Research Paper

Transcriptomic aging clock analysis identifies key genes in opioid dependence

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Keywords: aging, opioids, GWAS, transcriptomic clock
Received: April 17, 2026 **Accepted:** June 1, 2026 **Published:** July 27, 2026

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ABSTRACT

Opioid dependence is a complex disorder influenced by multiple factors, including aging and genetic factors. However, the underlying molecular mechanisms remain incompletely understood. This *in silico* study integrated transcriptomic profiling, transcriptomic aging clock modeling, and GWAS analysis of brain samples to investigate molecular mechanisms associated with opioid dependence. One-hundred sixty-one DEGs were identified, including 147 upregulated and 14 downregulated genes in individuals with opioid dependence, which are involved in immune and inflammatory pathways (TNF signaling pathway). Network centrality analysis highlighted *CCL2, CD44, THBS1, TIMP1, CD163, IL6*, *IL1B*, and *MYC* as potential hub genes. *FAM174B* was downregulated in older individuals with opioid dependence, whereas *ZNF256* was upregulated in younger individuals. A transcriptomic aging clock showed moderate predictive performance (r=0.686, MAE=5.16) and revealed significant differences in age-prediction residuals between younger and older groups, suggesting altered age-related transcriptional states in opioid dependence. Two genes (*PHYH* and *LUCAT1*) were strongly associated with age-prediction residuals. An analysis of six GWAS studies identified 223 SNP associations, including genome-wide significant variants in *ADGRV1, OPRM1, CNIH3, RGMA, GPRIN3, GAPDHP15, SRP72P1,* and *CTCF-DT* genes implicated in neuronal signaling and opioid pharmacology. Together, these findings highlight immune pathways, neuronal signaling, and aging-related molecular processes as key components of opioid dependence.

INTRODUCTION

Opioid dependence, a brain manifestation caused by chronic opioid abuse, is characterized by a chronic and relapsing condition that is difficult to cure [1]. It remains a major global public health crisis, with more than 60 million people globally reporting misuse of opioids [2]. Remarkably, the misuse of opioid drugs in the United States has increased dramatically over the past two decades (≥9 million people in 2020) [3] and has continued increasing. It is related to a high risk of overdose, infectious diseases, and psychiatric comorbidities [4], contributing substantially to morbidity, mortality, and socioeconomic burden [5].

Despite extensive research, the biological mechanisms underlying opioid dependence remain incompletely understood. Growing evidence suggests that opioid

exposure induces widespread molecular alterations in the brain and peripheral tissues, affecting neuronal signaling (e.g., the brain's reward system), innate and adaptive immunity, and metabolic pathways (e.g., lipid profile and glucose metabolism) [6–8]. Therefore, further investigation of the molecular mechanisms underlying opioid dependence is essential to improve our understanding of its impact on human health and to identify potential therapeutic targets.

Recent advances in transcriptomic technologies have enabled systematic exploration of gene expression changes related to substance use disorders. Bulk RNA sequencing (RNA-seq) and single-nucleus transcriptomic analyses studies have found transcriptional alterations in pathways involved in neuroinflammation, synaptic plasticity, and cellular stress responses in individuals with opioid use disorder (OUD) [9, 10]. Opioid dependence induces gene expression changes across key brain regions (e.g., cortex, amygdala, midbrain, etc.) [11]. These alterations are frequently linked to disruptions in circadian "molecular clock" pathways (e.g., CLOCK/BMAL1) [12, 13]. Chronic opioid exposure has been associated with glial reprogramming, interferon signaling activation, and persistent transcriptional changes during withdrawal and abstinence, including sex-specific effects [14, 15]. Collectively, these findings suggest that opioid-induced transcriptomic remodeling and circadian dysregulation may contribute to long-term neurobiological alterations underlying addiction and related phenotypes [16]. Furthermore, genome-wide association studies (GWAS) have identified genetic variants associated with opioid use disorder and related phenotypes, implicating genes related to neuronal signaling and addiction-related pathways [17]. However, integrating transcriptomic signatures with genetic susceptibility remains an ongoing challenge, and the molecular mechanisms contributing to opioid dependence are not fully characterized.

Aging represents another important factor influencing susceptibility to addiction and drug-related pathologies [18]. Emerging evidence suggests that substance use may accelerate biological aging processes through mechanisms involving inflammation, oxidative stress, and mitochondrial dysfunction [19, 20]. Transcriptomic aging clocks, which estimate biological age based on gene expression patterns, have recently emerged as powerful tools for assessing aging-related molecular changes [21, 22]. These approaches provide an opportunity to evaluate whether opioid dependence is related to accelerated biological aging and to identify genes that may contribute to this process. However, transcriptomic aging clocks specific to OUD remain underdeveloped. To address this gap, we constructed a transcriptomic age clock using elastic net regression trained on control samples and applied it to quantify age acceleration in opioid-dependent individuals.

In this study, we performed an analysis combining RNA-seq transcriptomic profiling, transcriptomic aging clock modeling, and analysis of GWAS to investigate the molecular landscape of opioid dependence. We first identified differentially expressed genes (DEGs) between healthy controls and individuals with opioid dependence, identified the hub genes, and characterized their functional pathways. We then constructed a transcriptomic aging clock to evaluate transcriptomic age-prediction residuals related to opioid dependence and to identify key genes correlated with age-related transcriptional states. Finally, we conducted an analysis of published GWAS to identify genetic variants and candidate genes associated with opioid dependence. Together, these analyses provide a comprehensive view of transcriptional alterations, aging-related signatures, and genetic susceptibility underlying opioid dependence. Because the transcriptomic dataset analyzed in this study was obtained from OUD patients while the GWAS studies reported opioid dependence, we use the term "opioid dependence" throughout this manuscript to refer broadly to opioid addiction–related phenotypes.

RESULTS

Differentially expressed genes between healthy controls and individuals with opioid dependence

To identify gene signatures associated with opioid dependence, we reanalyzed brain bulk RNA-seq data comparing healthy controls and individuals with opioid dependence. We identified 161 DEGs, including 147 upregulated genes and 14 downregulated genes in the opioid dependence group. A volcano plot illustrating the distribution of DEGs and a heatmap of the top 30 most significant genes are shown in Figure 1A, 1B.

To further characterize interaction patterns among DEGs, protein–protein interaction (PPI) network analysis was performed, followed by topological analysis based on degree, betweenness, and closeness centrality. We identified *CCL2, CD44, THBS1, TIMP1, CD163, IL6, IL1B*, and *MYC* as highly connected nodes (hub genes) within the network based on centrality measures.

A significant enrichment in several biological pathways and processes associated with immune and inflammatory responses was found. Notably, pathways including the TNF signaling pathway, inflammatory response, and cytokine binding were significantly

enriched, suggesting that immune and inflammatory mechanisms may contribute to opioid dependence. Disease enrichment analysis further indicated that these genes were associated with respiratory-related diseases, highlighting potential systemic effects of opioid dependence (Figure 1D, 1E).

Age-related gene signatures in opioid dependence

We next examined gene expression differences between younger (18–48 years) and older (49–68 years) individuals with opioid dependence compared with healthy controls. Age groups were defined based on chronological age while maintaining balanced sample distribution and adequate statistical power between groups. The transition at 48–49 years was selected because it approximated the cohort's median age and reflects a midlife stage commonly associated with aging-related molecular and transcriptomic alterations. Healthy controls were initially stratified into younger and older groups based on age; however, they were combined for downstream analyses due to limited sample size and served as a reference group for age-matched comparisons.

To identify age-related genes associated with opioid dependence, we performed differential expression analysis across the three groups (healthy control, younger opioid dependence, and older opioid dependence), focusing on genes showing significant age-dependent differences. Genes were ranked based on statistical significance (adjusted *p*-value) and effect size. The top selected 30 age-related genes potentially involved in opioid dependence are shown in Figure 2A. Among these genes, *FAM174B* (family with sequence similarity 174 member b) expression was significantly reduced in older individuals with opioid dependence (Kruskal-Wallis rank sum test followed by Dunn post hoc test, *p*-adjust = 0.0072), whereas *ZNF256* (zinc finger protein 256) expression was significantly increased in younger individuals with opioid dependence compared with healthy controls (*p*-adjust = 0.0118) (Figure 2B, 2C). FAM174B has been implicated in cellular homeostasis [23], whereas ZNF256 is involved in transcriptional regulation [24], suggesting potential roles in age-related gene expression changes. These findings suggest that age-related transcriptional differences may contribute to the biological heterogeneity of opioid dependence.

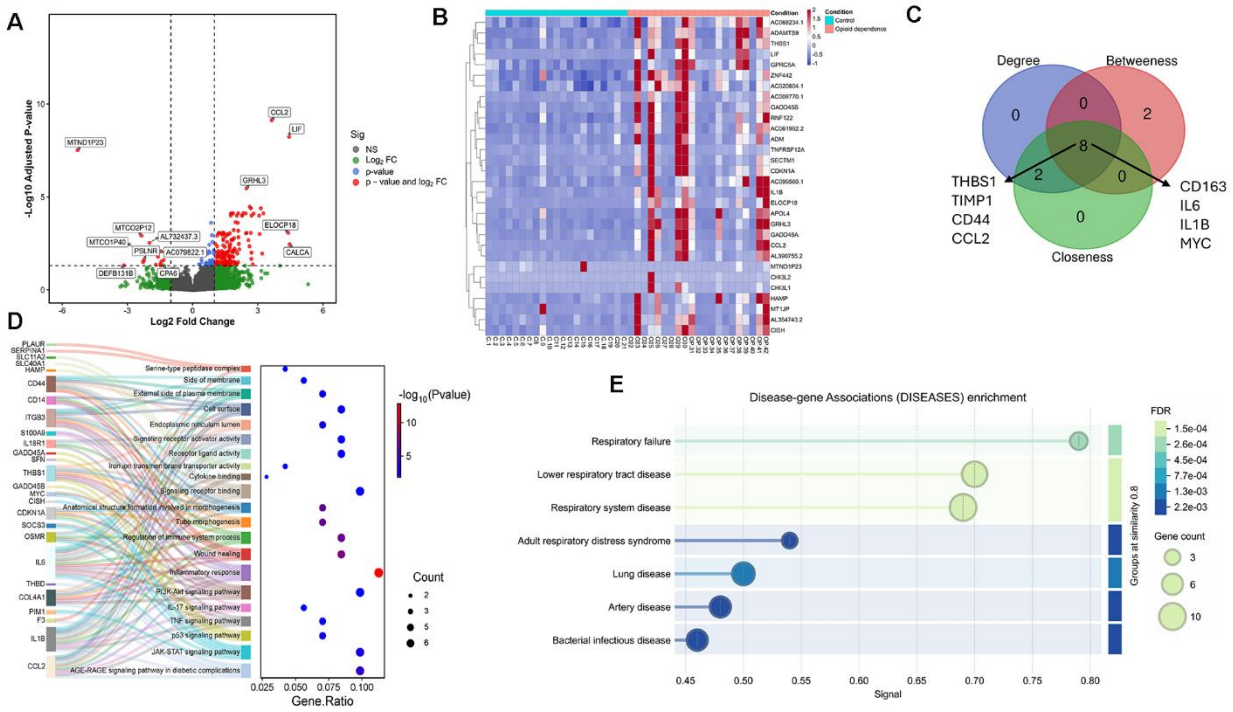

Figure 1. Differential gene expression analysis in opioid dependence. (**A**) Volcano plot showing differentially expressed genes (DEGs) between opioid dependence and controls. Red dots indicate significant genes, and green dots indicate non-significant genes based on the defined threshold. NS: not significant; FC: fold change. (**B**) Heatmap of the top 30 DEGs showing distinct expression patterns between the two groups. Rows represent genes and columns represent samples; colors indicate relative expression levels. (**C**) Venn diagram of hub genes identified using network centrality analysis, including degree, betweenness, and closeness. (**D**) Sankey and dot plots summarizing key findings from enrichment analysis using ToppGene Suite. Dot size represents gene count. (**E**) Bar plot showing the top 7 diseases associated with DEGs based on STRING v12.0 disease enrichment analysis.

Transcriptomic aging clock in younger and older opioid dependence

To evaluate the potential impact of opioid dependence on biological aging, we developed an elastic net–based transcriptomic age-prediction model trained exclusively on 21 healthy controls aged 30 to 68. We found that a moderate positive correlation was observed between predicted age and chronological age [r = 0.686, mean absolute error (MAE) = 5.16 years, Figure 3A and Supplementary Figure 1], indicating moderate predictive performance of the model. We next compared age acceleration between younger and older individuals with opioid dependence and healthy controls. The mean chronological age was 50.2 ± 9.1 years (range: 30–68) in healthy controls (n = 21), 36.3 ± 8.5 years (range: 18–45) in the younger opioid dependence group (n = 10), and 55.7 ± 6.2 years (range: 49–67) in the older opioid dependence group (n = 11). Transcriptomic age-prediction residuals differed significantly among the groups: 0 ± 1 years (range: −2.3 to 2.6) in healthy controls, 14.6 ± 4.9 years (range: 7.7 to 21.5) in the younger opioid dependence group, and −7.2 ± 6.0 years (range: −20.6 to 1.6) in the older opioid dependence group. These differences were statistically significant ($p < 0.0001$; Figure 3B), suggesting that opioid dependence may influence transcriptomic aging-associated signatures. Because the younger opioid dependence group was not fully age-matched with controls and the training cohort had a limited age distribution, these residuals should be interpreted cautiously. The observed differences may partially reflect calibration or extrapolation effects in addition to disease-associated transcriptional alterations, rather than definitive measures of biological aging.

To further investigate the molecular correlates of transcriptomic age-prediction residuals, we identified genes significantly correlated with these residuals. The top 30 age-associated genes with the largest absolute coefficients in the final elastic net model are shown in Figure 3C. To examine genes associated with age-related transcriptional states, Spearman correlations were calculated between each gene expression (logCPM) and transcriptomic age-prediction residuals across all samples. Among these, *PHYH* (phytanoyl-CoA hydroxylase) in younger (r = 0.43) and *LUCAT1* (lung cancer-associated transcript 1) (r = 0.56) exhibited correlations with age-prediction residuals (Figure 3D). Specifically, *PHYH* was downregulated in older individuals with opioid dependence compared to younger opioid dependence (*p-value* = 0.072), whereas

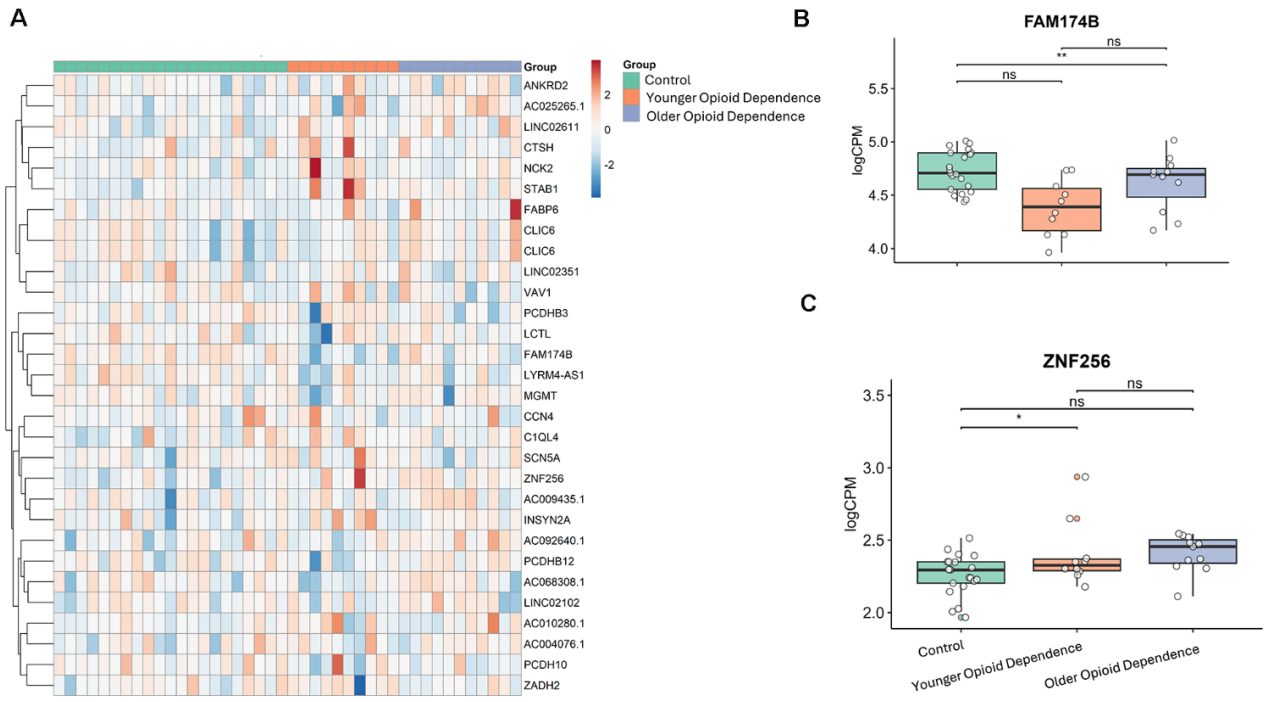

Figure 2. Differential gene expression analysis in younger and older individuals with opioid dependence. (**A**) Heatmap of the top 30 differentially expressed genes (DEGs) showing expression patterns across younger and older individuals with opioid dependence and healthy controls. (**B**, **C**) Boxplots showing the expression of the representative genes FAM174B (**B**) and ZNF256 (**C**) across the three groups. Statistical significance was assessed using the Kruskal–Wallis test followed by Dunn's post hoc test with false discovery rate (FDR) correction. $p < 0.05$; ns, not significant.

LUCAT1 was upregulated compared to older opioid dependence (*p-value* = 0.032) and controls (*p-value* = 0.052) (Figure 3E).

GWAS analysis

To further investigate the genetic factors underlying opioid dependence and to strengthen our transcriptomic findings, we performed a comparative analysis of previously published GWAS. Six independent GWAS of opioid dependence met the predefined inclusion criteria and were included in the analysis (Table 1). The studies were published between 2015 and 2022 and included a total of 362,176 participants, comprising 27,024 cases and 334,972 controls from six case–control studies (Supplementary Table 1).

After quality-control filtering, 223 unique single nucleotide polymorphism (SNP) associations were identified across the genome (Figure 4A). Of these, 13 SNPs (5.8%) reached genome-wide significance ($p < 5 \times 10^{-8}$), while 200 SNPs (89.7%) met the suggestive threshold ($p < 1 \times 10^{-5}$). The most significant association was observed for rs2366929, an intronic variant within the *ADGRV1* gene on chromosome 5 ($p = 2 \times 10^{-9}$, $-\log_{10}(p) = 8.7$, OR = 0.11). This SNP has been shown as a novel locus for opioid dependence [25]. *ADGRV1* (adhesion g protein-coupled receptor v1) has been previously implicated in neurological function and represents a compelling biological candidate for addiction susceptibility [25].

The second most significant variant was rs9478500 located in OPRM1 on chromosome 6 ($p = 3 \times 10^{-9}$). *OPRM1* encodes the mu-opioid receptor, the primary molecular target of opioid drugs and a well-established gene in opioid pharmacogenetics. Additional genome-wide significant loci were identified in *CNIH3, RGMA, GPRIN3, GAPDHP15, SRP72P1*, and *CTCF-DT* (Table 2).

Significant associations were distributed across all autosomes, with the highest density of SNPs observed on chromosomes 5 (22 SNPs), 6 (20 SNPs), and 12 (18 SNPs) (Figure 4B). Genome-wide significant associations ($p < 5 \times 10^{-8}$) were identified on

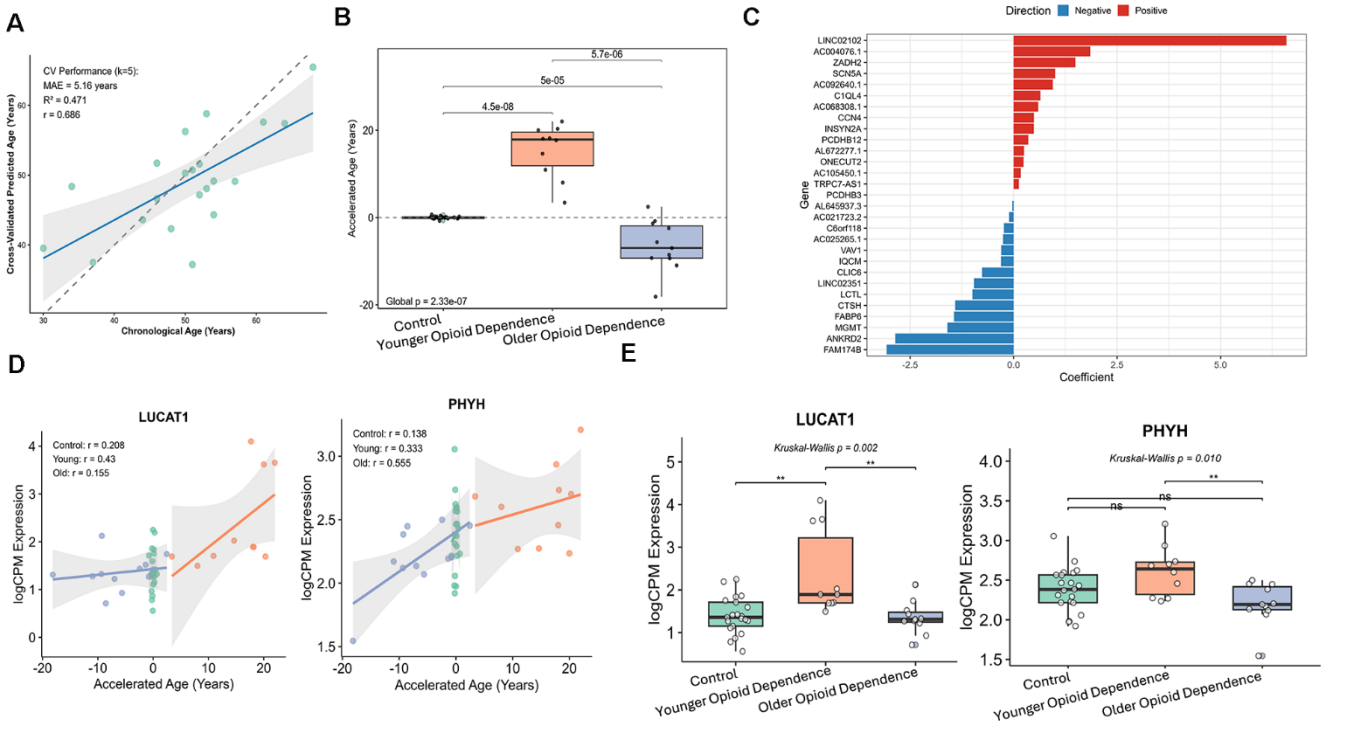

Figure 3. Transcriptomic aging clock analysis in opioid dependence. (**A**) Correlation between predicted transcriptomic age and chronological age using an elastic net regression model. Cross-validation was performed on the control group. CV: cross-validation; MAE: mean absolute error. (**B**) Comparison of age-prediction residuals among healthy controls, younger, and older opioid dependence groups. (**C**) A bar plot of the top 30 genes associated with age-prediction residuals identified by the transcriptomic aging clock. Negative: downregulated; Positive: upregulated. (**D**) Correlation of key genes associated with age-prediction residuals across the three groups. (**E**) Boxplots showing expression of key genes associated with age-prediction residuals across groups. Kruskal–Wallis tests followed by Dunn's post hoc tests with FDR correction. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

Table 1. Characteristics of GWAS cohorts.

PMID	First Author	Year	Cases	Controls	Ancestry	Top SNP	Top Gene
31792237	Yang BZ	2019	2,112/2,603	1,331/2,341	European/African American	rs2366929	ADGRV1
36207451	Gaddis N	2022	7,281/3,856	297,550/12,203	European/African American	rs9478500	OPRM1
26239289	Nelson EC	2015	1,167	161	European	rs10799590	CNIH3
31250797	Brick LA	2019	2,120	1,944	European	rs1970606	GAPDHP15
32099098	Polimanti R	2020	1,207/3,211	6,111/11,589	European/African	rs201123820	SRP72P1
34124712	Sherva R	2021	2,340/1,307	768/974	European/African American	rs4860439	LINC02619

SNP: single nucleotide polymorphisms.

chromosomes 1, 4, 5, 6, 15, 16, and 18. Chromosome 5 harbored the largest number of genome-wide significant SNPs (n = 6), all mapping to the ADGRV1 gene region (Figure 4B and Supplementary Table 2). Annotation of the 223 SNPs revealed that the majority were located in intronic regions, followed by intergenic variants (Figure 1C).

Gene-level analysis identified 15 genes harboring genome-wide significant or suggestive variants (Supplementary Table 3). *ADGRV1* showed the strongest signal, with nine independent SNPs, followed by OPRM1, which contained three significant genome-wide variants. Other notable genes included *RGMA, CNIH3, GPRIN3*, and *GRIN2B*, which are involved in neuronal signaling, synaptic transmission, and neural circuit development, and processes relevant to addiction biology.

Odds ratios for associated variants ranged from 0.06 to 5.55 (median = 0.89), indicating generally modest effect sizes consistent with the polygenic architecture of opioid dependence (Figure 4D). Power analysis demonstrated strong statistical power for detecting

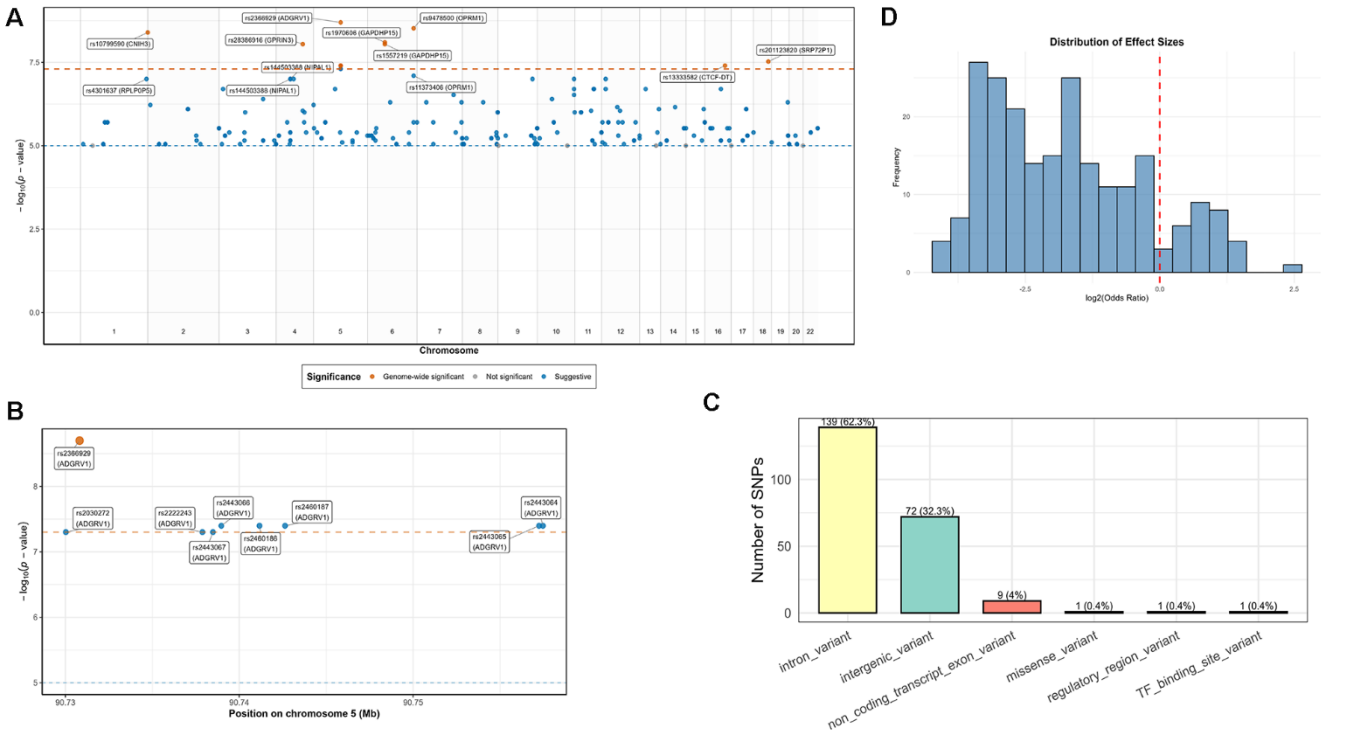

Figure 4. Overview of opioid dependence GWAS analysis. (**A**) Manhattan plot showing $-\log_{10}$(p-values) for all SNPs across chromosomes. The orange dashed line indicates genome-wide significance ($p = 5 \times 10^{-8}$), and the blue dashed line indicates suggestive significance ($p = 1 \times 10^{-5}$). The top 20 SNPs are labeled with gene names. (**B**) Chromosomal distribution of genome-wide significant SNPs (blue) and top SNPs (orange) on chromosome 5. (**C**) Classification of variant contexts across the dataset. (**D**) Distribution of effect sizes ($\log_2$-transformed odds ratios). The red dashed line indicates zero, representing no effect.

Table 2. Genome-wide significant associations ($p<5x\ 10^{-8}$).

Study	SNP	Chr	Position (bp)	Gene	Context	P-value	$-\log_{10}(p)$	Odds ratio
Yang BZ	rs2366929	5	90,730,817	ADGRV1	Intronic	2×10^{-9}	8.7	0.11
Gaddis N	rs9478500	6	154,057,088	OPRM1	Intronic	3×10^{-9}	8.52	1.14
Nelson EC	rs10799590	1	224,634,780	CNIH3	Intronic	4×10^{-9}	8.4	1.56
Brick LA	rs1970606	6	58,042,294	GAPDHP15	Intronic	8×10^{-9}	8.1	0.06
Brick LA	rs1557219	6	58,044,647	GAPDHP15	Intronic	9×10^{-9}	8.05	0.06
Gaddis N	rs28386916	4	89,432,039	GPRIN3	Intronic	9×10^{-9}	8.05	0.17
Polimanti R	rs201123820	18	49,498,978	SRP72P1	Intergenic	3×10^{-8}	7.52	5.55
Yang BZ	rs2443066	5	90,739,000	ADGRV1	Intronic	4×10^{-8}	7.4	0.1
Yang BZ	rs2460186	5	90,741,000	ADGRV1	Intronic	4×10^{-8}	7.4	0.1
Yang BZ	rs2460187	5	90,743,000	ADGRV1	Intronic	4×10^{-8}	7.4	0.1
Yang BZ	rs2443065	5	90,757,000	ADGRV1	Intronic	4×10^{-8}	7.4	0.1
Yang BZ	rs2443064	5	90,757,000	ADGRV1	Intronic	4×10^{-8}	7.4	0.1
Gaddis N	rs13333582	16	67,515,644	CTCF-DT	Intergenic	4×10^{-8}	7.4	0.8

common variant associations. Leave-one out sensitivity analysis of the five most significant SNPs showed that all remained genome-wide significant after sequential removal of each study (Supplementary Table 4), indicating that the results were not driven by any single dataset.

DISCUSSION

Our study identified significant transcriptional alterations and transcriptomic aging-associated signatures associated with opioid dependence, highlighted age-related gene expression differences, and revealed genetic variants linked to addiction susceptibility. These findings provide a perspective on the biological processes involved in opioid dependence.

We found that eight hub genes (*CCL2, CD44, THBS1, TIMP1, CD163, IL6, IL1B*, and *MYC*) are implicated in the pathogenesis of opioid dependence. For example, chemokines and cytokines (*CCL2, IL6*, and *IL1B*) play key roles in neuroinflammation and microglial activation induced by chronic opioid exposure, which can affect neuronal signaling and reward pathways [26]. *CD163* and *CD44* are related to immune cell activation and migration [27, 28], reflecting immune dysregulation associated with opioid dependence. Furthermore, *THBS1* and *TIMP1* regulate extracellular matrix remodeling [29, 30], and *MYC* controls transcriptional and metabolic responses to cellular stress [31]. Our enrichment analysis also highlights immune and inflammatory pathways, including the TNF signaling pathway and cytokine-related processes contributing to the pathophysiology of opioid dependence. We also found opioid dependence to be related to respiratory dysfunction, which is well known as 'opioid-induced respiratory depression', because opioids can depress the central nervous system's respiratory system, causing irregular breathing, shallow, or slow breathing [32]. Together, these findings partly support the role of neuroimmune signaling, inflammatory pathways, and respiratory function in opioid dependence. Importantly, our analysis also revealed age-related transcriptional differences among individuals with opioid dependence. Specifically, expression of *FAM174B* was reduced in older individuals with opioid dependence, whereas *ZNF256* was increased in younger individuals compared with healthy controls. Although the functional roles of these genes in addiction remain poorly understood, these findings suggest that age may influence transcriptional responses to opioid exposure [33]. Age-dependent molecular changes could contribute to heterogeneity in addiction susceptibility, treatment responses, or disease progression [34].

Recent evidence indicates that substance use disorders, including OUD, are associated with accelerated aging-related molecular alterations. More recently, brain-specific epigenetic clocks revealed that OUD accelerates aging through distinct molecular pathways, including transcriptional dysregulation, neuroinflammation, and mitochondrial dysfunction [35]. Notably, these clocks showed that different substances (opioids, alcohol, stimulants) drive aging via unique mechanisms, yet share common pathways such as oxidative stress and immune activation [35]. Parallel transcriptomic studies have identified aging-related gene expression signatures in SUDs, linking the FOS gene family to premature aging [36], and single-nucleus transcriptomics of striatum implicated DNA damage and neurodegeneration in OUD [37]. However, most work has relied on epigenetic clocks, and a trans-

criptomic clock specifically trained for OUD has not been developed. To address this gap, we constructed a transcriptomic age clock using elastic net regression trained exclusively on control samples and applied it to an opioid-dependent cohort to identify age-related transcriptional states and associated genes. We found that transcriptomic age-prediction residuals showed a moderate correlation with chronological age, indicating the model's reliable performance. Importantly, we observed significant differences in age acceleration among groups. Younger individuals with opioid dependence showed positive transcriptomic age-prediction residuals, while older individuals showed negative residuals relative to controls. Rather than representing straightforward acceleration or deceleration of biological aging, these findings may reflect nonlinear transcriptomic remodeling associated with opioid dependence. A key limitation of transcriptomic age-prediction in bulk brain tissue is that it reflects mixed cell populations rather than cell-intrinsic aging. Thus, age-prediction residuals may be influenced by opioid-associated changes in cellular composition, including glial activation, immune infiltration, and neuronal loss. These shifts, together with neuroimmune and extracellular matrix remodeling, can alter global expression profiles and bias transcriptomic age estimates. Therefore, the observed patterns likely reflect cellular composition changes rather than intrinsic cellular aging.

Several factors may contribute to age-related transcriptional states, including disease-associated alterations in brain cellular composition [38], such as shifts in glial cell proportions [39], or selective neuron loss [40]. Similar apparent “age deceleration” has been reported in neurodegenerative conditions, where loss of transcriptionally active neuronal populations leads to an apparent reduction in predicted biological age despite ongoing pathology [41]. Alternatively, chronic opioid exposure may induce adaptive transcriptional reprogramming in older individuals [42], partially normalizing age-associated gene expression patterns. Together, these findings suggest that opioid dependence is associated with age-dependent and nonlinear transcriptomic remodeling rather than uniform effects on biological aging trajectories. More broadly, opioid dependence may involve complex interactions among neuroimmune activation, synaptic plasticity, and adaptive transcriptional responses that differ across the lifespan. Importantly, the younger opioid dependence group was not fully age-matched with controls, which may influence transcriptomic age-prediction residuals. Therefore, the observed bidirectional pattern may reflect not only opioid-associated transcriptional remodeling, but also calibration effects, regression-to-the-mean, and differences in underlying cellular composition across age groups. On the other hand, the nonlinear age-associated pattern observed in this study likely reflects the coordinated influence of multiple biological aging processes rather than a single linear trajectory. In particular, age-related transcriptional changes may involve concurrent activation of inflammatory and immune-response pathways, alongside suppression of homeostatic, metabolic, and vascular maintenance programs. However, the current analysis does not formally deconvolute distinct mechanistic aging axes. Future studies incorporating pathway-level aging frameworks will be required to determine which aging programs are selectively activated or suppressed across cell types.

We also found several key genes correlated strongly with age-related transcriptional states (*PHYH*, *LUCAT1*). *LUCAT1* and *PHYH* are of particular interest due to their known roles in inflammatory regulation and lipid metabolism, respectively—processes closely linked to aging biology [43–45]. Although these genes are not established canonical markers of aging or opioid dependence, their significant association with age-related transcriptional states in our dataset suggests they may represent novel contributors to aging-related molecular alterations in opioid use. Dysregulation of these pathways has been widely implicated in aging-related neurological disorders. Together, these findings raise the possibility that chronic opioid exposure may contribute to altered molecular aging, potentially through mechanisms involving metabolic dysregulation, oxidative stress, and inflammatory signaling. Several hub genes identified in our transcriptomic analysis, including *CCL2, IL6, IL1B*, and *CD163*, are key regulators of neuroinflammatory responses that have also been implicated in aging-related processes [46–48]. The convergence of aging-associated genes and inflammatory hub genes in our analysis suggests that neuroimmune dysregulation may represent an important biological link between opioid dependence and biological brain aging. These results highlight the potential utility of transcriptomic aging clocks for understanding how substance use disorders influence aging-related transcriptional states and for identifying molecular targets to mitigate the long-term neurological consequences of opioid exposure.

In addition to transcriptomic analyses, our GWAS analysis identified genetic variants associated with opioid dependence across multiple independent studies. The most significant association was observed in *ADGRV1*, a gene encoding an adhesion G protein-coupled receptor involved in neuronal signaling. Previous studies have implicated *ADGRV1* in synaptic organization and neuronal communication, suggesting that alterations in this gene may influence neural

circuits relevant to addiction [49, 50]. Another key locus was identified in *OPRM1* (opioid receptor mu 1), which encodes the μ-opioid receptor, the primary pharmacological target of opioid drugs. Variants in *OPRM1* have long been associated with opioid sensitivity, dependence risk, and treatment outcomes, supporting the biological relevance of our findings [51]. Similar to other studies, we also found that variants in *CNIH3* (cornichon homolog-3) are a protective factor against the pathophysiology of opioid dependence [52]. Importantly, we found additional loci that were identified in genes such as *RGMA* (repulsive guidance molecule a) [53], *GPRIN3* (G protein-regulated inducer of neurite outgrowth 3) [54], and *GRIN2B* (glutamate ionotropic NMDA receptor subunit 2b) [55], further highlighting the importance of synaptic signaling and neuronal plasticity in addiction biology.

Limitations

This study integrated multiple complementary approaches, including transcriptomic analysis, transcriptomic age-prediction modeling, and genetic association data, providing a more comprehensive view of opioid dependence than any single method alone, which may contribute to opioid dependence through both regulatory and genetic mechanisms. However, several limitations should be considered. The transcriptomic analysis was based on a relatively modest sample size, which may limit the detection of subtle expression changes. The control cohort was older on average than the younger opioid dependence group, which may introduce bias in transcriptomic age estimation and contribute to extrapolation-related residual effects. In addition, potential confounding factors, including variability in opioid exposure history, polysubstance use, smoking status, comorbid medical or psychiatric conditions, medication use, postmortem interval, and lifestyle-related factors, were not uniformly available across datasets and may have influenced the observed transcriptomic and aging-associated signatures. The RNA-seq dataset was derived from bulk tissue, preventing the identification of cell-type–specific transcriptional alterations. Although we stratified individuals into "younger (18-48)" and "older (49-68)" groups, the overall age range was relatively limited, with the "older" group not representing advanced aging. As a result, the observed age-related effects may not fully capture transcriptional changes associated with more advanced aging, and different patterns might emerge in cohorts with a broader age distribution. Unlike chronological age, biological age is physiological and functional, which is not necessarily chronological. Our transcriptomic aging clock was modeled based on chronological age due to the unavailability of sufficient physiological data in our datasets. Future studies using single-cell transcriptomics or spatial transcriptomic approaches and a reliable model of biological age could provide deeper insights into the cellular mechanisms underlying opioid dependence. Furthermore, the GWAS analysis relied on previously reported associations from published studies rather than raw genotype data, which limited the ability to perform full genome-wide statistical modeling and fine-mapping analyses.

CONCLUSIONS

Our analysis identified key hub genes that may contribute to the neuroimmune and inflammatory mechanisms underlying opioid dependence and aging. Importantly, the application of a transcriptomic aging clock revealed age-related molecular alterations and suggests that opioid dependence may be associated with altered transcriptomic age-prediction patterns in an age-dependent manner. Together, these findings provide new insights into the molecular basis of opioid dependence and highlight candidate genes and aging-related pathways that warrant further investigation.

MATERIALS AND METHODS

Differential expression analysis

In this *in silico* study, the RNA-seq dataset was selected from the Gene Expression Omnibus (GEO) using the following criteria: (i) Human subjects with well-characterized opioid dependence diagnosis; (ii) Availability of raw RNA-seq counts and associated metadata (etc., age and opioid use); (iii) Inclusion of both healthy controls and opioid dependence individuals; (iv) Sample size sufficient for differential expression analysis (≥5 per group); (v) Publicly accessible data in a peer-reviewed dataset. Based on these criteria, GSE194368 was selected, comprising 42 samples (21 controls, 21 individuals with opioid dependence), which were further subdivided by age into younger and older opioid dependence groups.

Raw counts were analyzed in R (v4.5.2) using DESeq2 [56]. Genes with low expression (total counts <10 across all samples) were removed. Differential expression analysis was performed using the standard DESeq2 workflow with Control as the reference group, and p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR). Genes with adjusted $p < 0.05$ and $|\log_2 \text{fold change}| \geq 1$ were considered differentially expressed.

Variance-stabilizing transformed counts were used for visualization, including principal component analysis, heatmaps, and volcano plots. Enrichment analysis of

DEGs was performed using STRING v12.0 and ToppGene Suite, examining KEGG pathways and Gene Ontology categories. Hub genes were identified by using network centrality analysis, including three indicators (degree, betweenness, and closeness) [57]. To construct the protein–protein interaction (PPI) network, the list of candidate genes was uploaded to the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins), and interactions with a confidence score > 0.4 were retrieved. The resulting PPI network was then imported into Cytoscape (version 3.10.4) for further analysis. Within Cytoscape, network topology analysis was performed using the CytoHubba plugin to calculate degree, betweenness, and closeness centrality for each node. Genes with higher centrality values were considered to play more important roles in the network and were defined as hub genes. While such nodes may reflect increased connectivity and potential biological relevance within the network context, hub status is derived from network structure and does not, by itself, establish causal regulatory function. A Venn diagram was subsequently used to identify the overlapping genes among the top-ranked candidates from each centrality metric, and these common genes were selected as the final hub genes.

Transcriptomic aging clock

RNA-seq count data (GSE194368) and corresponding metadata were imported into R (v4.5.2). Counts were converted to integers, and genes with at least 1 count per million (CPM) in ≥5 samples were retained using the edgeR package. Library sizes were normalized with the trimmed mean of M-values (TMM) method, and $\log_2$-transformed CPM (logCPM) values were used for all downstream analyses.

The clock was trained exclusively on the 21 control samples. First, within the control group, the correlation between each gene's logCPM expression and chronological age was calculated using Pearson's correlation. Genes with |correlation| > 0.1 and a false discovery rate (FDR) < 0.3 (Benjamini-Hochberg correction) were selected as age-related features. If fewer than 20 genes met these criteria, the top 100 genes by absolute correlation were used. This set of genes formed the feature space for elastic net regression.

A five-fold cross-validation (CV) was performed on the control samples to evaluate the model and tune hyperparameters. For each fold, the training set was used to optimize the elastic net mixing parameter α (0.1–0.9, step 0.1) via an inner three-fold CV [58], selecting the α that minimized the mean squared error. A model was then trained on the training fold using the chosen α and the λ that minimized the inner-CV error, and predictions were made on the held-out test fold [59]. The cross-validated performance was summarized using mean absolute error (MAE), Pearson's correlation (r), and the coefficient of determination (R^2). After CV, the median α across the five folds was selected. A final elastic net model was trained on all 21 control samples using this α, with λ chosen by five-fold CV on the full control set. Coefficients with non-zero values were retained. The final model was applied to all 42 samples (control and opioid-dependent) to obtain predicted ages. Accelerated age was calculated as predicted age minus chronological age.

Differences in age-related transcriptional states among groups (Control, Younger Opioid Dependence, Older Opioid Dependence) were assessed using a conditional approach: normality of residuals within groups was tested with Shapiro–Wilk, and homogeneity of variances with Bartlett's test. If both assumptions were met, one-way ANOVA was used; otherwise, Kruskal-Wallis was applied. Pairwise comparisons were performed using either t-tests (with pooled SD) or Wilcoxon rank-sum tests, correspondingly, and p-values were not adjusted for multiple comparisons in the graphical output.

The top 30 genes with the largest absolute coefficients in the final elastic net model were selected for visualization. Heatmaps of their scaled logCPM expression were generated with sample annotations for group and age. For the nine most influential genes (by absolute coefficient), boxplots were constructed with Kruskal-Wallis global tests, and pairwise comparisons were evaluated with Dunn's test using Benjamini-Hochberg correction; significance (p-adjust < 0.05) was indicated in the plots. Scatter plots of expression versus chronological age were created for each group, with Pearson correlation coefficients shown.

To identify genes associated with age-related transcriptional states, Spearman correlations were calculated between each gene's logCPM expression and the continuous age-prediction residual variable across all samples. The top six genes (by absolute correlation) were visualized with boxplots (Kruskal-Wallis + Dunn's test with FDR correction) and scatter plots of expression versus accelerated age, stratified by group. Spearman correlation coefficients were reported for each group.

Comparative analysis of publicly available genome-wide association studies

We conducted a comparative analysis of GWAS on opioid dependence by accessing data from the NHGRI-EBI GWAS Catalog (www.ebi.ac.uk/gwas). The search was performed on 03/09/2026 using the terms "opioid

dependence," "heroin dependence," and "opioid use disorder." Studies were included if they met the following criteria: (1) case control study, (2) genome-wide association study design, (3) phenotype of opioid dependence or opioid use disorder, (4) human subjects, (5) published in peer-reviewed journals, and (6) reported summary statistics including effect sizes and p-values (Supplementary Figure 2). For each study, information including SNP identifier, chromosome position, associated gene, p-value, odds ratio, and risk allele frequency was extracted and compiled. SNPs with missing genomic coordinates or p-values were excluded. Genome-wide significance was defined as $p < 5 \times 10^{-8}$, and suggestive significance as $p < 1 \times 10^{-5}$. Chromosomal distribution and genomic context of SNPs were assessed using GWAS Catalog annotations. Effect sizes were summarized using odds ratios (OR), and effect size distributions were evaluated using $\log_2$-transformed OR values. Sensitivity analysis was performed using a leave-one-out approach for the five most significant SNPs to assess the robustness of associations.

Statistical analysis

All analyses were conducted in R (v4.5.2). Packages used in this study included DESeq2, edgeR, limma, glmnet, tidyverse, ggplot2, pheatmap, patchwork, ggpubr, and FSA. Multiple testing correction was performed using the Benjamini–Hochberg FDR method.

Data availability

This study utilized publicly available datasets GSE194368 and EFO_0005611. All data generated or analyzed during this study are included in this article and its Supplementary Tables. Additional processed results, including differential expression outputs, enrichment analyses, aging-clock results, and GWAS summary tables, are available from the corresponding author upon reasonable request.

Code availability

No custom software was developed for this study. All analyses were performed using publicly available bioinformatics tools as described in the Materials and Methods section. Software packages, analytical workflows, and key parameters are reported to ensure reproducibility. The scripts used for transcriptomic age-prediction modeling and GWAS analyses are provided as a Supplementary Code file.

Abbreviations

CMP: count per million; DEGs: differentially expressed genes; FDR: false discovery rate; GEO: Gene Expression Omnibus; GWAS: genome-wide association studies; MAE: mean absolute error; OUD: opioid use disorder; PPI: protein–protein interaction; RNA-seq: RNA sequencing; SNP: Single Nucleotide Polymorphism.

AUTHOR CONTRIBUTIONS

Hai Duc Nguyen: conceptualization, methodology, software, data curation, formal analysis, investigation, writing. Mary Peace McRae: writing. Sangkyu Kim: methodology, writing. Woong-Ki Kim: conceptualization, supervision, writing.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

This work was conducted at the Tulane National Biomedical Research Center (TNBRC RRID: SCR_008167) and supported by the TNBRC P51 Base Grant (P51OD011104) from the National Institutes of Health.

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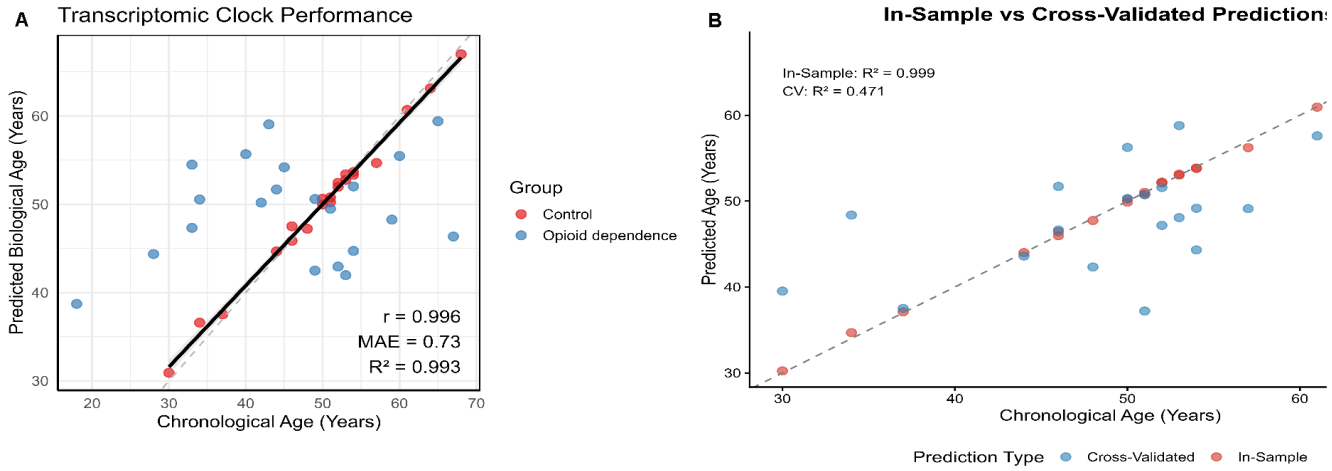
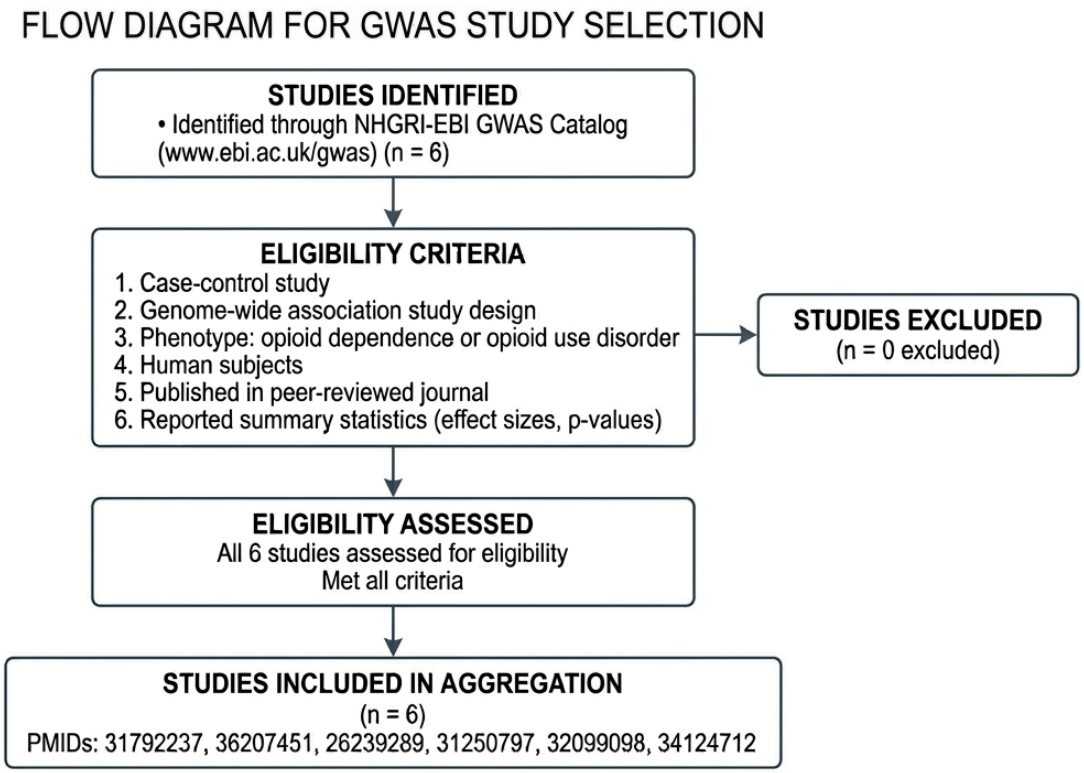
SUPPLEMENTARY MATERIALS

Supplementary Figures



Supplementary Figure 1. Transcriptomic aging clock performance in opioid dependence. (**A**) Correlation between predicted transcriptomic age and chronological age in control and opioid groups using an elastic net regression model without cross-validation. (**B**) Correlation between predicted transcriptomic age and chronological age in the control group (in-sample) and opioid group (out-of-sample or cross-validated) using an elastic net model with 5-fold cross-validation performed on the control samples. CV, cross-validation; MAE, mean absolute error.



Supplementary Figure 2. Flow diagram for GWAS study selection.

Supplementary Tables

Please browse Full Text version to see the data of Supplementary Tables 1–3.

Supplementary Table 1. Data characteristics for GWAS meta-analysis.

Supplementary Table 2. Chromosomal distribution of associations.

Supplementary Table 3. Top associated genes involved in opioid dependence.

Supplementary Table 4. Statistical power analysis from GWAS meta-analysis.

Study	Year	Cases	Controls	Power (OR=1.5, MAF=0.2)
Yang BZ	2019	4715	3672	100.00%
Gaddis N	2022	11137	309753	100.00%
Nelson EC	2015	1167	161	10.70%
Brick LA	2019	2120	1944	97.20%
Polimanti R	2020	4418	17700	100.00%
Sherva R	2021	3647	1742	99.90%
META-ANALYSIS		27204	334972	100.00%

Supplementary Code file

Please browse Full Text version to see the data of Supplementary Code file.