, TFAP2E) which harbor a total of 70 CpG sites, the methylation level of which is assessed by EpiTYPER technology (Agena Bioscience, San Diego, CA, USA). The regions were selected by Gensous *et al*.,[10] who analyzed the epigenetic profile of healthy subjects (aged 18-80 years) together with accelerated- and decelerated-aging subjects (Down syndrome and centenarians).[11,12] In the present work, peripheral blood samples were collected from donors and patients undergoing HSCT at the Advanced Cellular Therapies Program, IRCCS Bologna, Italy, between 2018 and 2021. Recipients' samples were collected at baseline and then at +30, +90, +180 and +360 days after HSCT. The study was approved by the local Ethics Committee (Comitato Etico Area Vasta Emilia-Romagna, file number: 151/2018/sper/AOUBo) and conducted according to the principles of the Declaration of Helsinki. (The trial was registered at clinicaltrials.gov: 03871296.) We analyzed DNA from at least one sample of 81 patients and 53 donors, for a total of 250 samples. Characteristics of the study population are shown in Table 1. The epigenetic age of HSCT donors and recipients was calculated applying a mathematical model built on our cohort of 276 healthy subjects (CTR), already described by Gensous *et al*.,[10] that were re-analyzed for the present study. The applied mathematical model assigns a different weight (coefficient) to the methylation value of the above mentioned 70 CpG sites (*Online Supplementary Figure S1*). The aging acceleration value (AA), i.e., the measure of whether individuals are aging faster or slower than their chronological age,[5] was determined for each subject as a continuous variable and then categorized as a dichotomous one as follows: negative value (AA-, anti-aging profile) when the inferred biological age is lower than the chronological one, and positive value (AA+, aging profile) when the inferred biological age is higher than the chronological one. (See *Online Supplementary Figure S2* for individual trends.) As expected, recipients were chronologically older than donors (median age: 55 *vs*. 29 years, Mann Whitney [MW] test, $P<0.0001$). At admission to the study, recipients had a clearcut prevalence of aging profile (AA+), while the donor profile was predominantly anti-aging (AA-), both when the AA value was considered as a dichotomous variable (% of AA+: 57.1 *vs*. 34.0, Fisher test, $P=0.059$) and when AA value was expressed as a continuous variable (median AA: +3.74 *vs*. -3.83, MW test, $P=0.023$) (Figure 1A). The AA value of peripheral blood leukocytes was then evaluated in a longitudinal analysis, starting at the pre-infusion timepoint up to one year after transplant. The samples collected from patients after HSCT showed full donor chimerism (i.e., 100% of cells were of donor origin) in FISH or STR analysis. The median AA value increased as follows: -2.00 at day +30, +0.94 at day +90, +0.1 at day +180, +3.14 at day +360 (Kruskal-Wallis test, $P=0.007$) (Figure 1B). At day +360, when all the recipient leukocytes are of donor origin (full donor chimerism), the median AA value was +3.14, compared to -3.83 at pretransplant (MW test, $P=0.0004$), i.e., very close to the AA value of the recipients at pre-transplant (AA: +3.14 *vs*. +3.74, MW test, $P=0.84$). These data allowed us to conclude that during the first year after transplant, there is an approximately 7-fold acceleration in cellular aging in transplanted cells. Similarly, the proportion of AA+ values showed a significant trend to increase from day +30 (45.6%)

up to day +360 (71.4%, Cramer's V=0.254, *P*=0.005) (Figure 2A). Notably, such an increase was more pronounced in the GvHD subgroup (Cramer's V=0.383, *P*=0.021; donor *vs*. recipient AA+ value at day +360: 25.0% *vs*. 78.6%, i.e., 3.14-fold, Fisher test, *P*=0.009) (Figure 2B) than in the non-GvHD group (Cramer's V=0.246, *P*=0.070; donor *vs*. recipient AA+ value at day +360: 37.8% *vs*. 67.9%, i.e., 1.80-fold, Fisher test, *P*=0.024) (Figure 2C). Interestingly, in our cohort, the GvHD donors had a slightly lower AA at baseline *versus* non-GvHD donors, and this may be part of an inherent difference between the two groups.

At variance with data in the literature,[13] we failed to find any statistical association between acute GvHD and chronological age or AA values, either in recipients (non-GvHD *vs*. aGvHD, median age: 53 *vs*. 60 years, MW test, *P*=0.106; median AA value: +3.94 *vs*. -1.76, MW test, *P*=0.650) or in donors (non-GvHD *vs*. GvHD, median age: 29 *vs*. 32 years, MW test, *P*=0.763; median AA value: -5.24 *vs*. -2.66, MW test, *P*=0.723). In univariate analysis, we found that AA value at admission to the study had a significant impact on overall survival (OS) (admission AA value, Hazard Ratio: 1.021, 95% Confidence Interval: 1.000-1.1042, *P*=0.047) while in the same cohort of patients, we found no significant impact of other relevant clinical variables on OS (chronological age of donor and recipient, Sorror Index, disease phase, intensity of conditioning regimen, donor type, age acceleration of donor).

To our knowledge, this is the largest study investigating the epigenetic age acceleration of patients undergoing allogeneic HSCT, and the first time an original tDNAMet has been applied, which proved to be capable of capturing relevant aspects of transplant biology. This study provides at least two important insights.

Firstly, tDNAMet was able to achieve the post-transplant age acceleration already described in previous studies.[8,9] Notably, in our case series, the level of age acceleration previously observed in a mean 8-year post-HSCT follow up was reached within one year.[8] Moreover, at variance with a previous report,[8] we did not detect any post-transplant rejuvenation of donor's cells once transplanted in recipients. Such results suggest that tDNAMet is extremely effective in measuring cell stress in the HSCT setting. We also observed that the post-HSCT age acceleration occurs to a higher extent in GvHD patients than in non-GvHD cases. This observation suggests that biological clocks are sensitive to both the cellular proliferation and leukocyte activation that likely occur during GvHD,[13,14] and that, in the long term, can lead to the pro-inflammatory drift known as inflammaging.[15]

The second point regards the assessment of the biological age of both donors and recipients before HSCT. On the one hand, tDNAMet analysis shows that donors are biologically younger than their chronological age, corroborating the rigorous selection that donors must undergo to ensure an exceptionally good health status. On the other hand, recipients show a consistent age acceleration phenotype, likely

Table 1. Characteristics of the study population.

Characteristic	Total N=81
Age recipient at HSCT in years Median (range)	55 (18-71)
Gender, N (%) Female Male	 32 (39.5) 49 (60.5)
HCT-CI Sorror score, N (%) 0 1 2 ≥3	 38 (46.9) 7 (8.7) 10 (12.3) 26 (32.1)
Disease, N (%) AL MDS/MPN LYM MM SAA	 53 (65.4) 16 (19.8) 9 (11.1) 2 (2.5) 1 (1.2)
Therapy lines before HSCT, N Median (range)	2 (0-5)
Time from diagnosis to HSCT in months Median (range)	11 (2-182)
Disease status at HSCT, N (%) Early Advanced	 55 (67.9) 26 (32.1)
Donor, N (%) MRD MUD MMUD HAPLO	 10 (12.3) 38 (46.9) 26 (32.1) 7 (8.6)
Donor age in years Median (range)	29 (19-63)
Source, N (%) PBSC BM	 69 (85.2) 12 (14.8)
Conditioning, N (%) MAC RIC	 44 (54.3) 37 (45.7)
GvHD prophylaxis, N (%) CSA+MTX/MMF+ATLG FK+MTX/MMF+ATLG FK+MMF+PT-CY	 70 (86.4) 4 (5.0) 7 (8.6)
aGvHD, N (%) No Yes, any grade	 52 (64.2) 29 (35.8)

aGvHD: acute graft-*versus*-host disease; AL: acute leukemia; ATLG: anti-T lymphocyte globulin; BM: bone marrow; CSA: cyclosporine; FK: tacrolimus; HAPLO: haploidentical donor; HCT-CI: Hematopoietic Cell Transplantation specific Comorbidity Index; HSCT: hematopoietic stem cell transplantation; LYM: lymphoma; MAC: myeloablative conditioning; MDS/MPN: myelodysplastic syndromes / myeloproliferative neoplasms; MM: multiple myeloma; MMF: mycophenolate mofetil; MMUD: mismatched unrelated donor; MRD: matched-related donor; MTX: methotrexate; MUD: matched-unrelated donor; PBSC: peripheral blood stem cells; PT-CY: post-transplant cyclophosphamide; RIC: reduced-intensity conditioning; SAA: severe aplastic anemia. Due to rounding, total % may not be 100.

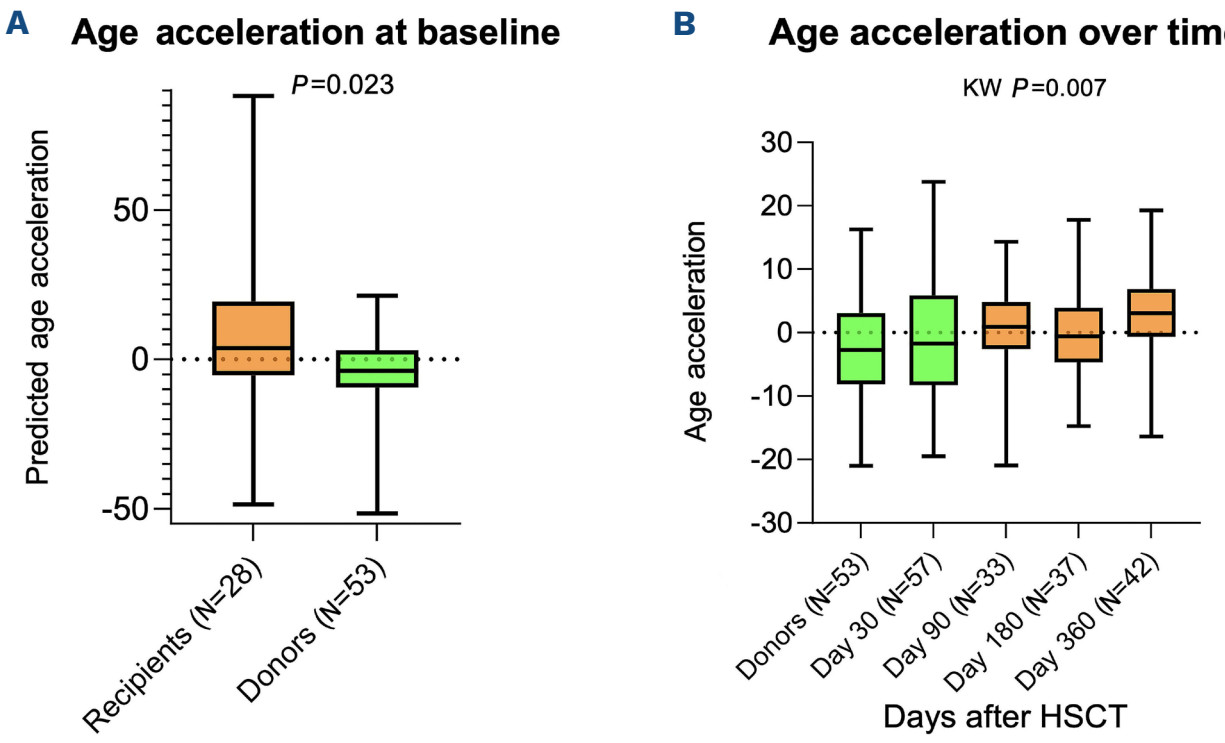

Figure 1. Age acceleration throughout the first year. Age acceleration expressed as a continuous variable in recipients *versus* donors (A) at baseline and (B) in recipients (donors' cells) throughout the first year post transplant; by Mann-Whitney and Cramer V test, respectively. KW: Kruskal-Wallis test.

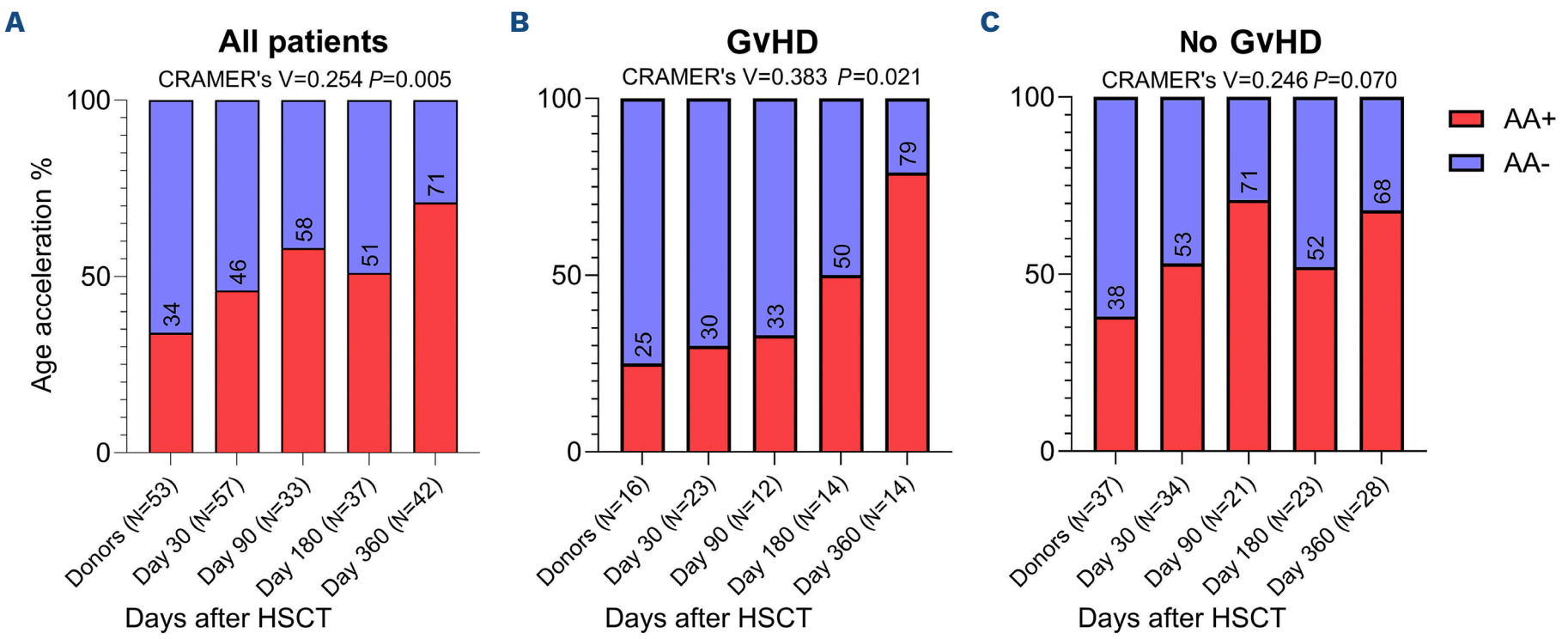

Figure 2. Age acceleration and acute graft-*versus*-host disease. Age acceleration (AA) expressed as percentage throughout the transplantation time in (A) all patients, (B) those who developed acute graft-*versus*-host disease (GvHD), or (C) those who did not develop acute GvHD.

due to the disease itself and the related treatments, that in turn correlates with OS. This last observation supports efforts to test epigenetic age acceleration at baseline in larger populations in order to investigate if it can play a role, as an additional tool to chronological age and the Sorror Index, in evaluating the patient's 'fitness' for transplantation, especially in the elderly.

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Contributions

FBo, PG and MB are responsible for study design and study coordination. MU, MB, FBo, MF and PG wrote the manuscript. PG, CP and KMK made epigenetic analysis. MU, MR, FDeF, FBa, EM, SF, MA and FBo carried out the clinical assessment. GS, DM, SNB, SDeM, ET, MN, NL, IS, BS and ED collected the samples. FBa and MU are responsible for data entry. MU, FI, MB and KMK carried out the statistical analysis. All authors reviewed the manuscript and approved the final version for publication.

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Data-sharing statement

This paper reports primary original data. Study data may be made available on reasonable request to the corresponding author.

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