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The Mechanisms Underlying the Intergenerational Transmission of Substance Use and Misuse: An Integrated Research Approach

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Abstract

Substance use and substance use disorders run in families. While it has long been recognized that the etiology of substance use behaviors and disorders involves a combination of genetic and environmental factors, two key questions remain largely unanswered: (1) the intergenerational transmission through which these genetic predispositions are passed from parents to children, and (2) the molecular mechanisms linking genetic variants to substance use behaviors and disorders. This article aims to provide a comprehensive conceptual framework and methodological approach for investigating the intergenerational transmission of substance use behaviors and disorders, by integrating genetic nurture analysis, gene expression imputation, and weighted gene co-expression network analysis. We also additionally describe two longitudinal cohorts — the Brisbane Longitudinal Twin Study in Australia and the Lifelines Cohort Study in the Netherlands. By applying the methodological framework to these two unique datasets, our future research will explore the complex interplay between genetic factors, gene expression, and environmental influences on substance use behaviors and disorders across different life stages and populations.

Keywords: Substance use; substance use disorders; genetic nurture; non-transmitted alleles; gene co-expression network

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Substance use (SU) and substance use disorders (SUDs) — including alcohol, nicotine, and cannabis use disorders — run in families (Bierut et al., 1998; Kendler et al., 2023; Merikangas et al., 1998). Offspring of parents with SUDs have an increased risk of SU and SUDs compared to offspring of parents without SUDs (Lieb et al., 2002; McGovern et al., 2023; Mellentin et al., 2016). Both genetic and environmental influences are critical in the intergenerational transmission of SU and SUDs (Kendler et al., 2012a; Rhee et al., 2003; Verhulst et al., 2015). Twin and family studies indicate that genetic factors explain approximately 50% of the phenotypic variance in SUDs (Deak & Johnson, 2021; Kendler et al., 2008). The most salient family environmental risks for SU and SUDs include parental SU, socioeconomic status (SES), parental divorce or death, parental attitudes and monitoring of SU, parental psychopathology, and disrupted family functioning (Barr et al., 2022; Finan et al., 2015).

Parental genetics, both transmitted and non-transmitted to offspring, may contribute to rearing environments, which in turn affect offspring SU. The correlation between the offspring's genome and their family environments complicates the separation of genetic and environmental influences, which have historically been studied independently. Therefore, the mechanisms through which parents contribute to their offspring's risk of SU and SUDs remain unclear. Furthermore, despite advances in genomewide association studies (GWAS) identifying multiple genetic variants associated with SU and SUDs (Hatoum et al., 2023; Johnson et al., 2020; Pasman et al., 2018; Polimanti et al., 2020; Saunders et al., 2022; Zhou et al., 2020), the molecular pathways linking genetic variants to SU and SUDs are still poorly understood.

Here, we provide a comprehensive methodological framework aiming to (1) disentangle directly transmitted parent-to-offspring genetics effects from the nontransmitted genetic effects on SU and SUDs, (2) measure the impact of both transmitted and non-transmitted parental genetics on offspring SU and SUDs via family environments, known as 'genetic nurture', and (3) elucidate the molecular pathways from genes to behaviour by leveraging family-based genomic data and gene expression networks. We note that the current article is primarily theoretical in nature. While we describe epidemiological characteristics of two longitudinal cohorts — the Brisbane Longitudinal Twin Study (BLTS) in Australia and the Lifelines Cohort Study (Lifelines) in the Netherlands — that will be used to apply these methods, we emphasize that this design paper does not include an application to

these data. Instead, our aim is to provide a comprehensive roadmap for our intended empirical investigations.

### Genes to Behaviors: Using Family-Based Genomic Data to Disentangle Intergenerational Transmission of SU and SUDs

In an intergenerational context, risk transmission pathways can be disentangled into (1) genetic transmission, where genetic variants for SUDs are directly passed from parents to children, and (2) genetic nurture, where parental genotypes associated with SUDs affect offspring outcomes indirectly via the rearing environments (Kong et al., 2018).

Several methods have been developed to disentangle direct genetic transmission and genetic nurture, including children of twins, half-sibs, and adoption designs and genotyped parent-offspring designs (Jami et al., 2021; McAdams et al., 2023). Among these, the adoption design works because biological parents do not provide the rearing environments for adoptees, which allows the separation of the biological parents' DNA and the environments that adoptive parents provide (Kendler et al., 2015; Kendler et al., 2016; Kendler et al., 2012b). Swedish adoption studies have shown that parenting styles significantly impact the risk for alcohol use disorder (AUD) in adoptees after adjusting for biological factors (Kendler et al., 2015). However, nonrandom placement, prior placements, placement timing, contact between adopted children and biological parents, and sample representativeness limit the generalization of adoption studies (Blackwood & Muir, 2010; Kendler et al., 2012b). Although these limitations can be addressed, there is no objective means of unconfounding parent-offspring genetics from parental behaviors and environments without measured parental and offspring DNA.

Methods analyzing genotyped mother-offspring pairs resolve the above issue partially by disentangling either maternal or paternal genetics effects from offspring phenotypes but not both simultaneously (Eaves et al., 2014; Jami et al., 2020; Qiao et al., 2020). Our method (Bates et al., 2019; Bates et al., 2018) (Figure 1), also developed by Kong et al. (2018), solves the above problems by relying on genotyped family trios to identify and separate parentally transmitted and nontransmitted alleles (Cordell et al., 2004; Wheeler & Cordell, 2007). The reassembled transmitted and nontransmitted parental genomes are used to construct transmitted and nontransmitted polygenic scores (PGSs, individual-level genetic liability for a trait). A nontransmitted PGS that is associated with offspring outcomes provides evidence of genetic nurture, which is unconfounded by the transmitted parent-to-offspring PGS.

The analysis of nontransmitted alleles has a long history in animal breeding (Walsh & Lynch, 2018) but was only recently leveraged to measure and interpret their effects on complex human traits (Bates et al., 2019; Bates et al., 2018; Kong et al., 2018), and particularly in genetic nurture of educational outcomes and psychiatric disorders (Frach et al., 2024; Martin et al., 2023; Shakeshaft et al., 2023; Tubbs & Sham, 2023; Wang et al., 2021). To our knowledge, genetic nurture studies with respect to SU and SUDs remain limited. One study found that parental PGS for smoking initiation explained unique variance in offspring frequency of tobacco and alcohol use after controlling for offspring's own PGS, providing evidence of genetic nurture (Saunders et al., 2021). A second study found that both transmitted and nontransmitted PGSs for AUD were associated with riskier alcohol outcomes via exposure to parental relationship discord and divorce (Thomas et al., 2023). However, previous studies primarily
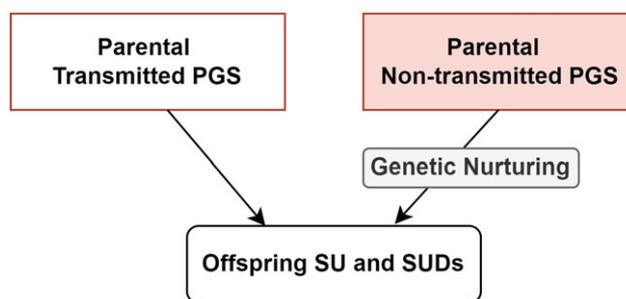
Figure 1. Pathways from parentally transmitted and nontransmitted polygenic risk score (PGS) to offspring substance use (SU) and substance use disorders (SUDs). Note: A significant pathway from nontransmitted PGSs to offspring SU and SUDs is evidence of genetically nurtured environments that are unconfounded by transmitted PGS. The model can be extended to the gene expression imputation data, whereby transmitted and non-transmitted module eigengene are substituted for the transmitted and nontransmitted PGSs, respectively.

require genotyping data for offspring and both parents (parent-offspring trios), which can reduce the sample size and potentially introduce selection bias (Martin et al., 2023). We therefore describe a novel haplotype-based method (see Methods) to differentiate transmitted and nontransmitted genomes in parent-offspring trios and pairs, thereby increasing the sample size and generalizability. This method has been validated and replicated genetic nurture effects on educational attainment in the Lifelines Cohort Study (Trindade Pons et al., 2024).

Genes to Functions: Using Functional Genomics to Identify the Molecular Mechanisms Underlying SUDs

The success of our method depends on well-powered GWAS to calculate PGSs from transmitted and nontransmitted parental genomes. Despite advances in GWAS, we do not fully understand the cascade of biological changes linking genetic variants to SU and SUDs. This can be addressed with gene expression (GE), which plays a critical role in the development of human diseases (Hindorff et al., 2009; Liu, 2011; Schadt et al., 2005). Expression quantitative trait loci (eQTL, Nica & Dermitzakis, 2013) are genetic variants (usually SNPs) that may contribute to disease susceptibility via influencing gene expression, consequently providing a direct link between GWAS and GE studies (Franke & Jansen, 2009; Liu, 2011). eQTL analyses can discern transcriptome adaptations (Bhattacharya & Mariani, 2009; Gu et al., 2002) and reveal the mechanisms by which genetic variants contribute to SU and SUDs (Lehrmann & Freed, 2008), depending on their genomic location (e.g., transcription factor binding sites, splice sites, or other regulatory regions). Since most GWAS variants reside outside protein-coding regions, eQTLs affect cell functions through subtle modification of gene transcription and translation (Shastry, 2009). Assessing eQTLs in linkage disequilibrium with SNPs associated with a trait is crucial to explain the functional significance of GWAS loci. However, most approaches for GE and eQTL analyses are limited to reduced statistical power, higher type I error (Langfelder & Horvath, 2008; Langfelder et al., 2013), and inability to capture genetic interactions underpinning psychiatric disorders (Zhi et al., 2013).

These limitations can be addressed by multivariate network-based methods such as weighted gene co-expression network analysis (WGCNA; Langfelder & Horvath, 2008; Oldham et al., 2008). WGCNA identifies genes driving traits by classifying gene sets into 'network modules' based on their expression and

connectivity patterns. These patterns are summarized in a single quantitative metric, the 'module eigengene' (ME), which can be used to test for associations with SU and SUD. However, WGCNA requires GE data that cannot be easily obtained from brain tissues in large samples. Unlike GWAS studies, which now reach sufficient sample sizes to detect small effects (Levey et al., 2023; Saunders et al., 2022), available GE data from brain tissues, including Gene Tissue Expression (GTEx; Lonsdale et al., 2013), is based on relatively small sample sizes.

In the last decade, GE imputation approaches, such as PrediXcan (Gamazon et al., 2015), summary data-based Mendelian randomization (Gusev et al., 2016), FUSION (Zhu et al., 2016), and our own JPEGMIX2 (Chatzinakos et al., 2018), have been developed to circumvent these limitations and identify trait-GE associations. GE imputation bridges the gap between large GWAS data and underpowered transcriptome studies by integrating genotypes and expression data collected on the same individuals from reference data such as GTEx (Gamazon et al., 2015). This approach builds predictive models to estimate heritable, genetically regulated components of GE, which can be stored as external weights to impute genetically regulated GE in independent samples (Gusev et al., 2016). However, most existing GE imputation methods suffer from low to moderate accuracy, often due to high genomic complexity in certain regions, or the tissue- and cell-type specificity of GE (de Leeuw et al., 2023; Wainberg et al., 2019). To address this, we aim to improve the accuracy of GE imputations and use them to evaluate higher order interactions between imputed expression data at the network level (see Methods). Our approaches enable the imputation of GE in both transmitted and nontransmitted genomes, organizing them into 'imputed gene network' for further analysis of associations with SU and SUDs.

Overall, this article aims to provide a comprehensive conceptual framework and methodological approach for investigating the intergenerational transmission of SU and SUDs, by integrating genetic nurture analyses, GE imputation, and WGCNA. We also additionally describe two longitudinal cohorts — the Brisbane Longitudinal Twin Study in Australia and the Lifelines Cohort Study in the Netherlands. By combining novel methodologies with unique datasets and advanced analytical techniques, we aim to provide new insights into how genetic and environmental factors shape SU, SUDs and associated disease risks across different life stages and populations.

Data Overview

To achieve the above aims, we will use phenotypic and genomic data from BLTS in Australia and Lifelines in the Netherlands. A detailed description of the BLTS SU and SUD phenotypic and genomic data are provided elsewhere (Couvy-Duchesne et al., 2018; Gillespie et al., 2013). Thus, only a brief description is provided below. A broad, general description of Lifelines is also provided elsewhere (Klijs et al., 2015; Scholtens et al., 2015; Sijtsma et al., 2022). Here, we provide a detailed report of all available Lifelines genomic data, SU and SUDs data.

Brisbane Longitudinal Twin Study

The BLTS was launched in 1992 to study melanocytic nevi and comprises over 7000 young adult twins, siblings and parents with longitudinal assessments when twins were aged 12, 14, 16, 21 and 25 years (Couvy-Duchesne et al., 2018; Gillespie et al., 2013; Wright & Martin, 2004). We rely on SU and SUD and

environmental risk data from the '19UP' online self-report survey of $N = 2876$ subjects comprising $n = 2142$ twins and $n = 734$ nontwin siblings (67% female, mean age = 25.9 years, $SD = 3.6$) (Couvy-Duchesne et al., 2018; Gillespie et al., 2013). In addition to demographic, general and mental health items, the survey assessed lifetime alcohol, nicotine and cannabis use, as well as *Diagnostic and Statistical Manual of Mental Disorders-IV* (DSM-IV) and DSM-5 criteria for alcohol and cannabis use disorders (American Psychiatric Association, 1994, 2013), and the Fagerström Test for Nicotine Dependence (FTND; Heatherton et al., 1991). As reported in detail elsewhere, the rates of lifetime alcohol, nicotine and cannabis use were 98.7%, 60.3% and 61.3% for males, 97%, 50.5%, 48.9% for females respectively (Gillespie et al., 2013). In terms of SUDs, the rates of lifetime DSM-IV cannabis abuse, cannabis dependence, alcohol abuse, and alcohol dependence were 17.0%, 9.8%, 40.2% and 35.4% for males, 7.7%, 4.6%, 29.2% and 22.6% for females respectively (Couvy-Duchesne et al., 2018). Such significantly higher rates of SUDs in males have been repeatedly reported in previous studies (Brady & Randall, 1999; Grant et al., 2015; Khan et al., 2013; McHugh et al., 2018). The 19UP study also included self-report indicators of parental and family environments, such as household SES, family functioning (parental marital history, parental and sibling absences and separations, parental involvement, and parental bonding), family and peer group deviance including peer SU, and religious behaviors (Table 1).

Lifelines Cohort Study

Lifelines is an ongoing multidisciplinary prospective population-based cohort study examining in a unique three-generation design the health and health-related behaviors of 167,729 persons living in the north of the Netherlands. The study employs a broad range of investigative procedures to assess biomedical, socio-demographic, behavioral, physical and psychological factors that contribute to the health and disease in the general population, with a special focus on multimorbidity and complex trait genetics. The data for the current project were collected in three waves: baseline (2006–2013), wave 2 (2014–2017) and wave 3 (2019–2023). The design and rationale for Lifelines have been described in detail elsewhere (Scholtens et al., 2015; Sijtsma et al., 2022).

Baseline data were collected from 167,729 participants aged from 6 months to 93 years. During the baseline recruitment period, individuals aged between 25 and 50 years were invited through their general practitioners to participate in the study. All persons who consented to participate were asked to provide contact details and to invite their family members, that is, partners, parents and children, resulting in a three-generation study. In addition, adults were also given the option of registering and participating online in the Lifelines study. The Lifelines website details ongoing research and data collection: https://wiki.lifelines.nl/doku.php.

Overall, among the baseline respondents, 49% of the participants ($n = 81,652$) were invited through their general practitioners, 38% ($n = 64,489$) via participating family members and 13% ($n = 21,588$) self-registered via the Lifelines website (Scholtens et al., 2015; Sijtsma et al., 2022). Participants were then invited for follow-up assessments every 5 years, including laboratory and biometrical assessments, and comprehensive questionnaires. Between assessments, follow-up questionnaires are completed approximately once every 1.5−2.5 years. In the current study, there are 143,595, 89,812 and 57,633 adult participants with available SU or SUD data from baseline, wave 2, and wave 3 respectively. The number of participants at wave 3

Table 1. Measurements of parenting behavior, family environments and substance use outcomes

Parenting and family environments	BLTS	Lifelines
Socioeconomic status		
SES (household)	×	×
Educational attainment (parents and offspring)	×	×
Employment history/status (parents and offspring)	×	×
Urban and rural parental home	×	×
Family functioning		
Parental marital history	×	×
Parental absence[a]	×	×
Parental involvement and availability [b]	×	×
Number of siblings	×	×
Parental bonding	×	×
Family and peer deviance		
Cotwin, sibling and parental history of drug use	×	×
Peer group deviance	×	–
Behavioral and emotional regulation		
adolescent behavioral and emotional regulation	–	×
Religious behaviors		
Religious upbringing (offspring's childhood)	×	–
Church attendance (offspring's childhood)	×	–
SU and SUD outcomes		
Nicotine		
Lifetime use, age at initiation or age at regular use	×	×
Frequency currently/when using the most	×	×
FTND criteria	×	×
Alcohol		
Lifetime use, age at initiation or age at regular use	×	×
Frequency in the past year	–	×
DSM-IV abuse/dependence or DSM-5 AUD criteria	×	×
Cannabis		
Lifetime use, age at initiation or age at regular use	×	×
Frequency in the past year /when using the most	×	×
DSM-IV abuse/dependence or DSM-5 CUD criteria	×	–

Note: [a]Due to divorce, separation, death, placed in foster care, or left early to live alone. [b]Includes step and adoptive parents.

will increase as the entire data release since summer of 2024. We note that some variables of interest have a considerable attrition rate, due to nonresponse in (one of) the follow-up assessments, withdrawal of participation or mortality (Sijtsma et al., 2022), which is often the case in large general population cohort studies.

Substance Use and Substance Use Disorders

In Lifelines, SU and SUDs were measured by self-reported questionnaires. Tobacco and alcohol use were measured across all three waves. Cannabis use were measured at wave 2 and 3. SUDs were measured at wave 3.

Substance Use

Tobacco use includes measures of smoking status (e.g., never/current/former smokers) and amount of tobacco consumed (Du et al., 2022). Cigarettes per day was defined as the average number of cigarettes smoked per day, either as a current or former smoker. Pack-years of smoking were calculated by multiplying the amount smoked per day (of the different tobacco products, including cigarettes/roll-ups, cigarillos, cigars, grams of pipe tobacco) by the number of years the person has smoked (e.g., 1 pack = 20 cigarettes). Alcohol use was assessed as part of a food frequency questionnaire (FFQ) developed by Wageningen University (Brouwer-Brolsma et al., 2022). Two questions referred to the frequency and quantity of alcohol consumed in the past month: 'How often did you drink alcoholic drinks in the past month?' (ranging from *Not in the last month* to *6–7 days per week*), and 'On days that you drank alcohol, how many glasses did you drink on average?' (from *1* to *12 or more*). These questions were split up for different kinds of alcoholic beverages (beer, alcohol-free beer, red wine/rose, white wine, sherry, distilled wine, other alcoholic beverages). Based on these questions, an average daily alcohol consumption score (gram per day) was calculated (Mangot-Sala et al., 2021).

Lifetime cannabis use were defined using two questions: (1) 'Have you ever used drugs? (Yes/No/I prefer not to answer that)', and if yes, (2) 'Have you ever used cannabis, such as weed, marijuana, hashish? (Yes/No)'. The answer categories were recoded to ever (1) versus never (0) used cannabis. Frequency of cannabis use was assessed using the questions, 'How often did you use cannabis in your entire life or in the past 12 months?' (from *0* to *40 times or more*).

Detailed sample characteristics are presented in Table 2. At baseline, the mean age of 143,595 adult participants was 44.5 years (*SD* = 12.8, range = 18−93) and 58.5% of the sample were women. The prevalence of ever smoking at baseline (52.1% for females, 56.6% for males) in Lifelines is higher than the prevalence of ever smoking (22.4% for females, 22.8% for males) in the Netherlands Twin Register study (NTR) based on young adult twins aged 18−25 years (Vink & Boomsma, 2011), but falls within the range of the general Dutch population based on the Dutch Central Bureau of Statistics (CBS, 2014). According to CBS, the prevalence of ever smoking among males was 31.6% under 23 years old, 55.4% in the 25−44 years group, 65.2% in the 45–64 years group and 79.1% in the ≥65 years group. Among females, this was respectively 26.1%, 47.8%, 60.8% and 52.2%.

At wave 2, the mean age of 89,812 adult participants was 50.3 years (*SD* = 13.0, range = 18−96). The 11.3% prevalence of lifetime cannabis use in Lifelines (9.5% for females, 13.8% for males) is comparable to the 12.3% in a general population sample aged 18−64 years from Netherlands Mental Health Survey and Incidence Studies (NEMESIS, 8.3% for females, 16.1% for males; Vega et al., 2002), which is a psychiatric epidemiological cohort study based on random sampling of individuals from the Dutch population register (Basisregistratie Personen). However, our estimate of lifetime cannabis use is lower than the 26.9% among subjects aged 18 to 65 years from the NTR (24.7% and 36.2% for females and males aged 21−40 years; Stringer et al., 2016; Vink et al., 2010). This may be due to differences in the sample characteristics, age ranges, regional differences in cannabis use, and different assessments for lifetime cannabis use. For example, in Lifelines, participants were asked (1) if they ever used drugs, and if yes, did they (2) ever use cannabis (weed, marijuana, hashish) in

Table 2. Descriptive characteristics of lifelines participants

Mean ± SD or n (%)	Baseline			Wave 2			Wave 3[a]		
Sex	Total	Female	Male	Total	Female	Male	Total	Female	Male
	143,595	84,003 (58.5)	59,592 (41.5)	89,812	53,120 (59.1)	36,692 (40.9)	57,633	34119 (59.2)	23514 (40.8)
Age (years)	44.5 ± 12.8	44.1 ± 12.7	45.2 ± 12.8	50.3 ± 13.0	49.7 ± 13.0	51.2 ± 13.1	56.1 ± 12.4	55.3 ± 12.3	57.3 ± 12.4
Nicotine use									
Smoking status									
Never smoker	66,019 (46.0)	40,208 (47.9)	25,811 (43.4)	41,206 (45.9)	25,523 (48.1)	15,683 (42.7)	25,785 (44.8)	15,662 (46.0)	10,123 (43.1)
Ever smoker	77,373 (54.0)	43,669 (52.1)	33,704 (56.6)	48,601 (54.1)	27,592 (51.9)	21,009 (57.3)	31,848 (55.2)	18,457 (54.0)	13,391 (56.9)
Current smoker	29,858 (20.8)	16,325 (19.5)	13,533 (22.7)	13,635 (15.2)	7515 (14.1)	6120 (16.7)	–	–	–
Former smoker	47,515 (33.1)	27,344 (32.6)	20,171 (33.9)	34,966 (38.9)	20,077 (37.8)	14,889 (40.6)	–	–	–
Start age (years)	16.5 ± 3.6	16.3 ± 3.5	16.7 ± 3.8	16.7 ± 4.04	16.5 ± 3.8	16.9 ± 4.4	16.6 ± 3.9	16.5 ± 3.6	16.8 ± 4.1
Packyears	11.6 ± 10.8	10.2 ± 9.7	13.3 ± 11.9	12.1 ± 11.3	10.7 ± 10.2	14.1 ± 12.4	12.2 ± 11.3	10.9 ± 10.6	14.2 ± 12.0
Cigarette per day	11.2 ± 6.6	10.4 ± 6.1	12.6 ± 6.9	10.9 ± 6.5	10.0 ± 6.0	12.0 ± 7.0	10.8 ± 6.6	10.0 ± 6.1	12.0 ± 7.0
Alcohol use									
Daily alcohol intake	6.8 ± 8.7	4.6 ± 6.4	9.9 ± 10.5	–	–	–	–	–	–
Drinking frequency									
Not this month	29,119 (20.3)	23102 (27.6)	6017 (10.1)	15,191 (17.0)	11,749 (22.2)	3442 (9.4)	12,260 (21.4)	9142 (26.9)	3118 (13.3)
1 day a month	9034 (6.3)	6590 (7.9)	2444 (4.1)	6651 (7.4)	4854 (9.2)	1797 (4.9)	3514 (6.1)	2576 (7.6)	938 (4.0)
2–3 days a month	21,663 (13.2)	12,551 (15.0)	6355 (10.7)	12,755 (14.2)	8587 (16.2)	4168 (11.4)	7911 (13.8)	5275 (15.5)	2636 (11.2)
1 day a week	21,704 (15.1)	12,115 (14.5)	9548 (16.1)	12,787 (14.3)	7172 (13.5)	5615 (15.3)	8158 (14.2)	4573 (13.5)	3585 (15.3)
2–3 days a week	34,222 (23.9)	15,666 (18.7)	1556 (31.2)	22,320 (24.9)	11,093 (20.9)	11,227 (30.6)	15,677 (27.3)	7769 (22.9)	7908 (33.7)
4–5 days a week	14,732 (10.3)	6825 (8.1)	7907 (13.3)	9645 (10.8)	4695 (8.9)	4950 (13.5)	5104 (8.9)	2472 (7.3)	2632 (11.2)
6–7 days a week	15,582 (10.9)	6969 (8.3)	8613 (14.5)	10,264 (11.5)	4826 (9.1)	5438 (14.8)	4787 (8.3)	2163 (6.4)	2624 (11.2)
Drinking quantity	2.7 ± 1.8	2.2 ± 1.3	3.3 ± 2.2	2.46 ± 1.61	2.07 ± 1.18	2.94 ± 1.91	2.28 ± 1.49	1.95 ± 1.11	2.70 ± 1.78
Cannabis use[b]	–	–	–						
Lifetime use, yes				10,038 (11.3)	5030 (9.5)	5008 (13.8)		–	–
Lifetime frequency								–	–
0 time				79,030 (88.7)	47,691 (90.4)	31,339 (86.2)			
1 time				628 (0.7)	425 (0.8)	203 (0.6)			
2–3 times				1620 (1.8)	998 (1.9)	622 (1.7)			
4–5 times				1255 (1.4)	702 (1.3)	553 (1.5)			
6–10 times				1367 (1.5)	748 (1.4)	619 (1.7)			
11–19 times				1107 (1.2)	558 (1.1)	549 (1.5)			
20–39 times				1185 (1.3)	574 (1.1)	611 (1.7)			

(Continued)

Table 2. (*Continued*)

Mean ± SD or *n* (%)	Baseline			Wave 2			Wave 3[a]		
Sex	Total	Female	Male	Total	Female	Male	Total	Female	Male
40 times or more				2938 (3.3)	1078 (2.0)	1860 (5.1)			
Past year frequency	–	–	–				–	–	–
0 time				87,067 (97.6)	51,860 (98.2)	35,207 (96.8)			
1 time				526 (0.6)	263 (0.5)	263 (0.7)			
2–3 times				484 (0.5)	230 (0.4)	254 (0.7)			
4–5 times				232 (0.3)	105 (0.2)	127 (0.3)			
6–10 times				175 (0.2)	77 (0.1)	98 (0.3)			
11–19 times				122 (0.1)	57 (0.1)	65 (0.2)			
20–39 times				141 (0.2)	50 (0.1)	91 (0.3)			
40 times or more				430 (0.5)	153 (0.3)	277 (0.8)			
SUDs[c]	–	–	–	–	–	–			
FTND							2.2 ± 2.2	2.1 ± 2.2	2.4 ± 2.2
Lifetime DSM-5 AUD[d]							8015 (14.7)	2873 (9.1)	5142 (22.6)
Diagnosis, yes									
Severity									
Non-AUD							35,923(66.1)	21,184 (67.1)	14,739 (64.7)
mild							5973 (11.0)	2175 (6.9)	3798 (16.7)
moderate							1465 (2.7)	503 (1.6)	962 (4.2)
severe							548 (1.0)	184 (0.6)	364 (1.6)

Note: *SD*, standard deviation. 1 packyear: using 20 cigarettes per day for 1 year, or using 1 cigarette per day for 20 years; Daily alcohol intake: grams/day; Drinking quantity: glasses/day. FTND: Fagerström Test for Nicotine Dependence. AUD: Alcohol Use Disorder.
[a]The number of participants at wave 3 will increase as the entire data release since summer of 2024.
[b]Data on cannabis use were assessed since the second assessment,
[c]Only the third assessment;
[d]The prevalence of DSM-5 AUD was based on 54,369 participants who reported ever drink alcohol and had DSM-5 AUD data available.

their entire life. The prevalence of lifetime cannabis use is likely underestimated in Lifelines due to underreporting if a participant did not classify cannabis as drugs. In NTR, the participants were asked what age they initiated cannabis use with answer categories: (1) 11 years and younger, (2) 12−13, (3) 14−15, (4) 16−17, (5) 18 years or older and (6) never. The answer categories were recoded to *Ever* (1) versus *Never* (0) used cannabis.

Substance Use Disorders

Alcohol and nicotine use disorders data were collected from self-reported questionnaires at wave 3. Alcohol use disorder (AUD) was assessed using the 11 diagnostic criteria from the DSM-5 (American Psychiatric Association, 2013). Lifetime DSM-5 AUD diagnosis required endorsing a minimum of two criteria in the 12 months preceding the questionnaire or previously. Consistent with DSM-5 criteria, lifetime AUD severity levels were categorized as non-AUD (<2 criteria), mild (2−3 criteria), moderate (4−5 criteria), or severe (≥6 criteria).

Nicotine dependence was assessed using the Fagerström Test for Nicotine Dependence (FTND; Heatherton et al., 1991). The FTND includes six items to evaluate the quantity of cigarette consumption, the compulsion to use and nicotine dependence; for instance, 'How soon after you woke up did you smoke your first cigarette?' The scores were summed to yield a total FTND score of 0−10, the higher the score, the stronger the nicotine dependence.

Finally, the 14.7% prevalence of lifetime DSM-5 AUD in Lifelines (9.1% for females, 22.6% for males) is comparable to the 12.8% in subjects aged 18−75 years from the NEMESIS (7.9% for females, 17.8% for males; Ten Have et al., 2023).

Genomic Data in Lifelines

Genotyping and Imputation

Lifelines participants were genotyped with the Illumina CytoSNP-12v2 array ($n = 15,400$), Infinium Global Screening Array® (GSA) MultiEthnic Disease Version 1.0 ($n = 36,339$) and FinnGen Thermo Fisher Axiom® custom array ($n = 28,249$) respectively. Quality control (QC) of marker and samples were performed separately per batch. The detailed pre-imputation QC criteria is described in the Lifelines wiki (http://wiki-lifelines.web.rug.nl/).

In brief, duplicated and monomorphic markers, markers with a low call rate, low minor allele frequency, or variants that deviated significantly from Hardy-Weinberg equilibrium were removed. Samples with a low call rate, heterozygosity outliers or were identified as mix-ups were filtered out. After QC, data from each array was imputed through the Sanger Imputation Service utilizing the Haplotype Reference Consortium panel. We selected a standardized collection of imputed markers with imputation quality scores equal to or above 0.8 across all batches. To minimize the impact of population stratification, we limited the samples to those of European ancestry, determined through principal component analysis with the 1000 Genomes reference. In cases of samples that were genotyped in multiple batches (duplicates), data from the latest batch was used.

For this project, samples included all genotyped offspring who had at least one parent genotyped. There were a total of 19,281 offspring available for analysis. This included 3217 complete trios and 16,010 duos with one genotyped parent. Since Lifelines aims to genotype all participants, these numbers are expected to increase over time.

Methods

Nontransmitted Alleles Inference

Unlike the previously applied pseudo-control method (Bates et al., 2018), which performs a marker-by-marker comparison to create non-transmitted parental genomes using genotyping data from offspring and both parents (parent-offspring trios), we will use a haplotype-based approach to differentiate transmitted and non-transmitted genomes in parent-offspring trios and pairs (Trindade Pons et al., 2024). Briefly, offspring's haplotypes were compared to the two (parent-offspring pairs) or four (parent-offspring trios) parental haplotypes using tiles of every 150 adjacent markers in a chromosome. Tile size of 150 markers was optimal for our genotyping data, given the SNP density of the genotyping array. Each tile overlapped by 50 markers with neighboring tiles on both sides, which allowed us to account for potential crossing-over events. These tiles were used to trace the best matching parental haplotype across all the tiles. For each tile, if the overall match between the offspring's haplotype and the best matching parental haplotype is less than 99.8%, the method assumes that there could be crossing-over and checks the match with the other haplotype from the same parent. For each available parent, the best match between parent and offspring tiles was used to determine which two parental tiles were transmitted to the offspring. After determining the transmitted tiles, the remaining parental tiles were stored in a separate data as non-transmitted alleles. For parent-offspring pairs, the nontransmitted alleles of the ungenotyped parent were set as missing (Trindade Pons et al., 2024). Once the transmitted and nontransmitted alleles are determined, they can be used not only to calculate separate PGSs but also for GE imputation. This allows us to generate neuronally derived GE networks for both transmitted and non-transmitted gene co-expression networks, which can then be tested for their impact on SU and SUDs.

Nontransmitted Gene Co-Expression Networks

Once identified and assembled into parental transmitted and nontransmitted genotypes, they can be used to impute GE. GE imputation will be performed using the PrediXcan software (Gamazon et al., 2015). To increase the accuracy of GE imputation, we developed a stringent imputation pipeline. Considering the much greater variability of gene expression, it is expected that there will be greater variability in the successful GE imputation, as compared to SNP imputation. In our GE imputation pipeline, the correlation is one of the many steps to ensure the reliability of accurate GE imputation. Furthermore, as we focus on the network interactions between the imputed genes, that is, carrying the cumulative signal across all genes in the network, we inherently have a greater tolerance to retain the potentially useful information from all genes. Specifically, the pipeline consists of the following steps: (1) the removal of all SNPs with NAs from our own genotype data; (2) ensuring genotype annotation is identical between the SQLite file and our own genotypes; (3) the use of SQLite weights from the GTEx final data release (Version 8, August 2019) to predict GE in our sample; (4) using regression models (with observed gene expression as the outcome and predicted gene expression as the predictor of interest) to filter out genes with low prediction accuracy across all subjects, that is, genes with predicted p values $\geq .05$; (5) the agreement between imputed and actual GE via Pearson correlation ($r \geq .10$); and (6) use the SQLite SNP weight files derived from specific brain regions such as nucleus

accumbens (NAc), putamen, prefrontal cortex (PFC), and amygdala. We chose to focus our GE imputation analysis on these brain regions comprising the mesocorticolimbic system, given their putative roles in SU and addiction (Koob & Volkow, 2016; Volkow et al., 2019). This system is central to reward processing, motivation, and executive function — all key processes implicated in SUDs. The NAc and putamen are critical components of the reward circuitry (Haber & Knutson, 2010; Volkow & Morales, 2015), the PFC is involved in decision-making and impulse control (Dalley & Robbins, 2017; Dixon et al., 2017), and the amygdala plays a role in emotion processing and drug-associated memories (Everitt & Robbins, 2016; Koob & Volkow, 2016). Numerous neuroimaging and postmortem studies have demonstrated altered structure and function in these regions in individuals with SUDs (Koob & Volkow, 2016; Volkow et al., 2016). By focusing on these specific areas, our aim is to capture gene expression patterns most relevant to the neurobiology of SU and addiction. With the GTEx final data release Version 8, we will substantially increase our ability to reliably impute gene expression, that is, from 3115 genes to 10,000−14,000 genes per brain region.

Regardless, to mitigate the limitations associated with GE imputation, we will implement several strategies: (1) applying stringent statistical thresholds with conservative multiple testing corrections, (2) interpreting our results conservatively and emphasizing the need for experimental validation, (3) conducting sensitivity analyses to assess the robustness of our findings to different methodological choices. By adopting this approach, we aim to provide a balanced and reliable interpretation of our results. We emphasize that while our findings may be consistent with causal relationships, they should be considered as generating hypotheses that would require replication across independent datasets and further experimental validation. These additional studies would aim to provide stronger evidence supporting or falsifying these hypotheses, rather than providing definitive proof of causality.

Following imputation, WGCNA will be performed on both transmitted and nontransmitted GE to identify co-expression networks and their corresponding MEs significantly correlated with SU and SUDs (Langfelder & Horvath, 2008; Langfelder et al., 2013; Zhang & Horvath, 2005). As mentioned above, WGCNA identifies higher order interactions between genes by assembling the imputed transcriptomes into 'imputed network modules'. Each network module in WGCNA is represented by its ME, which is a quantitative summary of the correlated expression and connectedness across all genes in a module. WGCNA can be applied to identify gene networks associated with any phenotype including sex, environment, and so forth. Here, our intention will be to focus only on gene networks correlated with each SU and SUDs while controlling for covariates such as sex and age. Post-hoc analysis will be used to identify sex-specific GE networks, followed by tests of network preservation. From our previous work (Vornholt et al., 2020, 2021), we expect to generate ~20 network modules per SU and SUD outcome with varying degrees of correlation, represented by their respective Pearson correlations and *p* values between SU and SUD outcomes and network modules. The validity of such identified module-trait associations will be further validated using the bootstrapped WGCNA approach (Gandal et al., 2018). The *p* values will be further corrected via Bonferroni, and modules significantly correlated with covariates excluded (Ponomarev et al., 2012).

Discussion

Our study advances research on SU and SUDs through several key innovations. First, we provide a methodological framework that integrates genetic nurture analysis, GE imputation, and WGCNA. This comprehensive approach will help unravel the complex interplay of genetic and environmental factors in SU and SUDs, potentially leading to more targeted interventions. Second, we leverage two unique datasets: the Brisbane 19UP Study from Australia, which offers rich phenotypic data on young adults during a critical developmental period for SU and SUDs; and the Lifelines Cohort from the Netherlands, as a large multigenerational study ideal for examining genetic and environmental factors across different life stages and family structures. These geographically and culturally distinct cohorts enable cross-population comparisons and enhance the generalizability of our findings. Third, our study design allows for meta-analyses, increasing statistical power to detect genetic effects. By incorporating detailed environmental data, we can examine gene-environment interactions across different life stages. Additionally, we integrate multi-omics data, including transcriptomic and epigenomic information, offering a multi-dimensional view of the biological processes underlying SU and SUDs.

Future Directions

Our future research will focus on SU and SUDs, utilizing a methodological framework that integrates genetic nurture analyses, GE imputation, and WGCNA. This approach, while tailored to SU and SUDs, has broad applicability across a wide range of complex traits and outcomes, including cardiovascular diseases, metabolic disorders, other psychiatric conditions, and non-medical traits such as educational attainment or personality.

We will apply three main methodological approaches across the Lifelines and Brisbane 19UP datasets. (1) Genetic nurture analyses: This will compare parental influence effects between the Netherlands and Australia, exploring how genetic nurture impacts SU, SUDs, and related outcomes across different cultural contexts, family structures, and socioeconomic backgrounds. (2) GE imputation: Using reference panels such as GTEx, we will explore how age-related changes in imputed gene expression patterns correlate with SU, SUDs, and associated health outcomes across the lifespan, particularly in the Lifelines. (3) WGCNA: We will impute GE data from both cohorts to identify co-expressed gene modules associated with SU and SUDs. This will also enable us to explore how environmental factors influence the relationship between these modules and health outcomes across different age groups.

By integrating these methods, we can address research questions such as how genetic nurture effects interact with specific gene co-expression network modules that influence SU and SUDs in different populations, and whether or not gene expression profiles mediate the relationship between polygenic risk scores and SU-related outcomes, while considering both direct genetic effects and indirect genetic nurturing effects.

Conclusions

The scientific premise for developing and applying our method to investigate transmitted and nontransmitted GE networks of SU and SUDs is compelling. To our knowledge, we are unaware of any studies using genotyped families for GE imputation to estimate the

impact of transmitted and nontransmitted GE networks, via parental and family environments, on SU and SUDs in offspring. This will provide the first molecular evidence to show how GE networks can 'genetically nurture' the risk of SU and SUDs by helping to foster risky and protective environments. For example, through which environments do these transmitted and non-transmitted GE networks impact on SU and SUDs? Furthermore, we can extend our research questions by deriving transmitted and non-transmitted maternal and paternal genotypes for GE imputation. This will enable us to determine, whether, for example, the paternally transmitted and nontransmitted GE networks are more salient, or whether their impacts vary according to the biological sex of each offspring. We anticipate that our integrated approach will provide a comprehensive understanding of the interplay between genetic factors, gene expression, and environmental influences on SU and SUDs across different life stages and populations. The insights gained from our research may not only advance our understanding of the etiology of SU and SUDs but also inform studies of other complex traits and outcomes.

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