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# Adolescent Alcohol, tobacco, and Marijuana Use and DMA Methylation in Young Adulthood

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# ADOLESCENT ALCOHOL, TOBACCO, AND MARIJUANA USE AND DNA METHYLATION IN YOUNG ADULTHOOD

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## ABSTRACT

Substance use, including the use of tobacco, alcohol, marijuana, and other drugs, is most likely to begin during adolescence or emerging adulthood. Substance use during these developmental periods has been linked with a number of negative physical, mental, and cognitive health outcomes. Some of these long-term negative effects of substance use during adolescence and emerging adulthood may be explained by epigenetic changes through a process called DNA methylation. However, much of the research on substance use and DNA methylation has focused on prenatal drug exposure and its relationship to differential methylation profiles during childhood and young adulthood. By contrast, little is known about the epigenetic impact of substance use during adolescence. The present study examined the associations between DNA methylation and alcohol, tobacco, and marijuana use during early adolescence and young adulthood. The analyses focused on three genes, *AHRR, NR2B,* and *COMT,* because their methylation has been implicated with substance use. Participants included 290 young adults from the Birmingham Youth Violence Study who also provided data in early adolescence ($M_{age}$ = 13.1), late adolescence ($M_{age}$ = 17.6), and young adulthood ($M_{age}$ = 27.4). Analyses included 60% females, 82% minorities primarily consisting of African Americans, and 18% European Americans. Substance use was self-reported at each developmental period. DNA methylation beta values were measured with salivary DNA in young adulthood and averaged for each gene. The relationships between alcohol, tobacco, and marijuana use

and DNA methylation were tested using multivariate regressions, adjusting for sex, ethnicity, smoking history during late adolescence and early adulthood, and age in young adulthood. The delta $R^2$ values when the alcohol, tobacco, or marijuana use were added in the model ranged from 0.00 to 0.02, all $p > 0.05$. Moreover, in the combined substance use models, the combination of tobacco, alcohol, and marijuana use did not significantly affect $R^2$ in any model ($p < 0.05$).

Keywords: Adolescence, DNA methylation, AHRR, NR2B, COMT, Substance use

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## Introduction

Substance use, including the use of tobacco, alcohol, marijuana, and other drugs, is most likely to begin during adolescence or emerging adulthood (Johnston et al., 2013; Poudel & Gautam, 2017). Substance use during these developmental periods has been linked with a number of negative physical, mental, and cognitive health outcomes, including substance use disorders, memory impairment, reduced pulmonary function, increased rate of obesity, general impairment of the Central Nervous System, and increased risk of psychosis (Doherty et al., 2009; Lubman et al., 2015; Tomas-Roig et al., 2017). Some of these long-term negative effects of substance use during adolescence and emerging adulthood may be explained by epigenetic changes through a process called DNA methylation, which may lead to changes in DNA expression (Prince et al., 2019; Sakharkar et al., 2019;). However, much of the research on substance use and DNA methylation has focused on prenatal drug exposure and its relationship to differential methylation profiles during childhood and young adulthood (Cecil et al., 2016; Knopik et al., 2012; Lee et al., 2015). By contrast, little is known about the epigenetic impact of substance use during adolescence. The present study examined the associations of alcohol, tobacco, and marijuana use during early adolescence and DNA methylation in young adulthood. The analyses focused on three genes, *AHRR, NR2B,* and *COMT,* because their methylation has been implicated with substance use (Gitik et al., 2018; Philibert et al., 2013; Ruggeri et al., 2015 Schrott et al., 2020).

## Adolescent Substance Use

Adolescent substance use is very common in the United States, with reports estimating that up to 60% of adolescents aged 12-17 have tried tobacco, alcohol, or illicit drugs (SAMHSA, 2018; YBRS, 2018). Lifetime prevalence rates of tobacco, alcohol, and illicit substance use in adolescents are estimated at 29%, 60%, and 36%, respectively (Merikangas et al., 2010; YBRS, 2018). Evidence also suggests that adolescents often use multiple substances concurrently (Purcell et al., 2021; Tomcyzk et al., 2016). Substance use during adolescence is linked to multiple sociodemographic factors, including age, sex, race, and socioeconomic status (SES). Specifically, males demonstrate higher rates of use and substance use disorders compared to females (Chen & Jacobson, 2012; Lipari, 2013; NSDUH, 2013). Regarding racial/ethnic differences, Hispanic youth have higher rates of alcohol and marijuana use in early adolescence, whereas Caucasian youth have higher rates of substance use in middle and late adolescence compared to Hispanic and African American youth (Benner & Wang, 2015; Chen & Jacobson, 2012; Ensminger et al., 2016). Finally, adolescents from higher SES backgrounds are more likely to engage in alcohol and illicit substance use, whereas adolescents from lower SES backgrounds are more likely to smoke (Andrabi et al., 2017; Leventha et al., 2015).

## Substance Use and DNA Methylation

The use of addictive substances may induce epigenetic modifications in the brain, leading to changes in reward behavior, psychomotor ambulation, and drug craving (Nielson et al., 2012). Epigenetics is defined as the modification of gene expression in response to environmental influences without altering the underlying DNA sequence (Busslinger & Tarakhovsky, 2014; Ecker et al., 2018; Pinel et al, 2018; Wen et al., 2016).

Epigenetic modification can result from multiple mechanisms, including DNA methylation, acetylation, phosphorylation, micro RNAs, histone modification, and chromatin accessibility (Duncan et al., 2014). The most studied form of epigenetics is DNA methylation, which broadly involves the insertion or elimination of a methyl group ($CH_3$), typically where consecutive cytosine bases are present (Seong et al., 2011). DNA methylation is part of central regulatory functions to ensure normal cellular processes, contributing to overall conventional development and variation of cell tissues (Gertz et al., 2012; Herb et al., 2012). DNA methylation begins with the DNA methyltransferase group (DNMT-1, -3a and -3b) moving a methyl group from the S-adenosylmethionine (SAM) to unmethylated cytosine-phosphate-guanine (CpG) sites (Jones et al., 2012). These alterations can occur in different parts of genes, including promoters, exons, and introns (Jones et al., 2012). The DNMT family are key enzymes in DNA methylation that block the splicing process from RNA polymerase II and transcription factors (Maunakea et al., 2013). Specifically, DNMT-1 copies methylation patterns from a parent strand of DNA, which helps retain the DNA methylation pattern throughout replication of daughter cells (Unternaehrer et al., 2015). Furthermore, DNMT-3a and -3b work together to generate new DNA methylation marks by methylating unmethylated CpG sites (Unternaehrer et al., 2015).

Animals who are given drugs such as alcohol, tobacco, and marijuana at various developmental periods demonstrate differences in DNA methylation (Cecil et al., 2015). Experimental animal studies have shown that prenatal substance use causes alterations in DNA methylation of the hypothalamic pituitary axis (Gangisetty et al., 2014; Govorko et al., 2012). For example, male rodents given alcohol prenatally showed hypomethylation

within the ventral tegmental area in the *Dlk1* gene, hypomethylation of the hypothalamic proopiomelanocortin gene (*Pomc*), and decreased DNA methylation along the promoter of the *BNDF* gene in their germ cells (Bekdash et al., 2013; Finegersh & Homanics, 2014). In turn, these alcohol-induced alterations in DNA methylation can affect gene expression within reward seeking regions of the brain (e.g., hypothalamus), as well as memory consolidation regions (e.g., hippocampus), which can lead to long-term neural changes underlying onset and perseverance of learning disabilities, addiction, and perpetuation of the hypothalamic-pituitary-adrenal (HPA) axis dysfunction (Gangisetty et al., 2014; Itzhak et al., 2014; Moore et al., 2012; Nestler, 2014).

In humans, the use of drugs such as alcohol, tobacco, marijuana, and opioids during multiple developmental periods is also associated with differential patterns of DNA methylation (Bick et al., 2012; Cadet, 2016; Cecil et al., 2015; Harlaar & Hutchinson, 2013). Specifically, some studies have found hypermethylation in the leukocyte cells of former opioid users and individuals using alcohol in a specific protein coding gene (*OPRM1*), known for mediating drug-produced stimulation of reward pathways related to opioid use (Doehring et al., 2013; Zhang et al., 2012). Much of past research on substance use and epigenetic modifications has focused on the effects of alcohol and tobacco use during prenatal development and adolescence, with minimal attention to marijuana use (Gartstein & Skinner, 2018; Hamilton & Nestler, 2019; Suter et al., 2011). It is important to identify specific epigenetic modifications related to each substance, including marijuana. In particular, epigenetic changes may help explain the development of cognitive deficits associated with marijuana use, including poorer performance on verbal intelligence, response inhibition, and spatial working memory

tasks (Grant et al., 2012; Nader et al., 2018; Pope Jr et al., 2002; Pope Jr et al., 2003). Long-term marijuana use during adolescence appears to result in reduced Gamma Aminobutyric Acid (GABA) levels in adulthood, increased risk of psychosis, deficits in memory, and increased social withdrawal (Anglin et al., 2012; Zamberletti et al., 2014). Thus, the following sections review existing research on the relationships between the use of alcohol, tobacco, and marijuana across various developmental periods and DNA methylation in both animals and humans.

### *Alcohol*

Alcohol is the most widely studied substance regarding DNA methylation, with most epigenome-wide examinations finding alterations in several genes in relation to alcohol use or exposure during prenatal, adolescent, and adult stages of development (Cecil et al., 2015). Epigenetic changes are found as early as in prenatal exposure to alcohol, with some animal studies suggesting that prenatal alcohol exposure leads to altered methylation and gene expression throughout oxidative stress pathways within the hippocampus that last into adulthood (Chater-Diehl et al., 2016; Chen et al., 2013; Kleiber et al., 2013; Popva et al., 2017). Other animal studies found that prenatal alcohol exposure results in greater *Pomc* methylation in a rodent hypothalamus, which is hypothesized to decrease gene expression (Bekdash et al., 2013; Govorko et al, 2012). Although little research has focused on prenatal alcohol exposure and DNA methylation in humans, several studies have shown increased methylation in protocadherin genes, specifically *PCDHB18*, in children who were prenatally exposed to alcohol (Laufer et al., 2015; Laufer et al., 2017).

Alcohol use in adolescence can also lead to lasting epigenetic changes (Chater-Diehl et al., 2016). Many animal studies have demonstrated that alcohol use during the adolescent period can lead to alterations in DNA methylation within the amygdala and hippocampus (Sakharkar et al., 2019). For example, adolescent rats who were given alcohol showed a decrease in mRNA and protein levels of lysine demethylase (Lsd1) in specific structures within the amygdala (Kyzar et al., 2017). It is theorized that these changes may increase behavioral anxiety phenotypes and alcohol use in adulthood (Sakharkar et al., 2019). Binge-like exposure to alcohol in rats during adolescence was also associated with hypermethylation of the catechol-O-methyltransferase (COMT) promoter, disrupting dopaminergic and GABAergic transmission in the medial prefrontal cortex during adulthood (Trantham-Davidson et al., 2016).

Moderate and excessive alcohol use in adulthood among humans and animals has been linked with differential DNA methylation in the glutamate receptor gene (*NR2B*), sodium transporter gene (*SLC5A6*), and TLR-pathway genes in the liver (Khachatoorian et al., 2013; Qiang et al., 2015; Srinivasan et al., 2014). Studies with adult rats who were fed alcohol showed a global DNA hypomethylation and reduced methylation in the *C-myc* gene, which has been linked with the development of hepatocellular carcinoma (Hamid et al., 2009; Lu et al., 2000; Similie et al., 1994). Among both human and animal adults, excessive alcohol use causes global hypomethylation of DNA due to alcohol's ability to inhibit DNA methyltransferases (*DNMT*s), and these changes are linked to alcoholic liver disease (Medici & Halsted, 2013; Zakhari, 2013). Furthermore, collection of human cells revealed that alcohol consumption in adults is related to epigenetic changes similar to those observed in mice with glioblastoma cancer, specifically

hypomethylation of the *Dnmt1* gene (Hegi et al., 2005; Hervouet et al., 2010; Holland et al., 2000).

***Tobacco***

Tobacco use and exposure throughout different developmental periods, ranging from prenatal to adolescence and adulthood, is related to changes in methylation throughout the body but especially in the respiratory system (Breitlin, 2013; Gao et al., 2016; Wu et al., 2019). Animal studies found that prenatal tobacco exposure leads to hypomethylation in genetic loci such as the *Cyp1a1*, which plays a role in lung inflammation (Chen et al., 2017; Suter et al., 2010). Human prenatal exposure to maternal smoking is associated with higher levels of DNA methylation in the brain-derived neurotropic factor (*BDNF*) gene, specifically the *BDNF-6* exon, during adolescence, potentially leading to long-term alterations in brain development and plasticity (Toledo-Rodriguez et al., 2010).

Rodent studies revealed that tobacco use in adolescence led to disruption of typical DNA methylation of the *Smarca2* and *Bahcc1* genes (Gitik et al., 2018). In humans, adolescent tobacco use was linked with increased DNA methylation at the aryl hydrocarbon receptor repressor (*AHRR*) and growth factor independent 1 transcriptional repressor genes (*GFI1*) (Philibert et al., 2012; Prince et al., 2019). Additionally, current adolescent smokers showed a decrease in DNA methylation at various CpG sites compared to adolescents classified as non-smokers (Han et al., 2019). Finally, longitudinal studies found an association between tobacco smoking in early adolescence and greater methylation of the *RASSF1A* gene in adulthood, which increases the risk of lung cancer (Marsit et al., 2005; Reuben et al., 2020). Furthermore, smokers who began

smoking before they were 19 years old were approximately four times more likely to exhibit hypermethylation of the *RASSF1A* gene compared to smokers who started smoking after age 19 (Kim et al., 2003).

Tobacco use in early to mid-adulthood also appears to lead to lasting DNA methylation effects that persist for the remainder of the lifespan (Anderson et al., 2021). Studies with mice revealed that nicotine use in adulthood induces methylation in the following genes, *Ash2l*, *Chsy3*, *Zcchc11*, *Cep192*, *Tmem107*, and *D2* receptor (Jung et al., 2016; McCarthy et al., 2018). Additionally, tobacco using human adults show hypomethylation in some genes, like the *AHRR* gene known for aiding in tumor suppression, compared to non-smoking human adults (Anderson et al., 2021; Gao et al., 2016). Furthermore, tobacco exposure in adulthood has been linked with lower levels of methylation in genes associated with overweight (*AVPR1B*), development of schizophrenia, autism, and depression (*CNTNAP2*), and development of diabetes (*KCNQ1*) (Alarcón et al., 2008; Enhörning et al., 2016; Gao et al., 2016; Yasuda et al., 2008).

***Marijuana***

While research on marijuana use and DNA methylation is limited, studies suggest that early use and exposure to marijuana and higher frequency of marijuana use are associated with changes in DNA methylation which may result in cognitive deficits (Becker et al., 2010; Fontes et al., 2011; Gerra et al., 2018; Meier et al., 2012). Animal studies found hypomethylation of the *Dlgap2* gene in male rodent sperm following marijuana exposure (Schrott et al., 2020). Additionally, prenatal marijuana exposure is related to decreased DNA methylation in the dopamine receptor D2 (*DRD2*) gene in the

human ventral striatum (Le Fol et al., 2009), with disrupted methylation of the *DRD2* gene being linked to higher addiction risk (Dinieri et al., 2011).

Research focused on DNA methylation and marijuana use in adolescence is quite limited and requires more investigation. Rodent studies found that marijuana use in adolescence resulted in increased DNA methylation in the *Glua1* gene in the Prefrontal Cortex (PFC) (Rubino et al., 2015). Some research with human adolescents suggested an association between marijuana smoking status and DNA methylation of the catechol-O-methyltransferase (*COMT*) gene, with non-daily marijuana smokers exhibiting higher levels of methylation than non-smokers and daily smokers (Caspi et al., 2005; Van Der Knaap et al., 2014). Differential methylation of the *COMT* gene has also been linked with alcohol use (Van Der Knaap et al., 2014; Trantham-Davidson et al., 2016), suggesting that methylation in some genes may be affected by multiple psychoactive substances.

Marijuana use in adulthood has also been linked to DNA methylation of the *CRN1* gene and cannabinoid receptor genes CB1 and CB2 (Gerra et al., 2018). Marijuana use in adult rodents leads to hypermethylation of the *Dlgap2*, *Drd2*, and *Ncam1* genes, which are associated with cancer, substance abuse diagnosis, and other mental health diagnoses such as schizophrenia (Gerra et al., 2018; Schrott et al., 2020). Rodents with methylated changes in the *Dlgap2* gene exhibit socially dysfunctional behaviors, including aggression (Jiang-Xie et al., 2014). Moreover, differential methylation of the *NCAM1* gene affects synaptic plasticity, neurodevelopment, and neurogenesis and has been associated with a diagnosis of schizophrenia or bipolar disorder (Atz et al., 2007; Sullivan et al., 2007). The *DRD2* gene encodes a dopamine receptor related to alcohol dependence, which may modulate cognitive processes within the prefrontal cortex

(Rinaldi et al., 2007). Finally, research suggests that adult human marijuana use is related to increased DNA methylation in keratin (*KRT*) 1 and 10 genes, as well as the transglutaminase 5 (*TGM5*) gene (Paradisi et al., 2007; Rotter et al., 2012).

**Present Study**

As reviewed above, previous research has shown robust associations between DNA methylation and substance use across multiple developmental periods in both animal models and humans. However, few studies have focused on long-term methylation changes resulting from adolescent alcohol, tobacco, or marijuana use in humans. Substance use in adolescence appears to produce longstanding cognitive and behavioral changes, which may be exacerbated by heightened brain plasticity during this developmental period. Additionally, research with humans needs to take into account the co-use of multiple types of substances that commonly occurs and may produce biased results if ignored. Thus, the present study sought to examine the associations of alcohol, tobacco, and marijuana use during adolescence with DNA methylation in young adulthood. Based on prior literature on the role of alcohol, tobacco, and marijuana use in DNA methylation, the focus was on average methylation in promoter regions of the *AHRR*, *NR2B*, and *COMT* genes. A better understanding of associations between specific types of substance use during the sensitive adolescent period and epigenetic modification in adulthood may aid in the understanding of the long-term effects of substance use. Ultimately, this line of research may inform prevention efforts to reduce adolescent alcohol, tobacco, and marijuana use and their negative consequences.

## Method

### Participants and Procedures

This project included 290 young adults ($M_{age}$ =27.4, SD =1.2; 60% female; 82% Minority Ethnicity, 18% White) who participated in Waves 2, 3, and 4 of the Birmingham Youth Violence Study (Mrug et al., 2008). The study originally recruited fifth-grade students from 17 public schools in the greater Birmingham, Alabama area using a two-stage probability sampling process. In 2003-2004, Wave 1 involved individual interviews with 704 children ($M_{age}$=11.8 years, SD=0.8) and their primary caregivers. Approximately 17 months after Wave 1, 603 children and their primary caregivers returned to complete Wave 2 interviews ($M_{age}$ =13.2 years, SD=0.9). At Wave 3, 502 adolescents were interviewed ($M_{age}$= 18.1 years, SD=1.0).

During Wave 4 (currently ongoing), the young adults provided informed consent, completed individual interviews, and provided a saliva sample for DNA analyses. The saliva samples were collected using the Oragene DNA Discovery Kit (OG-500). Participants were asked to rinse their mouths and abstain from eating or drinking 30 minutes prior to saliva collection. All study procedures were approved by the Institutional Review Board at the University of Alabama at Birmingham. This project included data from Waves 2, 3, and 4.

## Measures

At each wave, adolescents reported on their substance use using questions adapted from national surveys, including Add Health and YRBS (Brener et al., 1995; 2002). However, Wave 1 reports of substance use were not utilized in this study due to the young age of the youth and corresponding low rates of substance use.

### *Alcohol*

At each wave, adolescents were asked if they had more than a few sips of beer, wine, or liquor in the last 12 months (yes/no). Affirmative answers were followed by questions about the frequency and quantity of alcohol use in the past 30 days or 12 months. At Wave 2, adolescents were asked one question about how many days out of the past 30 days they had more than a few sips of either beer, wine, or liquor on a 7-point scale from 1 (0 days) to 7 (all 30 days). Then, adolescents were asked one question about the usual amount of either beer, wine, or liquor drank on a 5-point scale from 1 (less than one drink) to 5 (5 or more drinks).

At Waves 3 and 4, adolescents were asked separate questions about beer, wine/wine coolers, and liquor. For each type of alcohol, they specified the frequency of use in the past 12 months on an 8-point scale from 1 (a few times) to 8 (every day) and an average number of drinks in a given day on a 9-point scale from 1 (1 can or bottle/glass/drink per day) to 9 (more than 8 cans or bottles/glasses/drinks per day). To make the variables comparable across waves, responses from each type of alcohol were used to compute a single Quantity Frequency Index (QFI) representing the average amount of alcohol consumed in the last 30 days.

### *Tobacco*

At each wave, adolescents were asked if they had tried cigarette smoking in the last 12 months (yes/no). Affirmative answers were followed by questions about the frequency and quantity of cigarette use in the past 30 days or 12 months. At Wave 2, adolescents were asked how many days out of the past 30 days they smoked cigarettes (frequency) on a 7-point scale from 1 (0 days) to 7 (30 days). They were also asked how many cigarettes they smoked each day (quantity) on a 6-point scale from 1 (I did not smoke in the last 30 days) to 6 (more than 20 cigarettes per day). Waves 3 and 4 followed a similar structure to Wave 2, but instead asked about frequency and quantity of cigarette smoking in the last 12 months. To make the variables comparable across waves, a single variable was computed at each wave indicating the average number of cigarettes smoked per month (Ambatipudi et al., 2016; Zeilinger et al., 2013).

### *Marijuana*

At Wave 2, adolescents were asked about the frequency of their marijuana use over the past 12 months on a 7-point scale from 1 (0 times) to 7 (100 or more times). At Waves 3 and 4, adolescents were asked about the frequency of their marijuana use over the last 12 months on an 8-point scale from 1 (never) to 8 (every day).

### *Covariates*

Primary caregivers reported the adolescents' race/ethnicity and sex at Waves 1 and 2. Adolescents' age was calculated from their date of birth and date of interview at each wave. During Waves 1 and 2, parents reported family income (rated on a 13-point ordinal scale) and primary caregiver's education (rated on an 8-point scale). At Wave 4,

participants reported on their highest level of education and annual household income. An income-to-needs ratio was calculated for Waves 1 and 4 by dividing the reported household income in each respective wave by the poverty threshold for that family size during the year of data collection. An income-to-needs ratio is frequently used as an objective socioeconomic status indicator in longitudinal studies because it incorporates household size (Diemer et al., 2013; Dowsett et al., 2008; Roosa, et al., 2005). For analyses of alcohol and marijuana use, smoking at Waves 3 and 4 was also used as a covariate. Specifically, cigarette smoking within the last 12 months was dichotomized (yes = 1, no = 0) and summed across the two waves (Ambatipudi et al., 2016; Zeilinger et al., 2013).

**DNA Extraction and Methylation**

During Wave 4, saliva samples were processed using the PureGene extraction method (Qiagen) given manufacturer specifications. Samples were tested for quality using resuspension in 100-200μL of Tris-EDTA (10mM Tris-HCL, 50mM EDTA pH 7.5) and quantified using a revised OD260 technique on a Trinean Dropsense instrument with cDrop analysis software (Unchained Labs). Final samples yielded over 2.1 μg of high-quality DNA (OD260/280 above .45). The DNA extracted from the saliva underwent methylation analysis via Illumina Infinium MethylationEPIC BeadChip. The BeadChip includes 850,000 genome-wide methylation sites at single-nucleotide resolution selected by methylation experts. The EPIC chip probed >97% of genes, focusing on promoter and CpG-island CpGs, 3’ ends, and differentially methylated regions.

Quality control (QC) and normalization included probe QC, sample QC, background correction, within array-normalization, Type I and II chemistry modification, and chip/plate/batch adjustment. These QC tests consisted of checking probes for hybridizing and bisulfite conversion, p-value detection to test probe QC, removal of specious probes with cross-hybridization to sex chromosomes, and deletion of CpGs within or near probe sequence. Background correction, removal of experimental artefacts, unnecessary noise, and technical or methodical variation to normalize probes were performed. Normalization focused on between- and within-array normalization (Type I and Type II chemistry scaling) (Maksimovic et al., 2012). For this study, quantile preprocessing procedures (one sample at a time) and ssNoob (normalization of all samples) were used (Byun et al., 2009; Fortin et al., 2017). QC workflow was performed using the R package *minfi*, which includes complete QC to statistical testing for CpGs and differentially methylated regions (Aryee et al., 2014).

Adjustment for cellular heterogeneity was used given that saliva contains a heterogeneous mixture of leukocytes and epithelia cells with proportions that vary across individuals (Smith et al., 2015). Heterogeneous cells in a sample can lead to inter-individual variation of salivary DNA methylation profiles, which can bias the results of epigenetic analyses. Without accounting for heterogeneity, differential methylation at specific loci could reflect varying proportions of cell types instead of trait specific association (Jaffe & Irizarry, 2014; Schneider et al., 2010). The reference-based deconvolution method was used to correct methylation data for differences in cell composition (Houseman et al., 2012). Although originally developed for blood, this method has been applied to saliva (Langie et al., 2017; Smith et al., 2015).

Methylation Beta-values follow the beta-distribution given that they are computed as proportions. The Rank-Based Inverse Normal transformation were used to transform the methylation values (Zhang et al., 2019). In line with prior research (Zhang et al., 2015), average methylation of CpG sites were calculated within the promotor region for each gene - *AHRR, NR2B,* and *COMT*. CD8T cells, CD4T cells, natural kill (NK) cells, B cells, and monocytes were calculated using quantile-normalized data to infer saliva cell proportions. To control for cell mixture effects, the estimated cell proportions were used as covariates in methylation analyses.

**Statistical Analysis**

Descriptive statistics were examined for all variables. Attrition analyses were also conducted via independent sample t-tests and chi-square analyses comparing participants who remained in the study at Wave 4 and those who were lost to follow up. Pearson's correlations were used to examine bivariate associations among the substance use variables, average methylation levels in *AHRR*, *COMT,* and *NR2B* genes, and all covariates. None of the specific types of substance use (e.g., alcohol use) showed high correlations between Waves 2, 3, or 4, supporting their use as separate predictors in the main analyses.

For the main analyses, 18 hierarchical regression analyses in Mplus were used to test the relationships between predictors (adolescent alcohol use, tobacco use, or marijuana use at Waves 2 or 3), and outcomes (average methylation levels in the promoter regions of *AHRR, NR2B,* and *COMT* genes at Wave 4), adjusting for demographic covariates (age, income-to-needs ratio at Wave 4, sex, race/ethnicity, substance use at Wave 4, and income-to-needs ratio at Wave 1 and Wave 4). Averaged

methylation values from the *AHRR*, *COMT*, and NR2B genes were analyzed separately. Smoking history (combined across waves 3 and 4) was also used as covariates for each alcohol and marijuana use model.

Thus, Step 1 of each model included the following covariates: sex, ethnicity, age at Wave 4, substance use at Wave 4, income-to-needs-ratios at Waves 1 and 4, cell types, and, for alcohol and marijuana models only, smoking history. Step 2 included one type of substance use (alcohol, tobacco, or marijuana) at Wave 2 or Wave 3. Finally, a series of 6 hierarchical regressions included all types of substance use (alcohol, tobacco, and marijuana) for either Wave 2 or Wave 3 to examine the unique relationships between each type of substance use and average DNA methylation of *AHRR*, *COMT*, or NR2B genes. Smoking history was omitted from the combined analyses. In all models, Full Information Maximum Likelihood (FIML) was utilized to use all available data and reduce bias due to data missing at random (Choi et al., 2019).

## Results

### Preliminary Analyses

Of the 704 participants in Wave 1, 290 (41%) were retained in Wave 4 and had complete methylation data. This group comprised the analytic sample for this study. Participants who remained in the study at Wave 4 were more likely to be African American (82% vs. 75%, $X^2$ (1, $N$ = 704) = 5.11, $p < 0.05$) and female (60% vs. 39%, $X^2$ (1, $N$ = 704) = 31.47, $p < 0.01$) compared to those who were lost to follow up. Chi-squares and independent t-tests showed that these two groups did not differ in tobacco, alcohol, marijuana use at Waves 2 and 3 or income-to-needs ratio at Wave 1 ($p > 0.06$).

Of the 290 participants in the analytic sample, 204 (70%) had complete data on all variables and 5.5% of data points were missing. Participants who had complete data were more likely to be White (28% vs. 14%, $X^2$ (1 $N$ = 290) = 8.27, $p < 0.01$) and report more alcohol use at Wave 3 ($M$ = 7.55 vs, 4.03, $t$(237) = -1.41, $p < 0.05$) and tobacco use at Wave 4 ($M$ = 18.43 vs. 9.68, $t$(286) = -1.89, $p < 0.01$), compared to those who had missing data. Chi-squares and independent t-tests showed that these two groups did not differ in gender, income-to-needs ratio at Wave 1 or Wave 4, age at Wave 4, tobacco, alcohol, or marijuana use at Wave 2, tobacco or marijuana use at Wave 3, and alcohol or marijuana use at Wave 4 (all $p > 0.13$).

Descriptive analyses for predictors and covariates can be found in Table 1. The sample included 60% females, 81% African Americans, and 18% European Americans

with an average age of 27 years old. Substance use in the last 12 months was relatively low in Wave 2 ($M_{age}$ = 13.15) with 3% reporting any tobacco use, 11% reporting any alcohol use, and 2% reporting any marijuana use. At Wave 3 ($M_{age}$ = 17.63), 13% reported tobacco use, 49% reported alcohol use, and 20% reported marijuana use in the last 12 months. Finally, at Wave 4 ($M_{age}$ = 27.38), 25% reported tobacco use, 68% reported alcohol use, and 32% reported marijuana use in the last 12 months. Per the income-to-needs ratio, 43% of the sample was below the federal poverty level at Wave 1 and 25% were below the poverty level at Wave 4. The granulocyte (GRAN) cells (*Mean proportion* = 0.82, *SD* = 0.12) had the highest average proportion of cell types compared to B cells (*M* = 0.07, *SD* = 0.04), monocyte cells (*M* = 0.07, *SD* = 0.03), CD8 T cells (*M* = 0.01, *SD* = 0.03), CD4 T cells (*M* = 0.05, *SD* = 0.05), and NK cells (*M* < 0.001, SD = 0.001).

Table 2 shows correlations among outcome variables, predictors, and covariates. Substance use variables were weakly to moderately correlated across time. Average methylation at the *AHRR* gene was related to higher methylation at the *COMT* gene (*r* = 0.38) and lower methylation at the *NR2B* gene (*r* = -0.43), and the average methylation at the *COMT* gene was related to lower methylation at the *NR2B* gene (*r* = -0.29). There were no significant correlations between substance use variables and average methylation of *AHRR*, *COMT*, or *NR2B* genes. Female gender was related to lower alcohol, tobacco, and marijuana use in Wave 4. Smoking at Waves 2, 3, and 4 was related to lower income. The monocyte cell type weakly negatively correlated with Wave 3 tobacco use. All other cell types did not significantly correlate with demographics or substance use variables.

**Regression Models**

Table 3 shows covariates entered in the Steps 1 of the regression models accounted for 51% of variance in *AHRR* methylation, 11% in *COMT* methylation, and 37% in *NR2B* methylation. *AHRR* methylation was uniquely predicted by African American race (β = 0.12), older age (β = 0.10), and lower proportion of CD8T (β = -1.17), CD4T (β = -0.86), BCELL (β = -0.94), and GRAN (β = -2.27) cell types. Similarly, higher *NR2B* methylation was related to older age (β = 0.15) and lower proportions of CD8T (β = -0.66), CD4T (β = -0.88), BCELL (β = -1.18), MONO (β = -0.61), and GRAN (β = -2.38) cell types. Finally, *COMT* was associated with male sex (β = -0.24) and lower proportion of CD4T cell type (β = -0.78).

Steps 2 of the models, which contained individual substance use variables from each wave, did not explain additional variance in the methylation outcomes (delta $R^2$ value ranged from .00 to .02, all $p > 0.05$). None of the coefficients were statistically significant, although Wave 3 tobacco use approached significance for methylation of the *COMT* gene (β = 0.13, $p < 0.06$) and Wave 4 alcohol use approached significance for methylation of the *AHRR* gene (β = 0.08, $p < 0.06$). In the combined substance use models, the combination of tobacco, alcohol, and marijuana use did not significantly increased $R^2$ in any model ($p < 0.05$; Table 4).

## Discussion

The present study examined alcohol, tobacco, and marijuana use in early and late adolescence as predictors of average DNA methylation in young adulthood. Specifically, this study focused on average methylation in promoter regions of *AHRR*, *COMT*, and *NR2B* genes (Gitik et al., 2018; Philibert et al., 2013; Ruggeri et al., 2015 Schrott et al., 2020). Contrary to hypotheses, alcohol, tobacco, and marijuana use in adolescence or in young adulthood were not significantly correlated with average methylation of the *AHRR*, *COMT*, or *NR2B* genes. Similarly, regression analyses revealed that neither alcohol, tobacco, or marijuana use at any time point was uniquely related to average DNA methylation in each of the genes of interest.

### Alcohol, Tobacco and Marijuana Use and DNA Methylation

The results found in the current study contrast with previous research documenting effects of alcohol, tobacco, and marijuana use on DNA methylation of the *AHRR*, *COMT*, or *NR2B* genes (Gitik et al., 2018; Philibert et al., 2013; Ruggeri et al., 2015 Schrott et al., 2020). However, very few studies have tested the combined effects of adolescent alcohol, tobacco, and marijuana use on methylation of the *AHRR*, *COMT*, or *NR2B* genes. Broadly, some research on marijuana and tobacco use suggests differential methylation across the *AHRR* and *COMT* genes, but research is limited for alcohol use in relation to these genes (Osborne et al., 2020). Conversely, a significant amount of research reports a relationship between alcohol use and *NR2B* gene hypomethylation, but

little research has studied the effects for tobacco and marijuana use on *NR2B* methylation (Berkel & Pandey, 2017; Chandrasekar, 2013).

Prior research suggests that DNA methylation of the *AHRR* gene is a sensitive biomarker for smoking tobacco and marijuana, but no previous studies examined methylation of this gene in relation to alcohol use (Shenker et al., 2013). This study examined this relationship and did not find a significant association between adolescent alcohol use and methylation at the *AHRR* gene. Previous research has focused mainly on methylation of the *AHRR* gene in relation to tobacco use, finding robust patterns of demethylation in regular adult and adolescent tobacco smokers (Philibert et al., 2013; Prince et al., 2019; Shenker et al., 2013). The current study did not find a relationship between tobacco use and methylation at the *AHRR* gene, which could be due to a smaller sample size of 290 (compared to average sample size of 400 in other studies), the use of saliva rather than blood samples, and lower levels of tobacco use compared to prior studies. Similarly, one study found differential methylation in the *AHRR* gene based on adolescent marijuana use, but these associations did not extend to heavy marijuana users (Van der Knaap et al., 2014). The results of the current study did not show a relationship between adolescent marijuana use and methylation at the *AHRR* gene, which could be due to low reports of marijuana use or smaller sample size (N = 290 vs 463) in the present study.

Moreover, this study was one of the first to examine the relationship between adolescent alcohol use and methylation of the *COMT* gene, again showing no significant relationships which could be due to the limited sample size or the primary focus on normative levels of adolescent and adult alcohol use, rather than alcohol abuse or

dependence studied in prior work (Köhnke et al., 2003; Samochowiec et al., 2006). While methylation of the *COMT* gene has been associated with adult tobacco use, research is limited for adolescent tobacco use (Osborne et al., 2020; Xu et al., 2010). One previous study examined the relationship of *COMT* gene methylation and tobacco use, finding that non-daily use was associated with promoter methylation but daily use was not (Van der Knaap et al., 2014). The present study showed no significant relationships between adolescent tobacco use and *COMT* gene methylation.

One study linked high levels of DNA methylation in the *COMT* gene in adolescents with current tobacco use but not lifetime tobacco use (Prince et al., 2019), which suggests that a sustained and prolonged smoking history may help explain why adolescent tobacco use was not related to *COMT* gene methylation. Moreover, adult tobacco use has been linked to higher *COMT* gene methylation rates (Van der Knaap et al., 2014), further suggesting that older age, continued duration of smoking, and longer history of smoking may help explain why adolescent tobacco use was not related to *COMT* gene methylation in this study.

Finally, the present study showed no significant relationship between methylation of the *COMT* gene and adolescent marijuana use. One other study linked higher *COMT* gene methylation with non-daily marijuana use but not daily marijuana use, suggesting that quantity and frequency of marijuana use play a role in methylation (Van der Knaap et al., 2014). However, it is unknown if the epigenetic alterations persist after cessation of marijuana use (Szutorisz & Hurd, 2016). Lack of persistence in DNA methylation related to marijuana use over time may help explain why adolescent marijuana use was not related to *COMT* gene methylation in the present study.

Finally, research on adolescent tobacco and marijuana use in relation to methylation of the *NR2B* gene is quite limited. The results of this study suggest no significant relationships between *NR2B* gene methylation and adolescent tobacco or marijuana use. Moreover, the results of this study suggest that average methylation of the *NR2B* gene is not associated with adolescent alcohol use. However, the majority of prior research in this area has utilized adult rodent models to study the effects of alcohol addiction on *NR2B* gene demethylation at various CpG sites (Berkel & Pandey, 2017; Ponomarev, 2013). Adult rodent research has also shown a relationship between differential methylation of the *NR2B* gene and intermittent alcohol vapor exposure (Qiang et al., 2015). While rodent research has shown relationships between varying frequencies and quantities of alcohol exposure and differential methylation of the *NR2B* gene, the present study results may be explained by differences in age and duration of lifetime alcohol consumption in those studies vs. the present investigation.

**Covariates and Methylation**

In general, average methylation across *AHRR*, *COMT*, and *NR2B* genes showed associations with ethnicity and age. Older individuals had higher average methylation of the *AHRR* and *NR2B* genes, which is consistent with previous literature showing that aging is closely linked to epigenetic alterations (Johnson et al., 2012; Sedivy et al., 2008). Finally, African Americans had higher average methylation of the *AHRR* gene compared to whites. Previous research has reported that methylation of the *AHRR* gene may differ among ethnicities (Elliot et al., 2014; Philibert et al., 2020), suggesting that observed relationships may be driven by genetic variation or environmental exposures that vary by race.

**Limitations**

The current study has several limitations. The sample size of 290 is relatively small compared to other studies that found significant associations between adolescent substance use and DNA methylation of the *AHRR*, *COMT*, and *NR2B* genes (N=400-500) (Biermann et al., 2009; Van der Knaap et al., 2014; Zeilinger et al., 2013). It is common for effect sizes to be small in epigenetic studies, which requires larger sample sizes to reach adequate statistical power (Breton et al., 2017). An additional limitation is the limited retention rate of 41% from Wave 1 to Wave 4 at the time this study was conducted. African Americans and women were more likely to be retained across time, thus, the results may be less generalizable to Whites and men.

Substance use assessment is often coupled with limitations. For this study, substance use was measured with self-report scales which have been shown to have some limitations in accuracy (Hadland & Levy, 2016; Williams & Nowatzki, 2005). Self-report instruments are some of the most convenient, cost efficient, and widely used forms for measuring substance use (Hans et al., 1999), but they are subject to recall and report biases (Hadland & Levy, 2016; Ladis et al., 2019; Williams & Nowatzki, 2005). Moreover, adolescents may experience heightened discomfort disclosing substance use due to being underage for legal use of each substance and concerns about confidentiality of disclosing use of illegal substances, such as marijuana (Rosenkranz et al., 2012; Williams & Nowatzki, 2005). Furthermore, this study assumed that substance use during adolescence would affect DNA methylation across normative levels of use. However, substance use levels at Wave 2 were low, requiring dichotomization of Wave 2 tobacco, alcohol, and marijuana use. This dichotomization may have attenuated associations

between substance use and methylation. Furthermore, tobacco and alcohol use were measured on a quantity-frequency interval, whereas marijuana use was measured based on frequency only. These differences in measurement may have contributed to lower sensitivity to detect any effects of marijuana use on DNA methylation.

Additionally, DNA methylation research is still in relatively early stages. It should be noted that comparing results regarding substance use and DNA methylation across studies is complicated due to varying methods of analyzing epigenetic data, including large-scale epigenome-wide association studies (EWASs) with specific loci or average levels of methylation across a gene of interest (El-Maarri et al., 2007). Many studies examining adolescent substance use and DNA methylation utilize multiple testing across specific CpG sites in the promoter region of genes rather than average methylation across the promoter region as done in the present study. Research that utilized multiple CpG site testing tended to analyze 100 or more sites at a time which required a p-value correction to contain Type I error (e.g., Bonferroni correction) (Christiansen et al., 2021). While these studies may help identify specific CpG sites to focus on in future research, the strict Bonferroni corrections remain controversial for being too conservative, especially in smaller samples (Levin, 1996; Ranstam, 2016). Hence, the present study sought to utilize average DNA methylation across specific CpG promoter regions in each gene. The chosen method also has limitations because it is possible that small effects at individual sites existed but were not apparent in the averaged methylation values used in this study (Hagerty et al., 2020).

Additionally, it was not possible to assess changes in methylation across time because methylation was only measured during Wave 4 of the present study. Further,

DNA sequence variation was not used as a covariate in this study. Variations of specific genes can influence the levels of methylation and interact with environmental exposures, such as substance use (Qiu et al., 2015). For example, systemic lupus erythematosus and cigarette smoking were associated with greater methylation on the *AHRR* CG allele compared to the heterozygous CC and GG alleles (Saghaeian et al., 2021). Similarly, individuals who excessively consumed alcohol and had a CC genotype had a higher risk of differential *COMT* methylation than drinkers with one T allele (Kakino et al., 2016). Although studies show that *NR2B* variants have no effect on methylation levels of this gene (Nielsen et al, 2012; Qiu et al., 2015), the potential impact of genotypic variants and their interactions with substance use should be further investigated in future studies.

**Conclusions**

The results of this longitudinal study advance our understanding of relationships between alcohol, tobacco, and marijuana use during adolescence and average DNA methylation of *AHRR*, *COMT*, and *NR2B* in young adulthood. This was one of the first studies to examine combined substance use as well as individual substance use in adolescence in relation to DNA methylation. The current study did not detect significant relationships between average methylation in these genes and alcohol, tobacco, and marijuana use from adolescence to young adulthood. Future research should utilize larger samples with higher levels of substance use, as well as explore the role of polysubstance use at different developmental stages in relation to DNA methylation across development. Repeated assessments of DNA methylation across different developmental periods is essential to understand whether varying levels of substance use relate to changes in DNA methylation over more proximal time periods.

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Table 1
*Descriptives for Predictors and Covariates*

| | **N** | **%** |
|---|---|---|
| Female | 290 | 60.34 |
| African American | 290 | 80.70 |
| | **M** | **SD** |
| Age | 27.38 | 1.19 |
| W2 Tobacco Use | 0.03 | 0.16 |
| W2 Alcohol Use | 0.12 | 0.32 |
| W2 Marijuana Use | 0.02 | 0.15 |
| W3 Tobacco Use | 4.07 | 19.21 |
| W3 Alcohol Use | 4.55 | 13.70 |
| W3 Marijuana Use | 2.23 | 5.68 |
| W4 Tobacco Use | 12.24 | 36.02 |
| W4 Alcohol Use | 18.00 | 43.97 |
| W4 Marijuana Use | 3.72 | 7.16 |
| Income-to-needs ratio W1 | 1.68 | 1.40 |
| Income-to-needs ratio W4 | 2.24 | 1.48 |
| W3 & W4 smoking history | 0.37 | 0.64 |
| CD8T | 0.01 | 0.03 |
| CD4T | 0.05 | 0.05 |
| NK | 0.00 | 0.00 |
| BCELL | 0.07 | 0.04 |
| MONO | 0.07 | 0.03 |
| GRAN | 0.82 | 0.12 |

*Note.* W1 – Wave 1; W2 - Wave 2; W3 - Wave 3; W4 - Wave 4; $N = 239$ to 290.

Table 2

*Correlations Among All Variables*

| | 1. | 2. | 3. | 4. | 5. | 6. | 7. | 8. | 9. | 10. | 11. | 12. | 13. | 14. | 15. | 16. | 17. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Female | -- | | | | | | | | | | | | | | | | |
| 2. African American | .15** | -- | | | | | | | | | | | | | | | |
| 3. Age | .04 | .20** | -- | | | | | | | | | | | | | | |
| 4. W2 Tobacco Use | -.01 | .01 | .05 | -- | | | | | | | | | | | | | |
| 5. W2 Alcohol Use | .06 | .10 | .05 | .23** | -- | | | | | | | | | | | | |
| 6. W2 Marijuana Use | -.03 | .07 | .12 | .45** | .34** | -- | | | | | | | | | | | |
| 7. W3 Tobacco Use | -.08 | -.05 | .12 | .07 | .27** | .10 | -- | | | | | | | | | | |
| 8. W3 Alcohol Use | -.03 | .04 | -.02 | .12 | .25** | .14* | .18** | -- | | | | | | | | | |
| 9. W3 Marijuana Use | -.10 | .16* | -.01 | .15* | .14* | .22** | .39** | .36** | -- | | | | | | | | |
| 10. W4 Tobacco Use | -.19** | -.07 | .05 | .17 | .17** | .08 | .41** | .12 | .22** | -- | | | | | | | |
| 11. W4 Alcohol Use | -.12* | -.15* | -.16** | .06 | .08 | -.03 | -.02 | .21** | .09 | .22** | -- | | | | | | |
| 12. W4 Marijuana Use | -.24** | .12* | .00 | .05 | .12 | .08 | .16* | .28** | .26** | .28** | .33** | -- | | | | | |
| 13. Income-to-needs ratio W1 | -.09 | -.31** | -.36** | -.14** | -.08 | -.08 | -.18** | -.03 | -.07 | -.14* | .15* | .06 | -- | | | | |
| 14. Income-to-needs ratio W4 | -.14* | -.25** | -.21** | -.08 | -.12 | -.16* | -.20** | .01 | -.19** | -.13* | .28** | -.03 | .39** | -- | | | |
| 15. W3 & W4 smoking | -.11 | -.04 | .09 | .21** | .29** | .20** | .74** | .16* | .35** | .87** | .15* | .26** | -.18** | -.19** | -- | | |
| 16. *AHRR* methylation | .03 | .12** | .11 | -.06 | .00 | .01 | -.04 | .09 | .00 | -.07 | -.04 | -.01 | .00 | .09 | -.09 | -- | |
| 17. *COMT* methylation | -.20* | .07 | .02 | -.01 | .02 | .01 | .10 | .09 | .06 | .08 | .01 | .07 | -.03 | .07 | .10 | .38** | -- |
| 18. *NR2B* methylation | -.01 | .01 | -.27** | -.00 | -.03 | .03 | -.06 | -.11 | .06 | -.06 | .03 | .03 | .20** | .09 | -.09 | -.43** | -.29** |

*Note.* *$p < .05$, **$p < .01$. W1 – Wave 1; W2 - Wave 2; W3 - Wave 3; W4 - Wave 4; $N = 239$ to 290.

Table 3

*Hierarchical Regression Models Predicting AHRR, COMT, and NR2B Methylation from Covariates and Substance Use*

| | **AHRR** | | **COMT** | | **NR2B** | |
|---|---|---|---|---|---|---|
| Step 1 | β | SE | β | SE | β | SE |
| Female | -.08 | .05 | -.24** | .06 | -.08 | .05 |
| African American | .12* | .05 | .05 | .07 | -.07 | .06 |
| Age | .10* | .05 | -.01 | .06 | .15** | .05 |
| Income-to-needs W1 | .01 | .05 | -.07 | .07 | -.06 | .06 |
| Income-to-needs W4 | .07 | .05 | .07 | .06 | -.07 | .05 |
| W3 & W4 smoking history | -.07 | .05 | .12 | .07 | -.01 | .05 |
| CD8T | -1.17** | .21 | -.53+ | .28 | -.66** | .24 |
| CD4T | -.86** | .26 | -.78* | .35 | -.88** | .29 |
| BCELL | -.94** | .23 | -.40 | .30 | -1.18** | .25 |
| MONO | -.16 | .19 | -.04 | .25 | -.61** | .21 |
| GRAN | -2.27** | .65 | -1.41 | .87 | -2.38** | .73 |
| W4 Tobacco Use | .00 | .06 | .05 | .08 | -.01 | .06 |
| W4 Alcohol Use | .08 | .04 | -.00 | .06 | .05 | .05 |
| W4 Marijuana Use | .01 | .05 | -.04 | .06 | -.01 | .05 |
| Step 2a | | | | | | |
| W2 Tobacco Use | -.04 | .04 | .00 | .06 | -.01 | .05 |
| Step 2b | | | | | | |
| W2 Alcohol Use | .03 | .05 | -.00 | .06 | .04 | .05 |
| Step 2c | | | | | | |
| W2 Marijuana Use | .03 | .04 | .01 | .06 | -.01 | .05 |
| Step 2d | | | | | | |
| W3 Tobacco Use | -.08 | .05 | .10 | .08 | -.06 | .06 |
| Step 2e | | | | | | |
| W3 Alcohol Use | .01 | .05 | .02 | .07 | -.08 | .06 |
| Step 2f | | | | | | |
| W3 Marijuana Use | .03 | .05 | .04 | .07 | -.08 | .06 |

*Note.* $^{+}p \leq .06$, $*p < .05$, $**p < .01$. CD8T, CD4T, BCELL, MONO, and GRAN are cell types to control for cell heterogeneity. Smoking history was omitted from tobacco models. W1 – Wave 1; W2 - Wave 2; W3 - Wave 3; W4 - Wave 4; $N = 290$.

Table 4

*Hierarchical Regression Models Predicting AHRR, COMT, and NR2B Methylation from Covariates and Substance Use Combined*

| | **AHRR** | | **COMT** | | **NR2B** | |
|---|---|---|---|---|---|---|
| Step 1 | β | SE | β | β | SE | β |
| Female | -.07 | .05 | -.26** | .06 | -.08 | .05 |
| African American | .13* | .05 | .03 | .07 | -.06 | .06 |
| Age | .09* | .05 | -.00 | .06 | .15** | .05 |
| Income-to-needs W1 | .01 | .05 | -.08 | .07 | -.06 | .05 |
| Income-to-needs W4 | .09 | .05 | .04 | .06 | -.07 | .05 |
| CD8T | -1.19** | .21 | -.50 | .28 | -.67** | .23 |
| CD4T | -.89** | .26` | -.71* | .35 | -.88** | .29 |
| BCELL | -.93** | .23 | -.40 | .31 | -1.18** | .25 |
| MONO | -.18 | .23 | -.02 | .25 | -.61** | .21 |
| GRAN | -2.33** | .65 | -1.30 | .88 | -2.38** | .73 |
| W4 Tobacco Use | -.04 | .05 | .08 | .06 | -.03 | .05 |
| W4 Alcohol Use | .08 | .04 | .00 | .06 | .05 | .05 |
| W4 Marijuana Use | .00 | .04 | -.03 | .06 | -.01 | .05 |
| Step 2a | | | | | | |
| W2 Tobacco Use | -.07 | .05 | .01 | .07 | -.01 | .05 |
| W2 Alcohol Use | .01 | .05 | .01 | .06 | .06 | .05 |
| W2 Marijuana Use | .06 | .05 | .03 | .07 | -.03 | .06 |
| Step 2b | | | | | | |
| W3 Tobacco Use | -.08 | .05 | .08 | .08 | -.05 | .06 |
| W3 Alcohol Use | -.01 | .05 | .03 | .07 | -.09 | .06 |
| W3 Marijuana Use | .03 | .05 | .04 | .07 | -.05 | .06 |

*Note.* $^{+}p \leq .06$, $*p < .05$, $**p < .01$. CD8T, CD4T, BCELL, MONO, and GRAN are cell types to control for cell heterogeneity. W1 – Wave 1; W2 - Wave 2; W3 - Wave 3; W4 - Wave 4; $N = 290$.