Sex-specific and generational effects of alcohol and tobacco use on epigenetic age acceleration in the Michigan longitudinal study

Amir Carter[a,1], **Cristina Bares**[b,1], **Lisha Lin**[a,1], **Beth Glover Reed**[c], **Marjorie Bowden**[d], **Robert A. Zucker**[e], **Wei Zhao**[a], **Jennifer A. Smith**[a,f,2], **Jill B. Becker**[g,2,*]

[a]Department of Epidemiology, School of Public Health, University of Michigan, 1415 Washington Heights, Ann Arbor, MI 48109, United States

[b]School of Social Work, University of Michigan, 1080 South University, Ann Arbor, MI 48109, United States

[c]School of Social Work and Department of Women's and Gender Studies, University of Michigan, 1080 South University, Ann Arbor, MI 48109, United States

[d]Emerita, Social Work Department, Michigan Medicine, 1500 E. Medical Center Drive, Ann Arbor, MI 48109, United States

[e]Addiction Center & Departments of Psychiatry and Psychology, University of Michigan, 4250 Plymouth Rd, Ann Arbor, MI 48109, United States

[f]Survey Research Center, Institute for Social Research, University of Michigan, 426 Thompson St, Ann Arbor, MI 48104, United States

[g]Department of Psychology and Michigan Neuroscience Institute, University of Michigan, 205 Zina Pitcher Place, Ann Arbor, MI 48109, United States

Abstract

Background: Excessive alcohol and tobacco use are risk factors for poor health in both men and women, but use patterns and relationships with diseases and mortality differ between sexes. The impact of substance use on the epigenome, including DNA methylation profiles, may also differ by sex. It is also unknown whether parental substance use during childhood is associated with epigenetic changes that persist into adulthood. This study assessed the sex-specific effects

*Corresponding author at: Department of Psychology and Michigan Neuroscience Institute, University of Michigan, 205 Zina Pitcher Place, Ann Arbor, MI 48109, United States. jbbecker@umich.edu (J.B. Becker).
[1]These authors participated equally (co-first authors).
[2]These authors participated equally (co-senior authors).

of individuals' alcohol and tobacco use, as well as paternal alcohol and paternal/maternal tobacco use, on offspring's cellular aging as measured by epigenetic age acceleration.

Methods: Four measures of epigenetic age acceleration (HorvathAA, HannumAA, PhenoAA, and GrimAA), the difference between chronological age and inferred age based on DNA methylation, were estimated from saliva samples. Linear mixed models tested associations between alcohol/tobacco use and epigenetic age acceleration in parents and offspring.

Results: Current tobacco smoking was associated with a 4.61-year increase in GrimAA, and former tobacco smoking was associated with a 3.60-year increase in HannumAA after accounting for multiple testing ($p < 0.0125$). In males only, current tobacco smoking was nominally associated with a 2.19-year increase in HannumAA ($p < 0.05$), and this effect was significantly different than the female-specific effect ($p < 0.0125$). Paternal heavy alcohol use when the offspring was 12 or younger was associated with a 4.43-year increase in GrimAA among offspring ($p < 0.0125$).

Conclusions: This study found evidence of sex-specific effects of alcohol and tobacco use, as well as paternal heavy alcohol use, on epigenetic age acceleration.

1. Introduction

Alcohol and tobacco use are risk factors for poor health in both men and women, but their use patterns and relationships with diseases and mortality differ between sexes. Men are more likely to binge drink (defined as a pattern of alcohol drinking that brings a person's blood alcohol concentration to 0.08 g/dL or above), have higher rates of alcohol-related hospitalizations, and have higher rates of mortality from excessive alcohol use than women (Centers for Disease Control and Prevention [CDC], 2020). However, national survey data shows that while rates of alcohol use and binge drinking in males have remained steady, both have increased in females (White, 2020). Men also smoke tobacco at higher rates than women (Centers for Disease Control and Prevention [CDC], 2016). In 2016, 17.5% of men in the U.S. smoked tobacco compared to 13.5% of women (CDC, 2016). Despite these differences, women experience a larger burden of tobacco smoking-related disease and mortality (National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health, 2014) as well as a greater risk for alcohol-induced damage to the liver, heart, and brain (Agabio et al., 2016).

1.1. DNA methylation

DNA methylation is an epigenetic mechanism that cells use to control gene expression without making changes to the DNA sequence itself (Phillips, 2008). It involves the covalent addition of a methyl group to a cytosine base followed by a guanine base, known as a CpG site. Methylation can be influenced by behavioral factors across the life course, and methylation at some CpGs has been associated with alcohol (Dugué et al., 2021; Liu et al.,

2018) and tobacco use (Dugué et al., 2020; Joehanes et al., 2016). Methylation can also be used as a biomarker for aging, as methylation levels of many CpG sites are associated with chronological age and other biological or functional metrics of aging (Hannum et al., 2013; Horvath, 2013; Levine et al., 2018; Lu et al., 2019; Marioni et al., 2019).

1.1.1.   Epigenetic age acceleration—Several measures of epigenetic age based on methylation at specific CpG sites, known as epigenetic clocks, have been developed to estimate biological age. For these clocks, epigenetic age acceleration represents the relative difference between an individual's methylation-estimated biological age and their chronological age, acting as a measure of the degree of cellular aging beyond what is expected for a given chronological age. Each clock estimates a person's epigenetic age using a linear combination of specific CpG sites that have been selected because of their association with chronological age or other measures of aging. Epigenetic clocks differ from each other because they are constructed using different study populations, cell/tissue types, and statistical methods. Importantly, they may also be trained to capture methylation associated with different measures of aging (e.g., chronological age, aging biomarkers, or mortality biomarkers).

The most widely studied four epigenetic clocks are HorvathAge (Horvath, 2013), HannumAge (Hannum et al., 2013), PhenoAge (Levine et al., 2018), and GrimAge (Lu et al., 2019). Despite differences among the clocks, described in more detail below, epigenetic age acceleration estimated by each of these four clocks is associated with multiple age-related diseases (e.g., cardiovascular disease, diabetes, and cancer) as well as all-cause mortality, although the strength of these associations varies by clock (Oblak et al., 2021). Thus, epigenetic age acceleration is considered to be an important biomarker of aging.

1.1.2.   Differences among epigenetic clocks—First-generation epigenetic clocks, including HorvathAge and HannumAge, were developed, or trained, by selecting CpG sites most strongly associated with chronological age using blood or tissue samples. HorvathAge is estimated with 363 CpG sites identified using multiple tissue types from adults and children (Horvath, 2013), while HannumAge is estimated with 71 CpG sites identified using blood samples from adults (Hannum et al., 2013),

Second-generation clocks, including PhenoAge and GrimAge, were trained on age-related traits, or phenotypes, beyond chronological age. PhenoAge is estimated with 513 CpG sites selected to capture both chronological age and 9 aging biomarkers including albumin, creatine, C-reactive protein, and white blood cell proportions (Levine et al., 2018). GrimAge was designed to be a risk estimator for mortality, and is estimated using a combination of chronological age, sex, and 1030 CpG sites selected to capture one known predictor of mortality (cigarette smoking pack-years) and 7 mortality-associated plasma proteins (adrenomedullin, beta-2 microglobulin, cystatin C, growth differentiation factor 15, leptin, plasminogen activation inhibitor 1, and tissue inhibitor metalloproteinase 1) (Lu et al., 2019). Both PhenoAge and GrimAge were developed using methylation from adult blood samples.

1.1.3.   Associations among epigenetic age acceleration, health behaviors, and health outcomes—A variety of adverse exposures and health conditions including early life circumstances (e.g., low childhood socioeconomic status (Hughes et al., 2018)), biological indicators of chronic stress (e.g., allostatic load (Levine, 2020)), and comorbidity count (Lu et al., 2019) have been associated with various measures of epigenetic age acceleration. Associations with exposures and health outcomes tend to vary somewhat across the epigenetic clocks, which is not unexpected since the clocks likely measure only partially overlapping facets of cellular aging (Liu et al., 2020).

Given the important role of epigenetic age acceleration in cellular aging, it may serve as a key biomarker for understanding how health behaviors such as alcohol and tobacco use are biologically embodied, potentially leading to adverse health outcomes. Previous research has identified that those with alcohol dependence, alcohol use disorder, and/or habitual alcohol drinking show epigenetic age acceleration compared to those who do not (Fiorito et al., 2019; Kresovich et al., 2021; Luo et al., 2020; Rosen et al., 2018; Zhao et al., 2019), although effects are not consistent across all clocks and studies. Tobacco use has also been associated with various measures of increased epigenetic age acceleration in several studies (Fiorito et al., 2019; Levine et al., 2018). However, other studies have found a lack of association, especially with first-generation clocks (Quach et al., 2017; Zhao et al., 2019). GrimAA, which explicitly incorporates information about smoking into its derivation algorithm, tends to be more strongly and consistently associated with tobacco use than other measures (Zhao et al., 2019).

1.2.   Sex differences

It is important to examine whether the effects of alcohol and tobacco use on epigenetic age acceleration differ by sex, since we know that sex differences exist with respect to the patterning of these behaviors and their relationship with disease and mortality. For example, women who become addicted progress more quickly from initiation of alcohol use to seeking treatment than men (Becker and Koob, 2016). With respect to tobacco, women smoke fewer cigarettes per day than males (Peters et al., 2014), yet experience a higher burden of tobacco-related mortality. We are only beginning to understand whether these behavioral differences translate to differential effects on epigenetic aging, which may ultimately lead to health trajectories that differ by sex. In a study of five cohorts, the relationship between alcohol dependence and epigenetic aging did not differ by sex (Rosen et al., 2018). However, this study examined only one epigenetic age measure (HorvathAge).Zhao et al. (2019) found that the effects of tobacco use on GrimAA varied by sex, but not alcohol use. Specifically, the effect of current smoking on GrimAA was stronger in men compared to women. It is notable, however, that this study was conducted in a cohort of older African Americans with a high prevalence of hypertension and therefore may not be generalizable to a younger population with a low prevalence of hypertension. Given the limited research in this area, more studies that include younger participants from multiple races/ethnicities are needed to better understand the potential sex-specific effects of alcohol and tobacco use on cellular aging, as well as the effects of increased epigenetic age on health outcomes at older ages.

1.3. Intergenerational effects

Health behaviors such as alcohol and tobacco use not only directly affect the individual practicing these behaviors but may also influence their offspring (Kandel et al., 2015; Kerr et al., 2020). Intergenerational influences may extend to DNA methylation. Several studies have examined the association between maternal tobacco use during pregnancy and epigenetic aging in infancy, early childhood, or adolescence. For example, one study found that infants whose mothers smoked tobacco during pregnancy had higher HorvathAA than those whose mothers did not smoke (Javed et al., 2016). Another study observed this same association in early childhood and found that the difference in HorvathAA became larger as the children aged into adolescence (Simpkin et al., 2016). Another study found that exposure to maternal tobacco use during pregnancy and parental tobacco use during childhood (neither parent vs. both parents) were associated with the offspring's HorvathAA in childhood (de Prado-Bert et al., 2021). To our knowledge, no studies have evaluated maternal and paternal effects of alcohol use during childhood on offspring epigenetic age. Thus, additional research is needed to evaluate associations between paternal and maternal alcohol and tobacco use during childhood and age acceleration of their offspring in adulthood, or how the effects may differ by sex.

1.4. Importance of the current analysis

To test sex-specific effects of alcohol and tobacco use on four measures of epigenetic age acceleration, this study used data collected from parents and their adult offspring in the Michigan Longitudinal Study (MLS) (Zucker et al., 1996). We hypothesized that alcohol and tobacco use may have sex-specific effects on cellular aging that can be captured by epigenetic clocks. We also hypothesized that paternal and maternal alcohol or tobacco use during childhood may have long-term effects on cellular aging in the offspring as reflected by epigenetic clocks in adulthood. Understanding how these sex-specific and intergenerational effects are embodied may lend insight into the etiology and patterning of age-related chronic diseases and mortality, and may illuminate opportunities for prevention, early detection, or treatment.

2. Material and methods

2.1. Study sample

The Michigan Longitudinal Study (MLS) is a prospective longitudinal study of children raised in a family with a father who had alcohol use disorder or control families from the same neighborhoods (Zucker et al., 2000). Demographic information including education, occupation, family income, parents' occupation, and marital history was assessed by questionnaire (Zucker et al., 1996). The study sample was drawn from the participant families enrolled in the MLS who were returning for a scheduled follow-up visit during the recruitment period (approved IRB protocols HUM00042342 & HUM00039806 to RA Zucker). Once a family member consented, phone calls were made to additional members of the family to obtain the participation of both parents and at least one male and one female child. Twenty-one families met these criteria and comprised the current study sample.

2.2. Methylation measures

Saliva samples were collected from 92 parents and offspring between 11/10/16–01/24/18. Methylation from genomic DNA was measured on the Infinium MethylationEPIC BeadChip array. Raw fluorescence files were imported into *minfi* (v1.26.2) (Fortin et al., 2017), and initial quality control was performed using *ENmix* (v1.16.0) (Xu et al., 2017). Probes were excluded if: (1) they were on X or Y chromosomes, (2) they had detection p-value<0.05 in >5% of samples, (3) they had a single nucleotide polymorphism (SNP) with >5% minor allele frequency at the interrogation or base extension site, or (4) they were cross-reactive with   47 base pairs of homology with an off-target site (Pidsley et al., 2016). Samples with >5% of probes with detection *p*-value < 0.05 were also excluded ($n = 1$). Probe intensities were background-corrected and dye-bias corrected using RELIC (Xu et al., 2017). Inter-array normalization was performed and probe-type biases were corrected using BMIQ (Teschendorff et al., 2013).

Epigenetic clocks (HannumAge, HorvathAge, PhenoAge, and GrimAge) were calculated using the DNA methylation age online calculator (https://dnamage.genetics.ucla.edu/new) and were strongly correlated with chronological age (r ranged from 0.93 to 0.97, see Fig. 1). As has been observed in other studies (Cardenas et al., 2022; Horvath, 2013; Horvath and Raj, 2018), epigenetic age estimates from different epigenetic clocks for the same individual can be highly variable, and the mean epigenetic age for a sample may be shifted from the corresponding mean chronological age. Many factors can lead to different estimates including the statistical methods, study population, and cell/tissue types used to train each of the epigenetic clocks, as well as sample processing and storage, DNA quality, normalization, and batch adjustment in the study sample. However, epigenetic age acceleration, which was calculated by regressing epigenetic age against chronological age and taking the residual, would still be valid since it preserves the rank of individuals within the study sample. Measures of epigenetic age acceleration (HannumAA, HorvathAA, PhenoAA, GrimAA) were calculated within parents and offspring separately. Cell proportions, estimated using a reference-free method (Houseman et al., 2016), were included in all models to adjust for potential confounding effects.

2.3. Demographics and substance use

2.3.1. Demographic variables—We collected self-reported sex and race/ethnicity, age at the most recent psychosocial assessment (when current tobacco and alcohol use were assessed), and age when methylation samples were collected.

2.3.2. Alcohol use—Current alcohol use was assessed at the most recent psychosocial assessment, which preceded methylation measurement by an average of 0.6 years. A few participants had psychosocial assessments after the methylation measure. Given the short period of time between the two assessments (7 within a month and 4 within 6 months), it is likely that those psychosocial assessments were a good proxy of the assessment when the methylation measure occurred. Two measures of participants' current alcohol use were calculated. Cumulative alcohol use, defined as the number of alcoholic drinks per month, was calculated from participants' self-reports of the number of alcoholic drinks they had per day multiplied by the number of days spent drinking alcohol per month (averaged over

the past 6 months). Binge drinking (yes/no) was defined as going on a binge of constant drinking for two or more days during the last three years.

2.3.3.   Tobacco smoking—To assess tobacco use at each MLS psychosocial examination, participants were asked, "Have you smoked cigarettes during the past 12 months?". Those who answered "never" or "once or twice" were considered tobacco non-smokers. Those participants who answered "occasionally but not regularly", "regularly during this year", or "regularly now" were considered current tobacco smokers at that examination. Participants were coded as never, former, or current tobacco smokers based on responses across all psychosocial data collection timepoints over the course of the MLS study, with current tobacco smoking assessed at the most recent psychosocial examination (concurrent with current alcohol use assessment).

2.3.4.   Early life alcohol exposure—To assess alcohol exposure when the offspring were children, a variable based on paternal and maternal self-reports of alcohol use was created. This was a binary variable (yes/no) of whether the offspring had a parent who was a heavy alcohol user when the offspring was 12 years old or younger. Using the cumulative alcohol use variable, we calculated the average drinks per month for each parent across the psychosocial assessments from timepoints when the offspring was 12 years old or younger. CDC guidelines were used to define the cutoff for heavy alcohol use.[3] Mothers who consumed 30 or more alcoholic drinks per month and fathers who consumed 60 or more alcoholic drinks per month on average were classified in the heavy alcohol use group. Due to the small number of mothers who were defined as heavy drinkers ($n = 5$), we were only able to analyze paternal heavy alcohol use in this study.

2.3.5.   Early life tobacco exposure—To assess tobacco exposure when the offspring were children, a variable based on paternal and maternal self-reports of tobacco use was created using the definition of current tobacco smoking defined in 2.3.3 Whether the offspring had a parent who was considered a current smoker at any psychosocial assessment from timepoints when the offspring was 12 years old or younger was created (yes/no).

2.4.   Statistical analysis

After removing participants without dates for psychosocial data collection or DNA methylation measurement, 87 participants were available for analysis. We used linear mixed effects models to assess the association between cumulative alcohol use, binge drinking, or tobacco use (modeled using dummy variables for former and current smoking with never smoking treated as the reference group) with epigenetic age acceleration across all samples. We modeled epigenetic age acceleration as the outcome and the corresponding alcohol or tobacco exposure as the predictor variable adjusting for a generation indicator (parent or offspring), sex, race/ethnicity, age at methylation measurement, white blood cell proportions, and the time difference between last psychosocial assessment and methylation measurement. Family ID was modeled as a random effect to account for familial relationships. We

---

[3]CDC Guidelines define heavy alcohol use as two or more alcoholic drinks per day for men and one or more alcoholic drink per day for women.

next performed sex-stratified analyses to assess the corresponding association in men and women separately. Then, to examine whether the observed sex-specific effects (nominal or significant after Bonferroni correction) differed from each other statistically, we added a sex-by-alcohol use or sex-by-tobacco smoking term to the model and tested whether the interaction term was significant in the full sample.

In the offspring only, we examined associations between paternal heavy alcohol use and offspring epigenetic age using linear mixed models, adjusting for the same covariates as above. We then added the offspring's own alcohol use to the model. We tested maternal and paternal tobacco use analogously, with and without adjustment for the offspring's own tobacco use. We did not evaluate maternal alcohol use during the offspring's childhood due to a small number of observations for this exposure.

Since four epigenetic age acceleration estimates were tested against each exposure, we applied a Bonferroni-corrected alpha level to declare significance ($p < 0.0125$). However, since the four age acceleration measures are all biomarkers of aging and are moderately correlated, Bonferroni correction is likely too conservative in this case. For this reason, we present both sets of results, noting that nominally significant results ($p < 0.05$) should be interpreted cautiously. All analyses were conducted in R version 4.1.2. Linear mixed modeling was conducted using the *lmerTest* package.

3. Results

The overall sample of 87 participants (62% male; 66.6% white) had a mean age of 34.5 years at methylation measurement and 33.9 years at their last psychosocial assessment (see Table 1). The offspring ($n = 45$) had a mean age of 20.3 years at methylation measurement and 20.0 years at their last psychosocial assessment. The parents ($n = 42$) had a mean age of 49.7 years at methylation and 48.9 years at their last psychosocial assessment. The average number of alcoholic drinks consumed per month was 18.8, 10.7% reported binge drinking, and 68.4% reported having never used tobacco. A total of 26.8% and 12.5% of the offspring experienced exposure to paternal or maternal heavy alcohol use when they were 12 years or younger, respectively. In addition, 42.9% and 54.4% of the offspring had been exposed to paternal or maternal tobacco smoking, respectively.

No significant association between cumulative alcohol use and epigenetic age acceleration was observed in the overall sample (Table 2). In the sex-stratified analysis, we observed a nominal association between alcohol use and age acceleration as measured by GrimAA ($p = 0.047$) in females, but not in males. The female-specific effect differed from the male-specific effect as indicated by a sex-by-alcohol use interaction ($p = 0.019$), but was not significant after Bonferroni correction.

For former tobacco smokers, epigenetic age acceleration measured by HannumAA was 3.60 years ($p = 0.002$) higher than for tobacco non-smokers (see Table 3). Males who reported current or former tobacco use had a 3.71 and 2.19-year increase, respectively, in HannumAA compared to those who did not smoke tobacco ($p = 0.025$ and $p = 0.043$); these associations were not significant after Bonferroni correction, nor were they significant

in females. The male-specific effect was significantly different from the female-specific effect for current smokers ($p = 0.003$) but not former smokers. PhenoAA was 4.74 and 4.38 years higher in both former smokers ($p = 0.029$) and current smokers ($p = 0.015$) in the full sample, respectively, but was not significant after multiple testing correction. Current smoking was also nominally associated with PhenoAA ($p = 0.029$) in males only in the sex-stratified analysis, though interaction analysis showed that the effect was not different than in females. In the full sample, individuals who reported current tobacco use had 4.61-year higher GrimAA ($p = 2.7 \times 10^{-6}$) compared to individuals who never smoked, but no effect was observed for former smokers. In the sex-stratified analysis, Grim AA was 3.85 years ($p = 2.83 \times 10^{-4}$) and 7.37 years ($p = 0.003$) higher in female and male current smokers, respectively, and the sex-specific effect sizes were not significantly different from each other.

Table 4 shows the association between paternal heavy alcohol use during childhood and adult epigenetic age acceleration among offspring after adjustment for sex, race/ethnicity, age at methylation measurement, and white blood cell proportions, with family modeled as a random effect. Paternal heavy alcohol use was associated with a 4.43-year increase in GrimAA ($p = 0.010$) after multiple testing correction, and it remained nominally associated ($p = 0.046$) after further adjustment for offspring's own alcohol and tobacco use, and the time difference between the last psychosocial assessment and methylation measurement. There were no associations between paternal or maternal tobacco use during childhood and adult age acceleration among offspring.

4. Discussion

This study of families at risk for alcoholism found that a higher number of alcoholic drinks per month, current and former tobacco use, and paternal heavy alcohol use when the offspring was 12 years or younger were all nominally associated with increased epigenetic age acceleration as measured by at least one epigenetic clock, and that there were sex differences in some of the associations. Additionally, this is one of the first studies to investigate the role of paternal and maternal health behaviors on epigenetic aging of their offspring beyond early childhood. Specifically, paternal heavy alcohol use was associated with increased GrimAA in offspring, although the effect attenuated after adjusting for the offspring's own alcohol or tobacco use. Former smoking was robustly associated with HannumAA, but current smoking increased HannumAA only in males. Current smoking, however, was robustly associated with GrimAA in both sexes. Thus, epigenetic age acceleration plays an important role in cellular aging and is influenced by many factors including one's own and familial alcohol and tobacco use, which exhibit sex-specific patterns.

4.1. Associations between alcohol and tobacco use, and epigenetic age acceleration

Cumulative alcohol use was nominally associated with GrimAA, but not other epigenetic age acceleration measures, and only among females. While some studies have observed relationships between HorvathAA, HannumAA and/or PhenoAA and alcohol use (Fiorito et al., 2019; Luo et al., 2020; Quach et al., 2017; Rosen et al., 2018), others have not

(Kresovich et al., 2021; Zhao et al., 2019). Consistent with results from two other studies that examined all four age acceleration measures, GrimAA was the only measure even nominally associated with alcohol use in our study (Kresovich et al., 2021; Zhao et al., 2019). Notably, we observed a nominal association only in women, and both of the studies that observed a strong association between alcohol use and GrimAA were also conducted in samples consisting solely (Kresovich et al., 2021), or mostly, of women (Zhao et al., 2019). GrimAA is the strongest predictor of mortality out of the clocks examined here, and so it is perhaps unsurprising that it is most strongly associated with lifestyle factors strongly linked to mortality. However, we note that the association observed here was not significant after multiple testing correction, so additional large-scale representative studies of sex-specific effects are needed to verify this finding.

Further, in our study, current tobacco use was significantly associated with GrimAA and nominally associated with PhenoAA. This is expected, given that these epigenetic clocks are trained on measures of mortality or functional health metrics that are strongly associated with mortality (Levine et al., 2018; Lu et al., 2019). GrimAge also directly incorporates smoking pack-years into its derivation algorithm (Lu et al., 2019). Our study also found that current smoking was nominally associated with increased HannumAA in males but not females, and that former smoking was associated with HannumAA in the full sample. However, these results should be interpreted with caution as the sample sizes for current and former smokers were small, especially in sex-stratified analyses. A similar effect for current smoking was found with GrimAA, but not HannumAA, in Zhao et al. (2019), where the effect of current tobacco use on GrimAA was stronger in men than women in older African Americans.

4.2. Associations between paternal alcohol and paternal/maternal tobacco use, and epigenetic age acceleration in offspring

Previous research on the paternal and maternal effects of alcohol and tobacco use on offspring epigenetics has largely focused on alcohol or tobacco use during pregnancy and corresponding offspring age acceleration during gestation or shortly after birth. Our study found that paternal heavy alcohol use during early childhood is associated with increased adulthood epigenetic age acceleration among offspring, as assessed by GrimAA, suggesting that paternal alcohol use may influence the offspring epigenome beyond infancy and childhood. Importantly, alcohol use does not have a direct biological influence on age acceleration in the offspring, and thus the association we observed is likely to have happened indirectly through psychosocial stress and/or an adverse family environment. Further research is needed to better understand the mechanisms of this effect.

We found no association between maternal or paternal tobacco use during early childhood and offspring epigenetic age acceleration in adulthood. Findings in previous studies have been mixed, and most have focused on offspring epigenetic age at birth or in early childhood, making direct comparisons with our study difficult. Maternal tobacco use was positively associated with offspring HorvathAA at birth as well as changes in offspring HorvathAA from birth to childhood in both the Accessible Resource for Integrated Epigenomic Studies (ARIES) sample and the Human Early-Life Exposome project (de

Prado-Bert et al., 2021; Simpkin et al., 2016). However, this result was not replicated in some other studies, perhaps due to differences in sample inclusion and exclusion criteria (Simpkin et al., 2016). One study examining paternal tobacco use found no relationship with epigenetic age at birth (Khouja et al., 2018), although paternal tobacco use has been associated with methylation changes at specific CpG sites (Wu et al., 2019). More research is needed on the impact of paternal smoking exposures on adult methylation patterning as nearly all existing studies are in infants or children.

4.3. Differences in associations among clocks

Epigenetic clocks are designed to capture biological aging, but the associations in our study differed among clocks. Other studies have shown that these clocks are only weakly to moderately correlated with one another, suggesting that they may be capturing different facets of biological aging (Liu et al., 2020). This is perhaps not surprising due to the nature of their construction, with first-generation clocks (HorvathAge, HannumAge) focused on selecting CpG sites associated with chronological age and second-generation clocks (PhenoAge, GrimAge) focused on CpG sites related to morbidity and mortality. Associations with health risk factors and outcomes also vary across clocks (Bell et al., 2019). For example, second-generation clocks are more strongly associated with physical and cognitive functioning (Levine, 2020). A recent study using multi-omic data showed that the transcriptional (gene expression) profiles for HorvathAge, HannumAge, and PhenoAge shared common biological pathways including metabolism, immunity, and autophagy (Liu et al., 2020). However, the PhenoAge clock was more strongly related to cellular senescence and mitochondrial dysfunction than the other clocks evaluated. These differences in the biological aging processes captured by the clocks may partially explain the differences in their associations with health behaviors such as alcohol and tobacco use. Future studies that further disentangle the underlying molecular features captured by different clocks could help shed light on the interpretation of the results of this study.

4.4. Strengths and limitations

This study has several limitations. First, the small sample size prevented us from assessing the effects of maternal alcohol use or incorporating information about the severity of paternal or offspring alcohol use disorder into analyses. Second, we did not include information on socioeconomic status (SES), sociocultural stressors, or other behaviors that may differ by gender. SES was not included becausethere was little variability in indicators of SES among participants in this study. Third, methylation profiles vary by cell/tissue type. We examined saliva samples here, which may not be the most informative cell type for the aging process.

Despite these limitations, our study has several important strengths. Carefully collected longitudinal data across multiple generations in MLS families provides us a unique opportunity to assess the association between paternal alcohol use and paternal/maternal tobacco use during childhood and epigenetic age acceleration in adulthood. Another strength of this study is the examination of sex-specific effects of alcohol and tobacco use on epigenetic age acceleration in younger, healthy participants.

5. Conclusion and future directions

This is the first study to use longitudinal family data to examine associations between paternal alcohol use and paternal/maternal tobacco use during childhood and epigenetic age acceleration in adult offspring. While many studies focus on prenatal or neonatal paternal and maternal lifestyles and epigenetic aging, this study examines fills an important developmental gap in the effects of paternal and maternal lifestyle factors during early childhood and epigenetic age acceleration in adulthood among offspring. We have shown that paternal heavy alcohol use during childhood is associated with epigenetic age acceleration as assessed by GrimAA in adult offspring. Heavy alcohol use does not have a direct biological influence on the epigenome in the offspring, so future studies are warranted to understand whether this association was mediated through specific psychosocial stressors. Our findings regarding sex-specific effects of alcohol and tobacco use on epigenetic aging suggest the need for further assessment of the ways in which epigenetic age acceleration differs by sex and its patterning on morbidity and mortality. However, we note that the sample size of this study is limited, particularly for stratified analyses, and independent replication is necessary to confirm our findings. Thus, conclusions from this study should be interpreted cautiously. Importantly, future studies that incorporate health outcomes are needed to understand whether the observed associations between alcohol use, tobacco smoking, and epigenetic age acceleration underly the sex-specific relationships between those behaviors and adverse health outcomes. Future studies could also examine threeway interactions among sex, alcohol use, and tobacco use on epigenetic age acceleration. An increased understanding of how these sex-specific and intergenerational effects are embodied may further our knowledge of the etiology and patterning of age-related chronic diseases and mortality, which may ultimately lead to better prevention, more targeted detection of the early stages of diseases, and novel treatments.

Acknowledgments

Author disclosures: role of funding source

Abbreviation List

CDC	Centers for Disease Control and Prevention
DNAm	DNA methylation
DNAmAge	DNA methylation age
DNAmAA	DNA methylation age acceleration (epigenetic age acceleration)

GrimAA	GrimAge acceleration (epigenetic age acceleration estimated using the GrimAge method)
GrimAge	Epigenetic age estimated according to methods described in Lu et al. (2019)
HorvathAA	HorvathAge acceleration (epigenetic age acceleration estimated using the HorvathAge method)
HorvathAge	Epigenetic age estimated according to methods described in Horvath et al. (2013)
HannumAA	HannumAge acceleration (epigenetic age acceleration estimated using the HannumAge method)
HannumAge	Epigenetic age estimated according to methods described in Hannum et al. (2013)
MLS	Michigan Longitudinal Study
PhenoAA	PhenoAge acceleration (epigenetic age acceleration estimated using the PhenoAge method)
PhenoAge	Epigenetic age estimated according to methods described in Levine et al. (2018)

References

Agabio R, Campesi I, Pisanu C, Gessa GL, Franconi F, 2016. Sex differences in substance use disorders: focus on side effects. Addict. Biol 21 (5), 1030–1042. [PubMed: 27001402]

Becker JB, Koob GF, 2016. Sex differences in animal models: focus on addiction. Pharmacol. Rev 68 (2), 242–263. [PubMed: 26772794]

Bell CG, Lowe R, Adams PD, Baccarelli AA, Beck S, Bell JT, Horvath S, 2019. DNA methylation aging clocks: challenges and recommendations. Genome Biol. 20 (1), 1–24. [PubMed: 30606230]

Cardenas A, Ecker S, Fadadu RP, Huen K, Orozco A, McEwen LM, Rosero-Bixby L, 2022. Epigenome-wide association study and epigenetic age acceleration associated with cigarette smoking among Costa Rican adults. Sci. Rep 12 (1), 1–13. [PubMed: 34992227]

Centers for Disease Control and Prevention, 2016. National Health Interview Survey. National Center for Health Statistics.

Centers for Disease Control and Prevention, 2020. Excessive Alcohol Use is a Risk to Men's Health. Division of Population Health. National Center for Chronic Disease Prevention and Health Promotion.

de Prado-Bert P, Ruiz-Arenas C, Vives-Usano M, Andrusaityte S, Cadiou S, Carracedo Á, González JR, 2021. The early-life exposome and epigenetic age acceleration in children. Environ. Int 155, 106683. [PubMed: 34144479]

Dugué PA, Jung CH, Joo JE, Wang X, Wong EM, Makalic E, Southey MC, 2020. Smoking and blood DNA methylation: an epigenome-wide association study and assessment of reversibility. Epigenetics 15 (4), 358–368. [PubMed: 31552803]

Dugué PA, Wilson R, Lehne B, Jayasekara H, Wang X, Jung CH, Baglietto L, 2021. Alcohol consumption is associated with widespread changes in blood DNA methylation: analysis of cross-sectional and longitudinal data. Addict. Biol 26 (1), e12855. [PubMed: 31789449]

Fiorito G, McCrory C, Robinson O, Carmeli C, Rosales CO, Zhang Y, McKay GJ, 2019. Socioeconomic position, lifestyle habits and biomarkers of epigenetic aging: a multi-cohort analysis. Aging 11 (7), 2045 (Albany NY). [PubMed: 31009935]

Fortin JP, Triche TJ, Hansen KD, 2017. Preprocessing, normalization and integration of the Illumina HumanMethylationEPIC array with minfi. Bioinformatics 33 (4), 558–560. [PubMed: 28035024]

Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, Gao Y, 2013. Genome-wide methylation profiles reveal quantitative views of human aging rates. Mol. Cell 49 (2), 359–367. [PubMed: 23177740]

Horvath S, 2013. DNA methylation age of human tissues and cell types. Genome Biol. 14 (10), 1–20.

Horvath S, Raj K, 2018. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. Nat. Rev. Genet 19 (6), 371–384. doi:10.1038/s41576-018-0004-3. [PubMed: 29643443]

Houseman EA, Kile ML, Christiani DC, Ince TA, Kelsey KT, Marsit CJ, 2016. Reference-free deconvolution of DNA methylation data and mediation by cell composition effects. BMC Bioinf. 17 (1), 1–15.

Hughes A, Smart M, Gorrie-Stone T, Hannon E, Mill J, Bao Y, Kumari M, 2018. Socioeconomic position and DNA methylation age acceleration across the life course. Am. J. Epidemiol 187 (11), 2346–2354. [PubMed: 30060108]

Javed R, Chen W, Lin F, Liang H, 2016. Infant's DNA methylation age at birth and epigenetic aging accelerators. Biomed. Res. Int 2016.

Joehanes R, Just AC, Marioni RE, Pilling LC, Reynolds LM, Mandaviya PR, Aslibekyan S, 2016. Epigenetic signatures of cigarette smoking. Circ. Cardiovasc. Genet 9 (5), 436–447. [PubMed: 27651444]

Kandel DB, Griesler PC, Hu MC, 2015. Intergenerational patterns of smoking and nicotine dependence among US adolescents. Am. J. Public Health 105 (11), e63–e72.

Kerr DC, Tiberio SS, Capaldi DM, Owen LD, 2020. Intergenerational congruence in adolescent onset of alcohol, tobacco, and marijuana use. Psychol. Addict. Behav 839–851. [PubMed: 31916782]

Khouja JN, Simpkin AJ, O'Keeffe LM, Wade KH, Houtepen LC, Relton CL, Howe LD, 2018. Epigenetic gestational age acceleration: a prospective cohort study investigating associations with familial, sociodemographic and birth characteristics. Clin. Epigenet 10 (1), 1–9.

Kresovich JK, Martinez Lopez AM, Garval EL, Xu Z, White AJ, Sander DP, Taylor JA, 2021. Alcohol consumption and methylation-based measures of biological age. J. Gerontol. Ser. A

Levine ME, 2020. Assessment of Epigenetic Clocks As Biomarkers of Aging in Basic and Population Research. Oxford University Press US.

Levine ME, Lu AT, Quach A, Chen BH, Assimes TL, Bandinelli S, Li Y, 2018. An epigenetic biomarker of aging for lifespan and healthspan. Aging 10 (4), 573 (Albany NY). [PubMed: 29676998]

Liu C, Marioni RE, Hedman ÅK, Pfeiffer L, Tsai P-C, Reynolds LM, Tanaka T, 2018. A DNA methylation biomarker of alcohol consumption. Mol. Psychiatry 23 (2), 422–433. [PubMed: 27843151]

Liu Z, Leung D, Thrush K, Zhao W, Ratliff S, Tanaka T, Levine ME, 2020. Underlying features of epigenetic aging clocks *in vivo* and *in vitro*. Aging Cell 19 (10), e13229. [PubMed: 32930491]

Lu AT, Quach A, Wilson JG, Reiner AP, Aviv A, Raj K, Stewart JD, 2019. DNA methylation GrimAge strongly predicts lifespan and healthspan. Aging 11 (2), 303 (Albany NY). [PubMed: 30669119]

Luo A, Jung J, Longley M, Rosoff DB, Charlet K, Muench C, Horvath S, 2020. Epigenetic aging is accelerated in alcohol use disorder and regulated by genetic variation in APOL2. Neuropsychopharmacology 45 (2), 327–336. [PubMed: 31466081]

Marioni RE, Suderman M, Chen BH, Horvath S, Bandinelli S, Morris T, Relton CL, 2019. Tracking the epigenetic clock across the human life course: a meta–analysis of longitudinal cohort data. J. Gerontol. Ser. A 74 (1), 57–61.

National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health. (2014). The health consequences of smoking—50 years of progress: a report of the surgeon general. Centers for Disease Control and Prevention (US).

Oblak L, van der Zaag J, Higgins-Chen AT, Levine ME, Boks MP, 2021. A systematic review of biological, social and environmental factors associated with epigenetic clock acceleration. Ageing Res. Rev 69, 101348. [PubMed: 33930583]

Peters SA, Huxley RR, Woodward M, 2014. Do smoking habits differ between women and men in contemporary Western populations? Evidence from half a million people in the UK Biobank study. BMJ Open 4 (12).

Phillips T, 2008. The role of methylation in gene expression. Nat. Educ 1 (1), 1.

Pidsley R, Zotenko E, Peters TJ, Lawrence MG, Risbridger GP, Molloy P, Clark SJ, 2016. Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. Genome Biol. 17 (1), 1–17. [PubMed: 26753840]

Quach A, Levine ME, Tanaka T, Lu AT, Chen BH, Ferrucci L, Beasley JM, 2017. Epigenetic clock analysis of diet, exercise, education, and lifestyle factors. Aging 9 (2), 419 (Albany NY). [PubMed: 28198702]

Rosen AD, Robertson KD, Hlady RA, Muench C, Lee J, Philibert R, Lohoff FW, 2018. DNA methylation age is accelerated in alcohol dependence. Transl. Psychiatry 8 (1), 1–8. [PubMed: 29317594]

Simpkin AJ, Hemani G, Suderman M, Gaunt TR, Lyttleton O, Mcardle WL, Horvath S, 2016. Prenatal and early life influences on epigenetic age in children: a study of mother–offspring pairs from two cohort studies. Hum. Mol. Genet 25 (1), 191–201. [PubMed: 26546615]

Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, Beck S, 2013. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics 29 (2), 189–196. [PubMed: 23175756]

White AM, 2020. Gender differences in the epidemiology of alcohol use and related harms in the United States. Alcohol Res. Curr. Rev 40 (2).

Wu CC, Hsu TY, Chang JC, Ou CY, Kuo HC, Liu CA, Yang KD, 2019. Paternal tobacco smoke correlated to offspring asthma and prenatal epigenetic programming. Front. Genet 10, 471. [PubMed: 31214241]

Xu Z, Langie SA, De Boever P, Taylor JA, Niu L, 2017. RELIC: a novel dye-bias correction method for Illumina Methylation BeadChip. BMC Genom. 18 (1), 1–7.

Zhao W, Ammous F, Ratliff S, Liu J, Yu M, Mosley TH, Smith JA, 2019. Education and lifestyle factors are associated with DNA methylation clocks in older African Americans. Int. J. Environ. Res. Public Health 16 (17), 3141.

Zucker RA, Ellis DA, Fitzgerald HE, Bingham CR, Sanford K, 1996. Other evidence for at least two alcoholisms II: life course variation in antisociality and heterogeneity of alcoholic outcome. Dev. Psychopathol 8 (4), 831–848.
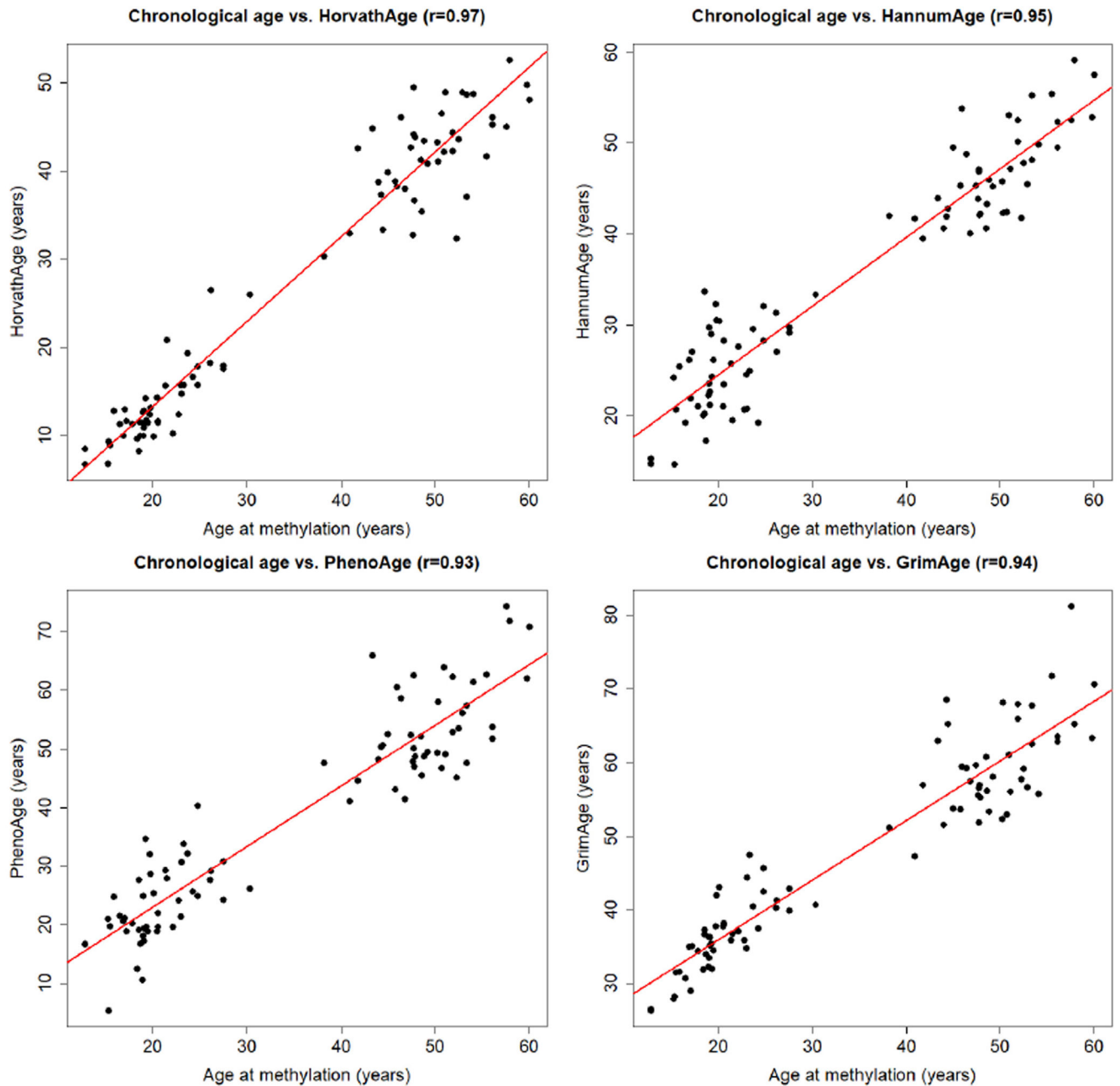
Fig. 1.
Scatterplot of chronological age and epigenetic clocks (overall sample, *N* = 87), the
Michigan Longitudinal Study (MLS).

Table 1

Descriptive characteristics of Michigan Longitudinal Study (MLS) participants.

	All (N = 87)	Parents (N = 42)	Offspring (N = 45)
Male	54 (62.0%)	21 (50%)	33 (73.3%)
Race/ethnicity			
White	58 (66.6%)	30 (71.4%)	28 (62.2%)
Black	11 (12.6%)	5 (11.9%)	6 (13.3%)
Mixed race	5 (5.7%)	3 (7.1%)	2 (4.4%)
Hispanic	13 (14.9%)	4 (9.5%)	9 (20.0%)
Age at methylation measurement (years)	34.5 (15.4)	49.7 (5.0)	20.3 (3.9)
Age at last psychosocial assessment (years)	33.9 (15.1)	48.9 (4.5)	20.0 (4.0)
Time difference between last psychosocial assessment and methylation measurement (years)	0.6 (1.2)	0.8 (1.5)	0.3 (0.6)
Binge drinking (N = 66) [a]	7 (10.7%)	1 (2.8%)	6 (20%)
Cumulative alcohol use (drinks/mo) (N = 80) [b]	18.8 (31.7)	18.4 (34.5)	19.2 (28.8)
Tobacco use (N = 79) [c]			
Never	54 (68.4%)	19 (52.8%)	35 (81.4%)
Former	11 (13.9%)	10 (27.8%)	1 (2.3%)
Current	14 (17.7%)	7 (19.4%)	7 (16.3%)
Paternal and maternal lifestyle factors			
Paternal heavy alcohol use (N = 41)	—	—	11 (26.8%)
Maternal heavy alcohol use (N = 40)	—	—	5 (12.5%)
Paternal tobacco use (N = 35)	—	—	15 (42.9%)
Maternal tobacco use (N = 33)	—	—	18 (54.4%)

N (%) or mean (SD) is reported.

[a] Sample for binge drinking included N = 36 parents and N = 30 offspring.

[b] Sample for cumulative alcohol use included N = 42 parents and N = 38 offspring.

[c] Sample for tobacco use included N = 36 parents and N = 43 offspring.

Table 2

Association between cumulative alcohol use and epigenetic age acceleration in the Michigan Longitudinal Study (MLS).

	All[a] (N = 80)		Female[b] (N = 32)		Male[b] (N = 48)	
	Beta	p	Beta	p	Beta	p
HorvathAA	0.01	0.711	0.02	0.546	0.005	0.771
HannumAA	0.01	0.654	2.92×10^{-4}	0.991	0.02	0.095
PhenoAA	0.04	0.067	0.06	0.172	0.05	0.127
GrimAA	0.01	0.420	**0.05**	**0.047**	−0.01	0.643

[a]Model: DNAmAA = Cumulative alcohol use + sex + age at methylation measurement + race/ethnicity + white blood cell proportions + time difference between last psychosocial assessment and methylation measurement + generation indicator (parent/offspring) + family ID (random effect).

[b]Model: DNAmAA = Cumulative alcohol use + age at methylation measurement + race/ethnicity + white blood cell proportions + time difference between last psychosocial assessment and methylation measurement + generation indicator (parent/offspring) + family ID (random effect)

$p < 0.05$ is in bold

Beta indicates the change in mean age acceleration (years) for each additional alcoholic drink per month after covariate adjustment.

Table 3

Association between tobacco use and epigenetic age acceleration in the Michigan Longitudinal Study (MLS).

	All[a] (N = 79)				Female[b] (N = 30)				Male[b] (N = 49)			
	Former (n = 11)		Current (n = 14)		Former (n = 7)		Current (n = 4)		Former (n = 4)		Current (n = 10)	
	Beta	p	Beta	P	Beta	p	Beta	p	Beta	p	Beta	p
HorvathAA	0.59	0.667	−0.13	0.910	4.45	0.120	1.81	0.554	−1.52	0.427	0.29	0.815
HannumAA	**3.60**	**0.002** *	0.09	0.924	4.03	0.066	−1.70	0.502	**3.71**	**0.025**	**2.19**	**0.043**
PhenoAA	**4.74**	**0.029**	**4.38**	**0.015**	2.33	0.561	5.96	0.177	5.25	0.138	**5.10**	**0.029**
GrimAA	0.75	0.478	**4.61**	**2.7×10^{-6}** *	2.01	0.132	**7.37**	**2.83×10^{-4}** *	2.59	0.129	**3.85**	**0.003** *

[a] Model: DNAmAA = Former smoker + current smoker + sex + age at methylation measurement + race/ethnicity + white blood cell proportions + time difference between last psychosocial assessment and methylation measurement + generation indicator (parent/offspring) + family ID (random effect).

[b] Model: DNAmAA = Former smoker + current smoker + age at methylation measurement + race/ethnicity + white blood cell proportions + time difference between last psychosocial assessment and methylation measurement + generation indicator (parent/offspring) + family ID (random effect) $p < 0.05$ is in bold

Beta indicates the difference in mean age acceleration (years) for current or former tobacco users vs. tobacco non-users after covariate adjustment.

*
Indicates the associations that remained significant after correction for multiple testing ($p < 0.0125$).

Table 4

Association between paternal heavy alcohol use and epigenetic age acceleration among offspring in the Michigan Longitudinal Study (MLS).

	Reduced Model[a] (N = 41)		Full Model[b] (N = 33)	
	Beta	**p**	**Beta**	**p**
HorvathAA	−2.25	0.085	−2.59	0.086
HannumAA	−1.67	0.196	−0.64	0.737
PhenoAA	0.37	0.887	−0.78	0.802
GrimAA	**4.43**	**0.010**[*]	**3.75**	**0.046**

[a]Reduced Model: Age acceleration = Paternal heavy alcohol use + sex + offspring's age at methylation measurement + race/ethnicity + white blood cell proportions + family ID (random effect).

[b]Full Model: Age acceleration = Paternal heavy alcohol use + sex + offspring's age at methylation measurement + race/ethnicity + offspring alcohol drinking status + offspring tobacco use + time difference between age at methylation and age at psychosocial assessment + white blood cell proportions + family ID (random effect). $p < 0.05$ is in bold.

Beta indicates the difference in mean age acceleration (years) for offspring whose fathers were heavy alcohol users vs. those whose fathers were not heavy alcohol users after covariate adjustment.

[*]Indicates the associations that remained significant after correction for multiple testing ($p < 0.0125$).