Differential methylation clock ages across buffy coat (BC), peripheral blood mononuclear cells (PBMC), and saliva in individuals approaching midlife

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Abstract

Understanding epigenetic aging prior to midlife is gaining interest as a potentially intervenable period to address factors that influence health and cognitive aging. Epigenetic changes associated with aging may point to differential biological aging rates; however, methylation profiles may not be substitutable across tissues. We compared DNA methylation in three tissues collected in 91 siblings and twins from the Colorado Adoption/Twin Study of Lifespan behavioral development and cognitive aging (CATSLife1): saliva, buffy coat (BC), and peripheral blood mononuclear cells (PBMC). Overall, across five methylation clocks and two blood-derived and one saliva-derived tissues, moderate to strong associations between chronological age and methylation ages were observed. Moreover, PBMC methylation age values correlate more strongly with BC values (Spearman r = 0.66 – 0.87), whereas saliva showed weaker correlations with either form of blood-derived measures (Spearman r = 0.25 – 0.69) although still moderate to strong magnitudes. Saliva demonstrated significantly older methylation ages across four of five clocks, whereas PBMC and BC did not differ. Twins were more strongly correlated for BC and PBMC derived clocks with weaker and inconsistent patterns among Saliva clocks. DunedinPACE age acceleration showed no significant tissue differences and on average demonstrated the largest divergence of similarity between monozygotic (MZ) versus dizygotic (DZ) twins (rMZ= .56, rDZ= .21). In summary, saliva-derived methylation is not a direct substitute for blood-derived methylation whereas blood-derived methylation values were comparable across buffy coat and peripheral blood mononuclear cell tissues.

Keywords

methylation clocks, age acceleration, tissue specificity, established adulthood, twins

Key Policy Highlights

- Saliva-derived methylation is not a direct substitute for blood-derived methylation.
- Saliva-derived methylation clocks demonstrated relatively older methylation ages relative to blood-derived methylation clocks.
- Blood-derived methylation clocks were comparable across buffy coat and peripheral blood mononuclear cell tissues.
- Twin similarity was higher for blood-derived methylation clocks and newer generation methylation clocks.
- An age-acceleration clock that tracks rate of ageing was comparable across blood- and saliva-derived tissues, with the highest twin pair similarity among identical twins supporting the importance of tool development.

Introduction

Individual differences in DNA methylation patterns may signal important variation in gene expression in response to exposures and may index accelerated aging. DNA methylation may increase or decrease gene expression over time (Singal & Ginder, 1999), where attachment of a 5-methyl group to cytosine-phosphate-guanine (CpG) sites leads to reductions or silencing of specific gene activity (Mattei, Bailly, & Meissner, 2022). The methylation process is a vital component of development and has wide implications across aging. While DNA sequence remains relatively stable as we age (Schumacher, Pothof, Vijg, & Hoeijmakers, 2021), DNA methylation patterns differ by tissue type and are subject to alterations across time within tissue (Gopalakrishnan, Van Emburgh, & Robertson, 2008; Jones, Goodman, & Kobor, 2015; Miller et al., 2023; Robertson, 2005; Slieker, Relton, Gaunt, Slagboom, & Heijmans, 2018). The choice of tissue is often determined by ease of tissue sampling, with blood and saliva commonly used for methylation research (Langie et al., 2017). The choice of tissue is a salient consideration and may illuminate possible mechanisms if proximally or directly associated with phenotypes of interest. Many studies have stored saliva or buffy coat samples as opposed to whole blood, and such biobanked samples could be a boon for research into DNA methylation, especially where comparable (Dou et al., 2018; Ghamrawi et al., 2021; Langie et al., 2017; Staunstrup et al., 2017). In this study we seek a better understanding of methylation differences by tissue type comparing multiple methylation clocks across different commonly sampled tissue types collected across twins and siblings.

A wide variety of methylation clocks exist to estimate one's methylation age compared to their established chronological age. Methylation clocks measure methylation activity across selected CpG sites to calculate a single summary value that may provide insights into biological aging and possible risks of aging-related disease and mortality (Jones et al., 2015). Chronological age is positively and strongly correlated with methylation age in most cases (X. Li et al., 2020). Deviations from chronological age, either lower or higher, indicates relatively slower or faster biological aging and lesser or greater susceptibility to disease, respectively. Higher methylation age may signal the need for potential interventions to alter behavior, disease susceptibility, or disease progression (Gopalakrishnan et al., 2008; Robertson, 2005). Many different methylation clocks exist with metrics ranging from biological age to one's relative rate of aging, and differ as to the number of CpG sites measured, the locations of CpG sites, the chronological ages of those on which the clock was trained, and the tissue type or types used to derive the clock (Y. Wang, Grant, Zhai, McDonald-Maier, & Schalkwyk, 2024).

Saliva is a common biological medium in methylation research (Birknerova et al., 2022; Del Toro et al., 2024; Langie et al., 2017; Smith et al., 2015), particularly due to its ease in collection and relatively low cost. With respect to blood, PBMC are often the tissue of choice in methylation work testing possible disease markers, disease progression, as well as aging (Cheishvili et al., 2024; Friso et al., 2013; Y. Li et al., 2010; Steegenga et al., 2014; Stefanowicz et al., 2012; T. Wang et al., 2023). Understanding whether and when significant differences in methylation measurements exist across the commonly collected biological mediums of saliva, BC, and PBMC can assist

researchers with determining which sample type can offer comparable results weighed against accessibility of each biological medium.

PBMC consist primarily of lymphocytes and monocytes (Navas, Giraldo-Parra, Prieto, Cabrera, & Gómez, 2019). BC contains a condensed layer of PBMC components as well as granulocytes, platelets, and any contaminating red blood cells (Martins et al., 2024). Saliva is likewise a composite tissue for epigenetic analysis containing both epithelial cells and leukocytes (Theda et al., 2018). Quality control work in preparing methylation data for analysis includes estiamting cell counts to reduce inter-sample variability within tissue type (Teschendorff & Horvath, 2025). Nonetheless, cross-tissue differences remain pertinent, related to their respective physiological roles.

The comparability of methylation clocks within and across tissue types is unclear, especially in informative samples such as those that may be stored in large biobanks. Early work noted key differences in methylation between saliva, BC, and PBMC, warranting further work to better understand the methylation differences (Langie et al., 2017; Staunstrup et al., 2017). Recent comparison studies are small in sample size and focused on platform differences (Tay et al., 2025), employ only two tissues types (e.g., whole blood, saliva) (Zarandooz & Raffington, 2025), and/or compare multiple tissues across a wide age range (Apsley et al., 2025). For example, comparisons of buccal epithelial, saliva, dried blood spots, BC, and PBMC samples (Apsley et al., 2025), suggested salient differences between the three blood versus two oral tissue types; however, sample sizes per tissue type varied between 35 - 81 individuals and ages ranged from 9 to 70 years of age, with BC available in children only and PBMC in adults

only. The best performing clock was the pan-tissue Skin and Blood Clock (Horvath et al., 2018).

The Colorado Adoption/Twin Study of Lifespan behavioral development and cognitive aging (CATSLife), is a genetically and environmentally informative study, including both monozygotic and dizygotic twins, as well as biological and adopted sibling pairs, allowing the dissection of genetic and environmental factors that contribute to age-related outcomes. Multiple biological samples are being collected longitudinally including blood and saliva. The goal of the present study is to determine the comparability of tissue source to inform future methylation studies. Specifically, we test differences among five common methylation clocks with methylation results on BC, PBMC, and saliva in 91 individuals approaching midlife (28 to 41 years). Our sample includes a subset of 64 twins (32 pairs) which allows us to evaluate the relative similarity of monozygotic (MZ) to dizygotic twin (DZ) pairs. We predicted that BC and PBMC derived clocks will more closely align with each other than with saliva derived clocks. Moreover, we expected that second and third generation clocks, trained on multiple tissues or multiple aging related biomarkers (Belsky et al., 2022; Horvath et al., 2018; Zhang et al., 2019), would show stronger associations across sample mediums (Apsley et al., 2025; Zarandooz & Raffington, 2025). Last, we expected that MZ twins would show greater similarity than DZ twins (Jylhava et al., 2019; Reynolds et al., 2020), with greater twin similarity for more recently developed clocks.

Methods

Samples from 91 participants were selected as a subset of the Colorado Adoption/Twin Study of Lifespan behavioral development and cognitive aging

(CATSLife1) conducted between 2015 to 2021 (Wadsworth et al., 2019). All research was conducted according to the APA's ethical standards and approved by the Institutional Review Boards at the at the University of Colorado Boulder [14-0421] and the University of California, Riverside [HS 14-073]. Written informed consent was obtained from each participant. Among these 91 participants, 84 individuals were from sibling or twin pairs including 20 sibling pairs (7 biological pairs, 3 adoptive pairs), 14 dizygotic (DZ) twin pairs, and 18 monozygotic (MZ) twins pairs.

DNA was isolated from either saliva, buffy coat or peripheral blood mononuclear cells (PBMC) samples provided by the participants. Two mL of saliva were collected from each participant in a 15 mL conical tube and a cotton swab containing dried reagents was added bringing the solution to 1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0 final concentration. The saliva was then stored at room temperature until DNA was extracted. Blood samples were collected by venipuncture into Vacutainer CPT blood collection tubes (BD, for PBMC) and Monoject EDTA (K3) blood collection tubes (Covidien, for BC). The samples were processed following the manufacturer's instructions. PBMC were lysed one mL lysis buffer (1% SDS, 50 mM Tris pH 8.0, 10 mM EDTA) and both PBMC and buffy coats were stored at -80C after processing on the day they were collected.

DNA was isolated from 500 µL of thawed buffy coat using Maxwell 16 Blood DNA Purification Kits (Promega) on the Maxwell 16 instrument (Promega) following the manufacture's protocol. DNA was prepared from both the PBMC and saliva using a protocol modified from Haberstick & Smolen (Haberstick & Smolen, 2004). Briefly, DNA from 600 µL of either saliva or PBMC in lysis buffer were RNase and proteinase K

treated followed by precipitation with two volumes of isopropyl alcohol and pelleted, washed with 1mL 70% ethanol, air dried and resuspended in 110 µL of TE. The concentration of purified DNA was quantified utilizing PicoGreen, and then adjusted to 30-50 ng/µL in 1x TE. DNA methylation was measured on the EPIC v1 array (Illumina) by the Johns Hopkins University Genetic Resources Core Facility. Quality control and analysis was then performed in R using packages *minfi, wateRmelon*, and *sva* (Fortin, Triche, & Hansen, 2017; Leek, Johnson, Parker, Jaffe, & Storey, 2012; Pidsley et al., 2013) to conduct normalization, estimate cell counts, and adjust for batch effects.

Methylation analysis was completed by calculating methylation clocks using the *dnaMethyAge* package in R (Y. Wang et al., 2024). We estimated rank-based Spearman correlations of methylation age among the sample mediums, as well as their correlations with chronological age, for each of the five methylation clocks. To test differences in methylation clocks by sample medium that were measured across the same individuals and individuals in the same family (sharing genetic and environmental influences), we used a mixed-effects model using the *nlme* package's *lme()* function in R (Pinheiro, 2021). For each of the five different methylation clocks, methylation age was the outcome for each model, with sample medium and chronological age at the time of the blood draw (centered at the mean age, 30.90 years) as fixed effects. The unique family ID was set as a random effect, producing a between-family standard deviation while the residual standard deviation indexed the within-family (individual) random effect and any measurement error. Analyses with twin pairs used age acceleration estimates from *dnaMethyAge* (Y. Wang et al., 2024), where positive values index faster aging and negative values index slower aging.

Post-QC methylation data have been deposited in NIAGADS where participant directives have allowed [doi and accession number will be included upon acceptance].

Results

The full overall sample (n=91) was roughly evenly split by sex (46 female, 45 male) with 80 participants self-reporting their race as White and 11 as Multiple/other, and 80 participants self-reporting their ethnicity as non-Hispanic and 11 as Hispanic. Age at the time of blood draw ranged from 28.07 - 41.13 years with an average age of 30.90 years (SD = 3.81 years). Differences in ages at the time of the blood draw were typically over two years for non-twin sibling pairs (Range = 0.08 - 5.38, mean = 2.86, median = 2.59, SD = 1.56) and near zero for the MZ and DZ twin pairs (Range = 0 - 0.52, mean = 0.04, median = 0.00, SD = 0.10). Quality control led to the removal of 9 saliva samples, while all BC and PBMC samples passed quality control. Therefore, final sample sizes were 91 for both PBMC and BC, and 82 for saliva. Results of probe coverage by methylation clock are shown on Table 1. All clocks had over 90% of probes present for analysis, with four out of five clocks at 94.9% of probe coverage or higher. Core clocks of GrimAge and PhenoAge were not included in the analysis due to being trained on individuals well beyond the age range of our sample (Levine et al., 2018; Lu et al., 2019).

Table 1. Probe Coverage Across Major Clocks.

Clock	Tissue(s) Trained	Total Probes	Probes Present	Percent Present
Hannum	Whole blood [a]	71	65	91.5%
Horvath	27 tissues [b]	353	335	94.9%
Horvath2	8 tissues [c]	391	382	97.7%
ZhangQ	Blood, saliva [d]	515	509	98.8%
DunedinPACE	Blood [e]	173	173	100.0%

[a] -(Hannum et al., 2013); [b] -(Horvath, 2013); [c] -(Horvath et al., 2018); [d] -(Zhang et al., 2019); [e] -(Belsky et al., 2022);

Spearman rank correlation by sample medium and across methylation and phenotypic ages are shown in Figure 1. Overall, BC-derived and saliva-derived methylation clock ages, across different clocks, showed marginally higher correlations with chronological age, compared to PBMC-derived clock ages (Fig. 1). Spearman rank correlations showed strong correlations between BC and PBMC methylation ages (correlation range 0.66 – 0.87), whereas saliva showed weaker correlations with BC (correlation range 0.45 – 0.69) and PBMC (correlation range 0.25 – 0.55).

In the random-effects model results using lme, which account for between-family effects, the estimated fixed effect intercept reflects the expected BC-derived methylation age for Hannum, Horvath, Horvath2, and ZhangQ clocks. These results suggest that PBMC methylation age effect is not significantly different from zero, or the equivalence BC and PBMC ages reflected in the intercept. However, there was a significant effect of saliva tissue on methylation clocks, with estimated methylation ages tending to be older for four of five clocks (Table 2). Saliva was significantly different from the blood-based methylation clocks when using Hannum (B_{Saliva} = 12.514, p = <0.001), Horvath (B_{Saliva} = 7.423, p = <0.001), Horvath2 (B_{Saliva} = 16.411, p = <0.001), and ZhangQ (B_{Saliva} = 3.768, p = <0.001). Chronological age at the time of the blood draw was significantly positively associated, as expected, where one elapsed chorological year predicted 0.645 to 0.968 increased years in methylation age.

The DunedinPACE clock measure is scaled as a rate of aging (Belsky et al., 2022) with expected values of 1.0 if individuals' rates are on par with chronological year; values over 1.0 suggesting accelerating rates of aging and values less than 1.0 index a slower rate of aging. The average intercept suggested accelerated aging across the

sample ($B_{intercept}$ = 1.304, p = <0.001). However, there were no significant differences in aging rates of PBMC- versus BC-derived estimates (B_{PBMC}= 0.003, p = .789) or saliva-derived versus BC-derived estimates (B_{Saliva}= -0.007, p = .486), supporting overall comparability. Chorological age was likewise non-significant.

Table 2. Linear Mixed-effects Model Fit of Fixed Effects, Methylation Clocks by Sample Medium.

Methylation Clock	*B*	*SE*	df	*p*-value	Between Family SD	Within Family SD
Hannum						
Intercept [BC=ref]	36.023	0.365	212	<0.001	1.606	2.692
PBMC	0.136	0.399	212	0.734		
Saliva	12.514	0.411	212	<0.001		
Age	0.645	0.072	212	<0.001		
Horvath						
Intercept [BC=ref]	21.830	0.471	212	<0.001	2.117	3.417
PBMC	0.386	0.507	212	0.447		
Saliva	7.423	0.522	212	<0.001		
Age	0.797	0.094	212	<0.001		
Horvath2						
Intercept [BC=ref]	18.420	0.396	212	<0.001	1.699	2.963
PBMC	0.085	0.439	212	0.847		
Saliva	16.411	0.452	212	<0.001		
Age	0.811	0.078	212	<0.001		
ZhangQ						
Intercept [BC=ref]	26.164	0.346	212	<0.001	1.643	2.411
PBMC	0.217	0.357	212	0.545		
Saliva	3.768	0.368	212	<0.001		
Age	0.968	0.070	212	<0.001		
DunedinPACE						
Intercept [BC=ref]	1.304	0.011	212	<0.001	0.057	0.070
PBMC	0.003	0.010	212	0.785		
Saliva	-0.007	0.011	212	0.486		
Age	0.003	0.002	212	0.224		

Note. BC= Buffy Coat; PBMC= peripheral blood mononuclear cells; SD = standard deviation.

Age acceleration estimates were used for twin pair comparisons for all Hannum, Horvath, Horvath2, and ZhangQ clock measures to focus on similarity in aging rates, where positive values index faster aging and negative value index slower aging, and more analogous to the DunedinPACE clock. On average, Spearman rank correlations among MZ pairs were higher than for DZ pairs across all Buffy Coat age acceleration estimates (rMZ=.55, rDZ=.33) and generally comparable for PBMC (rMZ=.37, rDZ=.45) (see Supplemental Table 1). The patterns of MZ and DZ Spearman rank correlations for age acceleration estimates are lower and less consistent for saliva (Average rMZ=.18, rDZ=.37), albeit the number of pairs is smaller. Across all three tissue types, the second and third generation age acceleration estimates (Horvath2, ZhangQ, DunedinPACE) show, on average, higher MZ versus DZ correlations (rMZ=.55 versus rDZ=.43), driven primarily by DunedinPACE showing the greatest divergence (Average rMZ= .56, Average rDZ= .21). In Figure 2, we illustrate twin pair similarity for age acceleration metrics for 26 to 32 twin pairs (14 - 18 MZ pairs, 12 - 14 DZ pairs), displaying DunedinPACE and the ZhangQ age acceleration estimates, where twin 2's age acceleration is regressed on twin 1's age acceleration. The steepness of the regression lines indexes the relative similarity of MZ versus DZ twins. For DunedinPACE, the MZ regression line is visually steeper than for the DZ pairs suggesting greater similarity. For the ZhangQ age acceleration, the regression lines for MZ pairs are comparable to DZ pairs suggesting little difference in similarity by zygosity.

Discussion

We evaluated methylation ages across different tissues to gauge the equivalence of blood and saliva tissues commonly sampled and biobanked. Strong correlations of

methylation ages with phenotypic age were observed across all tissue types. However, blood-derived PBMC and BC methylation age estimates were more correlated with each other than with saliva-derived estimates. Saliva-derived methylation ages were significantly older relative to the blood-derived methylation ages, whereas PBMC and BC methylation ages were indistinguishable from one another. The age-acceleration clock, DunedinPACE, was the exception showing no tissue type differences. Patterns of MZ and DZ similarity were stronger for blood-based clocks than saliva-based clocks, and for second and third generation compared to first generation clocks.

Our results are among the first to demonstrate that methylation clock ages do not significantly differ between BC and PBMC in a larger sample of adults. Moreover, BC and PBMC derived clocks more closely aligned with each other than with saliva derived clocks. Prior work (Apsley et al., 2025) evaluated BC in children only and PBMC in adults only but generally observed higher methylation ages in oral-tissues than blood-based tissues. The lower correlation of clocks estimated from PBMC and BC derived tissues with saliva is consistent with a small study of 16 individuals focused on platform comparability (c.f., supplemental data EPIC v1,Tay et al., 2025) and between PBMC and BC with buccal and saliva (Apsley et al., 2025). Notably, however, in our current study the third-generation DunedinPACE age acceleration clock showed no significant differences between biological mediums.

We expected that second and third generation clocks would show greater associations across sample mediums, with the Horvath2, ZhangQ and DunedinPACE clocks generally showing higher correlations than Hannum and Horvath first generation clocks. Within and across each biological tissue, second generation ZhangQ clock

ages showed the strongest correlations as well as with chronological age. Similarly, Zarandooz and Raffington (2025) reported stronger correlations of whole blood versus saliva derived methylations ages comparing second and third generation clocks to first generation clocks in a recent study of 107 individuals aged 5 to 74 years. However, we observed that absolute methylation ages were divergent across clocks. Hannum, trained on whole blood(Hannum et al., 2013), showed notably higher estimated methylation ages compared to other clocks for blood-derived BC and PBMC (Table 2). Both Horvath clocks had younger methylation ages compared to chronological age across all samples, but noticeably so in the BC and PBMC (Table 2). Overall, further insights into the age differences for both blood derived and saliva mediums are needed. Age-related CpG sites specific to saliva are of increasing interest (Xiao et al., 2025), and calls for cell-specific work is advancing which may produce clocks that work across tissue type that share common cell types (Teschendorff & Horvath, 2025).

Twin similarity patterns were commensurate with the overall findings, showing patterns of greater pair similarity for blood-based than saliva-based clocks. MZ pairs were on average more similar than DZ pairs for second generation than first generation clocks across tissue type, with patterns largely consistent with publications on clock-related CpGS and second-generation clocks (Jylhava et al., 2019; Reynolds et al., 2020). In the current work, the greatest divergence in MZ and DZ similarity was observed for the 3rd generation DunedinPACE clock (Belsky et al., 2022), suggesting genetic influences on the pace of biological aging. Nonetheless, larger sample sizes are needed to estimate formal biometrical estimates of heritability and environmentality

and is part of our planned work when methylation is completed on all CATSLife samples.

In choosing between tissue types for methylation analysis, it is important to evaluate the proximity of blood versus saliva tissues to biological pathways of interest. While it might be presumed that blood-based methylation is more proximal than saliva-based methylation to brain-related mechanisms, saliva may be a reasonable medium given it likewise contains immune cell types (Theda et al., 2018). Moreover, biomarkers related to neurodegenerative outcomes, including p-tau and beta-amyloid components, are detectable in salivary tissues (Nijakowski, Owecki, Jankowski, & Surdacka, 2024). A caution is that while saliva is an attractive medium, in our study saliva samples were generally of a lower quality with nine samples removed during the QC steps. The differences in sample storage (-80C for buffy coats and PBMC lysate and room temperature for saliva for up to five years) could explain this difference in quality. As previous work has discussed (Staunstrup et al., 2017), overall DNA quality and potential contamination can be factors in utilizing saliva samples for methylation analysis.

It is important to note our study is not without limitations. Our sample set includes individuals within established adulthood and where chronological age ranges were less than 15 years apart. Also, while we show notable differences between saliva compared to PBMC and BC, additional blood-derived and other biological mediums may be more appropriate for methylation analysis. Future work including additional biological mediums, wider chronological age ranges of participants, longitudinal comparisons, and addressing cell-specific patterns, would each add to a greater understating of methylation profiles.

Our study offers insights on the comparability of biological tissues. While saliva is a less invasive method of collecting a biological medium for methylation, the costs of potential losses of samples due to poorer quality should be factored into decisions. Additional considerations include the consistently elevated saliva-based methylation ages predicted across individuals compared to blood-derived methylation results. Notably, one newer generation age acceleration clock, scaled as a rate rather than absolute age, showed comparability across all three tissues. Broadly, our results support comparability of BC-derived methylation to PBMC-derived methylation and thus for banked buffy coat samples it may be an effective medium to address blood-based methylation when PBMC tissues are not available.

Acknowledgments

Funding details

This work was supported by the National Institute On Aging of the National Institutes of Health under Award Number R01AG046938. LME is supported by Hevolution/AFAR AFAR New Investigator Awards in Aging Biology and Geroscience Research HEV~NI23013.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Declaration of interest

The authors report there are no competing interests to declare.

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Figure 1. Spearman rank correlations among methylation ages and with phenotypic age by sample medium.

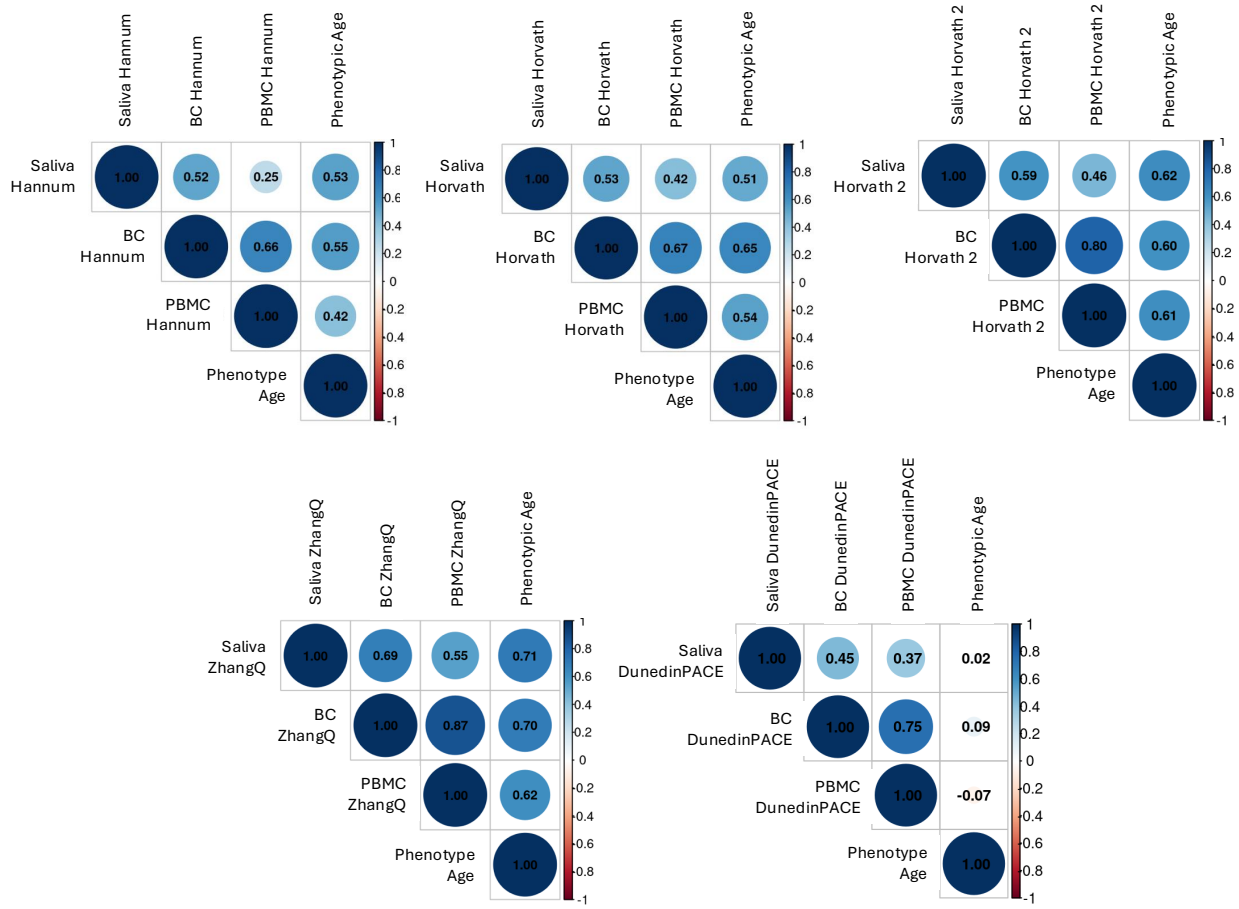
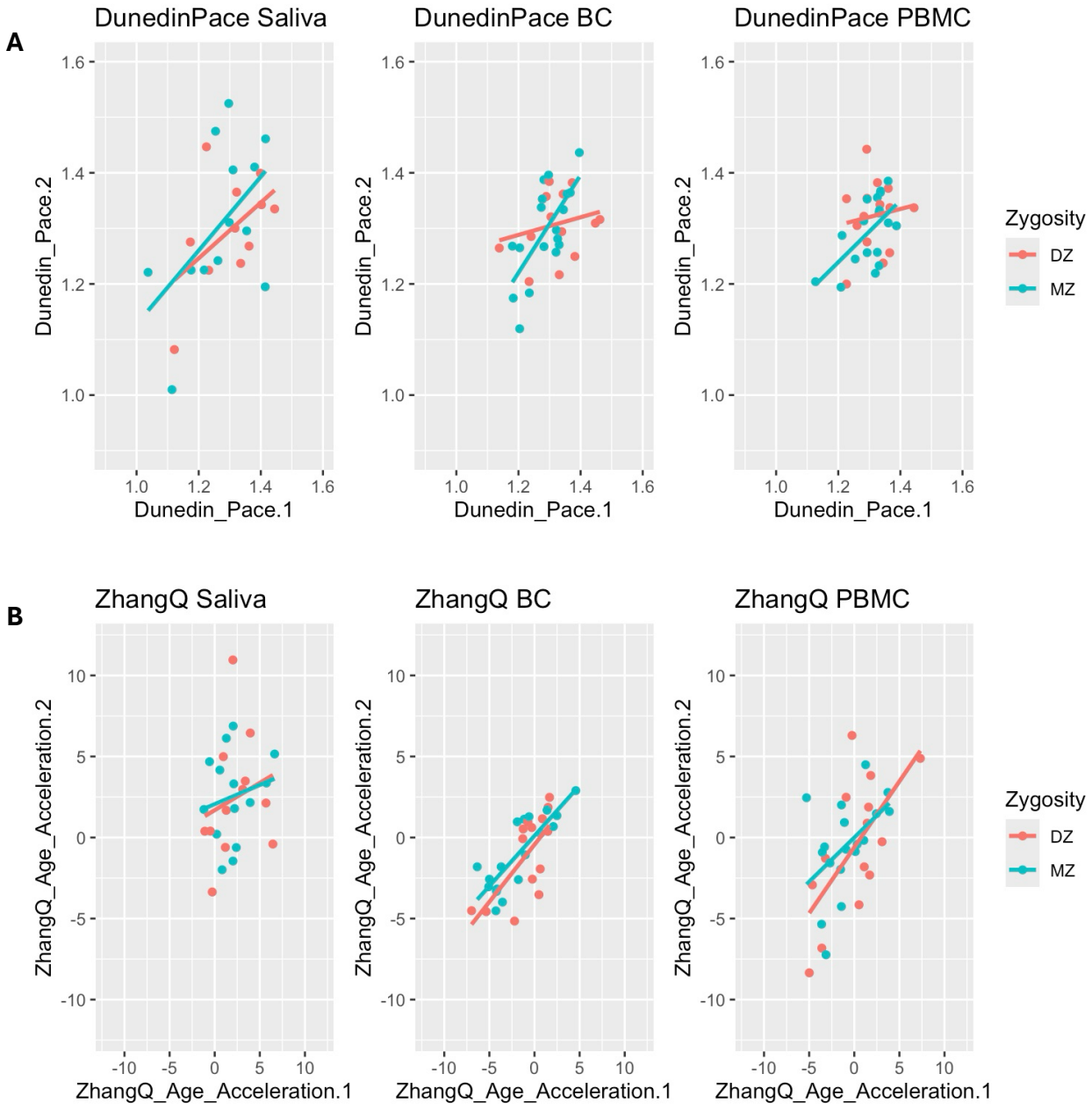


Figure Note. BC=Buffy Coat; PBMC=peripheral blood mononuclear cells.

Figure 2. Regression of Twin 2 Age Acceleration on Twin 1 Age Acceleration by Zygosity and sample medium: (A) Dunedin Pace, (B) ZhangQ clock acceleration.



Note. BC=Buffy Coat; PBMC=peripheral blood mononuclear cells; MZ=monozygotic; DZ=dizygotic.