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[Shailesh Panday](#) , [Rachel Lyman](#) , [Vijay Shankar](#) , [Emil Alexov](#) \*

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## Article

# Genetic Variants Linked to Opioid Addiction: A Genome Wide Association Study

Shailesh Kumar Panday <sup>1</sup>, Vijay Shankar <sup>2</sup>, Rachel Ann Lyman <sup>2</sup> and Emil Alexov <sup>1,\*</sup>

<sup>1</sup> Department of Physics and Astronomy, Clemson University, Clemson, SC 29634, USA

<sup>2</sup> Center for Human Genetics, Clemson University, Greenwood, SC 29646, USA

\* Correspondence: ealexov@clemson.edu

**Abstract:** Opioid addiction disorder (OAD) affects millions of people worldwide. While it is known that OAD originates from many factors, including social and environmental factors, the role of genetic variants in developing the disease has also been reported. This study aims to investigate the genetic variants that may be associated with the risk of developing OAD upon exposure. Twenty-three subjects who had previously been given opioid-based painkillers to undergo small surgical treatment were recruited at Prisma Health Upstate clinic and elsewhere. Eleven of them were considered non-persistent opioid users (controls), and 12 of them were persistent opioid users (cases) after an initial surgery and at the time of sample collection. The subjects were asked to provide saliva samples, which were subjected to DNA sequencing at Clemson University Center for Human Genetics, and variant calling was performed. The GWAS for genes known to be associated with OAD resulted in 13 variants (intronic or SNV) with genome-wide significance (raw p-value < 0.01) and two missense variants, rs6265 (Val66Met in BDNF isoform a) and rs1799971 (Asn40Asp) in *OPRM1* previously reported in the literature. Furthermore, extending the GWAS to find all the genomic variants and filtering the variants to include only variants found in cases (persistent opioid users) but not in controls (non-persistent opioid users) resulted in 11 new variants (p-value < 0.005). Considering that OAD is a complex disease and the effect may come from different variants in the same genes, we performed a co-occurrence analysis of variants on the genes and identified four genes, *LRFN3*, *ZMIZ1*, *RYR3*, and *OR1L6* with three or more variants in the case subjects but not in control individuals.

**Keywords:** case-control genome-wide association study; protein-protein interaction network and genetic variants; functional enrichment analysis

## 1. Introduction

Opioid addiction disorder (OAD) affects millions of people worldwide, and it has immense and multifaceted adverse socio-economic impacts affecting individuals, families, communities and economies [1,2]. The impacts involve drug overdose deaths [3], increased healthcare costs [4], low productivity [5], heightened criminal justice involvement [6,7], and broader societal challenges. Human studies consistently show that the heritability of opioid addiction is substantial [8,9]. Twin studies estimate the heritability of opioid use disorder (OAD) to be between 40% and 60% [8,9]. Family studies also suggest that first-degree relatives of individuals with OAD have a higher risk of developing the disorder [10]. Identifying genetic variants and genes linked to elevated risks can be used to develop a better understanding of the biology of OAD. It can be further utilized in screening people for their genetic susceptibility to OAD and to avoid prescription of opioid-based painkillers to limit the risks of developing OAD. The two popular approaches to association studies are (a) Candidate gene studies, which are hypothesis-driven studies that analyze genes with known connections to a phenotype, and (b) Genome-wide association studies (GWAS), which are statistical analyses of polymorphisms across a genome for association studies. Both approaches have been used to study genetic association with OAD in the past [11–26].

Several candidate gene studies aim to identify the genetic variants related to OAD [11–21] have reported several genetic variants on genes. For example, the mu-opioid receptor gene (*OPRM1*), and the variant rs1799971 (A118G), have been associated with differences in opioid effects and addiction risk [14,15,20]. Studies show that individuals with the G allele may experience altered drug effects and

a higher risk for OAD [27]. Likewise, the dopamine receptor D2 gene (*DRD2*) has been implicated in the reward system's role in addiction. Variants like the Taq1A polymorphism are associated with an increased risk of addiction due to their impact on dopamine receptor availability and function [11,12,18,19]. The delta-opioid receptor (DOR), encoded by the *OPRD1* gene, has been investigated in the context of opioid addiction, though it is less studied compared to the mu-opioid receptor [16]. Research indicates that DOR plays a role in modulating mood, anxiety, and stress responses, all of which are factors in addiction vulnerability. Variants in the *OPRD1* gene have been linked to altered responses to opioids, suggesting that DOR could influence individual susceptibility to OAD [16,17]. Brain-derived neurotrophic factor (BDNF) is a neurotrophin critical for brain plasticity, learning, and memory [28]. Alterations in BDNF expression and function have been associated with various neuropsychiatric conditions, including addiction [29]. Studies suggest that BDNF plays a role in the brain's reward system and its response to opioids. Genetic variations in the *BDNF* gene may influence the risk of developing OAD by affecting neural plasticity and resilience to stress [13,19].

A handful of case-control genome-wide association studies (GWAS) have also been reported using varied cohorts consisting of populations of varied ethnicity, sex, and whether the controls were exposed to opioids [22–26]. For example, Nielsen DA et al. performed a GWAS on subjects of European origin with 104 heroin-dependent patients and 101 controls [22]. They compared the frequencies of 10,000 SNPs but failed to find any significant associations. However, they extended the cohort consisting of subjects of African and European descent and a larger pool of 100,000 SNPs in methadone-maintained 325 heroin addicts and 250 controls [23]. After the multiple testing corrections, only a single intergenic variant, rs10494334, was found significant in European subjects, while none in subjects of African descent.

Gelernter J et al. reported a GWAS on 5432 African American and 6877 European American subjects [24]. Analysis using the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV) symptom counts for opioid dependence or case-control status was performed on sub-groups and meta-analysis on the entire cohort. In the final meta-analysis, a variant rs62103177 in *KCNG2* was found to be genome-wide significant. Other variants as rs60349741 in *KCNC1* and rs114070671 in *APBB2* had genome-wide significance in combined analysis but only nominally in meta-analysis [24].

Defining controls merely based on the DSM definition has a caveat that the subjects not exposed to opioids cannot develop OAD, even though they carry the risk-associated variants. In a study of European Americans consisting of 1290 cases and 1768 opioid-exposed controls, an SNP, rs12442183, 110 kb downstream to *RGMA*, was found to be significantly associated with OAD [25]. Further, microarray data analysis suggested that rs12442183 is an expression quantitative trait locus (eQTL) for the *RGMA* gene. However, previously reported variants on *KCNG2*, *KCNC1*, and *APBB2* didn't have a significant association despite the study using the overlapping sample with the previous study [25,30].

In another study comparing the daily injection opioid users (cases: n=1167) vs. the opioid abusers who never injected opioids daily (controls: n=161), a variant rs1436175 in the gene *CNIH3* showed genome-wide significance. Five other variants, rs10799590, rs12130499, rs298733, rs1436171, and rs1369846, in *CNIH3*, reached genome-wide significance in a meta-analysis of the discovery cohort and two independent populations [26,30].

The studies mentioned above [22–26], consolidated together, indicate that clear external replication of GWAS findings is rare. The following factors contribute to this situation: (a) OAD is a complex psychiatric disease with relatively low heritability, and there is no single variant with a large effect size that can be detected in small cohorts (b) previous OAD GWAS were relatively small compared with those for legal substance use disorders and (c) in published work relevant to opioid use, there was considerable phenotypic heterogeneity across samples [30], and (d) OAD is likely highly polygenic and individual loci contributions are too small for detection in studies with limited sample size.

With the above issues in mind, we present a case-control study of twenty-three individuals who had been previously given opioid-based painkillers to undergo small surgical treatment and were recruited at Prisma Health Upstate clinic and elsewhere. Eleven of them were considered non-

persistent opioid (controls), and twelve of them were persistent opioid users (cases) after an initial surgery and at the time of the sample collection (details in section 4.1). The subjects were asked to provide saliva samples, which were subjected to DNA sequencing at Clemson University Center for Human Genetics, and variant calling was performed (details in section 4.2). The variants are analyzed to identify their association with OAD.

## 2. Results

The first task of our study is to check if previously reported variants can be found in our cohort. For this purpose, from the literature, we compiled a list of genes harboring variants reported to have associations with OAD (Table 1). Then, we present the results of variants found on the listed genes in our present study. Two of the variants with coding consequence (rs6265 in *BDNF* and rs1799971 in *OPRM1*) show some indication of the association to OAD, though the p-values are not small enough (raw p-values are  $< 0.3$ ) to call these significant. Then the search is extended to variants with no direct coding consequences on these genes, and several such variants on *DRD3*, *KCNG2*, and *NRXN3* are found with (significance p-values  $< 0.01$ ) for the association with OAD (section 2.1). The next task is to search for genetic variants across the whole genome, where the alternate alleles are exclusively found in cases but not in any of the control subjects (section 2.2). We hypothesize that several variants on the same gene may be working together to compromise the function of a gene, and to test it, we performed a co-occurrence analysis of variants on the genes. In co-occurrence analysis, multiple variants on the same genes are assumed to have additive impairments on the function of the gene, and these variants are grouped and analyzed for their functional consequences. This analysis identified *LRFN3*, *ZMIZ1*, *RYR3*, and *OR1L6* with three or more variants on them and alternate alleles frequency sum greater or equal to 14 (section 2.2.1), which potentially may have an association with OAD.

**Table 1.** List of genes reported in literature harboring variants associated with OAD risk.

Gene	Chr <sup>a</sup>	Gene BP(beg) <sup>b</sup>	Gene BP(end) <sup>c</sup>	Variant (rsID)	Sample population	Opioid exposed controls	Reference
OPRM1	chr6	154039240	154132356	rs1799971	EuA <sup>d</sup> , AfA <sup>e</sup>	Yes	[14,15,20]
OPRD1	chr1	28812170	28871267	rs2236861	EuA, AfA; EuA, AfA	Yes; No	[15,16]
DRD2	chr11	113409605	113475398	rs1799978	Eu <sup>f</sup> , NaAm <sup>g</sup> , Asn <sup>h</sup> , Yes Per <sup>i</sup>		[19]
BDNF	chr11	27654893	27700455	rs6265	Eu <sup>f</sup> , NaAm <sup>g</sup> , Asn <sup>h</sup> , Yes Per <sup>i</sup>		[19]
APBB2	chr4	40810027	41214542	rs114070671	EuAm <sup>k</sup> , AfAm <sup>m</sup>	No	[24]
KCNG2	chr18	79797938	79900100	rs62103177	EuAm, AfAm	No	[24,31]
KCNC1	chr11	17734781	17783057	rs60349741	EuAm, AfAm	No	[24]
CNIH3	chr1	224616317	224740554	rs1436175, rs10799590, rs12130499, rs298733, rs1436171, rs1369846	EuAu <sup>n</sup>	Yes	[26]
RGMA	chr15	93035271	93089211	rs12442183	EuAm	Yes	[25]
DRD3	chr3	114127580	114179052	rs324029 rs2654754	and EuAm, AfAm	No	[32,33]
DRD4	chr11	637269	640706	rs1800955	Chinese males	No	[34,35]
NRXN3	chr14	78170373	79868291	rs8019381 <sup>p</sup>	Caucasians	No	[36,37]

<sup>a</sup> Chromosome; <sup>b</sup> Gene's beginning base position; <sup>c</sup> Gene's ending base position; <sup>d</sup> European ancestry; <sup>e</sup> African ancestry; <sup>f</sup> European; <sup>g</sup> Native South/North American; <sup>h</sup> Asian; <sup>i</sup> Persian; <sup>k</sup> European-American; <sup>m</sup> African-American; <sup>n</sup> European-Australian; <sup>p</sup> Associated to substance use disorder

The OAD is a complex phenotype, and it may not result from a single variant or single gene, but a network of genes-/protein-interactions is perturbed due to several variants on multiple genes. To investigate it, we also performed the network analysis of protein-protein interaction (PPI) and network

analysis of the genes harboring single or multiple variants suggesting an association with OAD. We identified a large, connected component of PPIs involving more than half of the genes of interest (GOI) (section 2.3). Finally, we perform a gene function enrichment and pathway analysis to identify the molecular processes, biological function and interaction pathways that may be affected due to the presence of variants in the cases but not in controls and discuss the results (section 2.4).

### 2.1. Variants on Genes Reported in the Literature with Association to OAD

We begin our association analysis to OAD with the set of genes reported in the literature to be associated with OAD. Thus, we analyzed gene variants (Table 1) to test their association with OAD in our samples. We extracted the variants within extended gene loci, for each gene, an extended region with 5 kb upstream and 5 kb downstream, including the gene boundary, is defined. The effects of variants are predicted using the Variant Effect Predictor (VEP) version 111 [38] about human genome assembly GRCh38.

A total of 6903 variants, including 67 novel, variants effects were predicted, consisting of one *stop\_gained*, one *inframe\_insertion*, one *inframe\_deletion*, 58 *missense\_variant* and 56 *synonymous\_variant* with coding consequences. Further association testing is performed using PLINK v1.90b7.2 [39] of these variants. However, none of the variants with coding consequences could reach the genome-wide association significance (raw p-value < 0.01). In contrast, 13 variants (intronic or SNV) showed genome-wide significance (Table 2). The validation of intronic variants' association with OAD requires further study. Considering this, we focused on only variants with the most severe consequence (coding consequences), but none showed significance for genome-wide association to OAD. However, two variants, rs6265 (C>T at 11:27658369) on BDNF, a missense coding variant (Val66Met in BDNF isoform a, previously reported [19]), and rs1799971, a missense variant on OPRM1 (118A>G) changing Asn40Asp in mu-opioid receptor (also previously reported [14,15,20]), have a raw p-value < 0.30, after relaxing the p-value to call association. Here, we understand that a raw p-value of 0.30 is not significant to ascertain an association. However, this very weak indication aligns with the previously reported studies. The large p-value of these variants' association with OAD can be attributed to the small cohort used here.

**Table 2.** The non-coding consequence variants reaching significance (raw p-value < 0.01) with association to OAD in the extended genes loci that are listed in Table 1.

Chr <sup>a</sup>	Variant <sup>b</sup> or rsID	BP <sup>c</sup>	Gene	F_A <sup>d</sup>	F_U <sup>e</sup>	Conseqn. <sup>f</sup>	$\chi^2$	p-value	OR <sup>g</sup>
3	TGAAA>T	114142739	DRD3	0.0909	0.5455	iv <sup>h</sup>	10.48	0.0012	0.0833
18	rs76838079	79873271	KCNG2	0.3182	0	iv	8.324	0.0039	NA
3	rs73232565	114124222	-	0	0.2727	-	7.527	0.0061	0
11	rs3051820	17785864	-	0.2727	0.6818	-	7.379	0.0066	0.175
4	rs1011069	41217734	-	0.2727	0	-	6.947	0.0084	NA
14	rs143010574	79227804	NRXN3	0.2727	0	gutv <sup>i</sup> , iv	6.947	0.0084	NA
4	rs7695309	41216892	-	0.3636	0.0455	-	6.844	0.0089	12
14	rs7145683	79241818	NRXN3	0.3636	0.0455	gutv, iv	6.844	0.0089	12
14	G>GA	79242068	NRXN3	0.3636	0.0455	gutv, iv	6.844	0.0089	12
14	rs12889183	79360638	NRXN3	0.5	0.1364	iv	6.705	0.0096	6.333
14	rs11625994	79364803	NRXN3	0.5	0.1364	iv	6.705	0.0096	6.333
14	rs8008332	79365491	NRXN3	0.5	0.1364	iv	6.705	0.0096	6.333
14	rs2167150	79367835	NRXN3	0.5	0.1364	iv	6.705	0.0096	6.333

<sup>a</sup> Chromosome; <sup>b</sup> rsID if found is listed otherwise the variant is listed in this column in form X>Y, where X is reference allele while Y represents alternate allele; <sup>c</sup> Base position; <sup>d</sup> minor allele frequency in cases; <sup>e</sup> minor allele frequency in controls; <sup>f</sup> variants' consequence; <sup>g</sup> odds ratio; <sup>h</sup> intronic variant; <sup>i</sup> genic upstream transcript variant

The variants with no direct coding consequence found significant in extended gene loci of genes listed in Table 1 are shown in Table 2. These variants may have an impact on the genes by altering

the expression of the genes. We found some variants, though not the listed variants, in genes *DRD3* [32,33], *KCNG2* [24,31], and *NRXN3* have been reported in the literature to have an association with OAD [36,37].

## 2.2. Genes with Alternate Allele Exclusively in Cases but Not in Controls

We extended our search to include all the genomic variants found in the samples. The variant effect is predicted using VEP version 111 [38] for 20,910 variants found in the sample. Afterward, all the variants are ranked based on the severity of the predicted consequence and filtered to satisfy two requirements: (a) only variants for which all controls have homozygous reference alleles, and (b) only variants in the OAD group have the alternative alleles. A variant's association with phenotype OAD is considered significant when the raw p-value is less than 0.05. It resulted in 158 variants, with significance within 126 genes. In the case of these variants with significance, the alternate allele frequency ranged from 11 to 4 in case samples and zero in controls. A list of such variants with association to OAD with only high-significance (raw p-value < 0.005) is provided in Table 3.

**Table 3.** The variants across the genome are ranked by consequence and filtered as per significance associated with OAD.

Chr <sup>a</sup>	Variant <sup>b</sup> or rsID	BP <sup>c</sup>	Gene	C_A <sup>d</sup>	Conseqn. <sup>e</sup>	$\chi^2$	p-value
12	rs773026868	50352078	FAM186A	11	fsv <sup>f</sup>	13.25	0.000272
11	rs60494098	9091455	SCUBE2	10	mv <sup>g</sup>	12.94	0.000321
1	rs10907376	223394461	CCDC185	9	mv	11.31	0.000769
13	C>A	29324631	MTUS2	8	mv	9.778	0.001766
7	rs3750050	77627396	PTPN12	7	mv	8.324	0.003912
7	rs1046515	140694787	ADCK2	7	mv	8.324	0.003912
16	rs308925	77735937	NUDT7	7	mv	8.324	0.003912
2	A>G	207613128	METTL21A	7	mv	8.324	0.003912
6	rs1048886	70579486	SDHAF4	7	mv	8.324	0.003912
16	rs3869427	69954416	CLEC18A	7	mv	8.324	0.003912
1	rs147489167	248574363	OR2T34	7	mv	8.324	0.003912

<sup>a</sup> Chromosome; <sup>b</sup> rsID if found is listed otherwise the variant is listed in this column in form X>Y, where X is reference allele while Y represents alternate allele; <sup>c</sup> Base position; <sup>d</sup> alternate allele count in cases; <sup>e</sup> varinats' consequence; <sup>f</sup> frameshift variant; <sup>g</sup> missense variant

So far none of the genes listed in Table 3 has been directly linked to addiction. However, in a study of attention-deficit hyperactive patients, gene *MTUS2* showed the highest variation frequency [40]. Studies have suggested the association of *MTUS2* with psychological disorders [40]. The suggested association of *MTUS2* to the psychological disorder may indirectly relate to addiction-like traits, but further exploration in this regard is needed to test this possibility.

### 2.2.1. Co-Occurrence of Variants on Genes and Association to OAD

So far, our focus has been on finding common genetic variants that may be associated with OAD. However, the OAD phenotype may result from the altered function of one or more proteins, and alteration could be caused by different variants within the same proteins, not necessarily identical variants in the affected individuals. It is also possible that there are multiple mechanisms and disruptions of pathways that can result in OAD. This led us to focus on proteins or genes and their combinations, instead of individual variants. Considering it, we analyzed the co-occurrence of multiple variants within the same gene/protein. The alternate alleles count of all gene variants exclusively in cases is aggregated by summing it, among the total 126 genes harboring variants associated with OAD, considering the significance (raw p-value < 0.05). Eighteen genes have multiple variants exclusively

in cases, including 12 genes with two variants, three genes with three variants and three genes with greater than three variants.

The following genes harbor the most severe consequence (transcript ablation, splice acceptor variant, splice donor variant, stop gained/lost, frameshift variant, start lost, transcript amplification, feature elongation, or feature truncation) variants with alternate allele frequencies sum greater or equal to 10, *ZMIZ1*, *LRFN3*, *OR1L6*, *RYR3*, *PWWP2B*, *ZNF92*, *CYP4F12*, *FAM186A*, *SCUBE2*, *NUTM2D*. The number of variants, count of alternate alleles in cases and list of predicted consequences for the variants are provided in Table 4. Considering the significant raw p-values of the genes listed in Table 3 or Table 4 consisting a total 18 genes is compiled, we will call these as genes of interest (GOI) hereon for further analysis.

**Table 4.** Details of co-occurring variants on genes, where the sum of alternate allele count in cases are greater or equal to 10 but zero in controls.

Chr <sup>a</sup>	Gene	#variants <sup>b</sup>	list of C_A <sup>c</sup>	list of Consequences <sup>d</sup>
10	ZMIZ1	7	5, 5, 5, 4, 4, 4, 4	fsv <sup>e</sup> , mv <sup>f</sup> , fsv, fsv, fsv, fsv, fsv
19	LRFN3	6	5, 5, 5, 5, 5, 5	id <sup>g</sup> , pav <sup>h</sup> , ii <sup>k</sup> , fsv, fsv, fsv
9	OR1L6	4	4, 4, 4, 4	mv, mv, mv, mv
15	RYR3	3	5, 5, 4	fsv, fsv, sg <sup>m</sup> & fsv
10	PWWP2B	3	4, 4, 4	sg, mv, fsv
7	ZNF92	2	6, 6	mv, mv
19	CYP4F12	3	4, 4, 4	mv, mv, sdv <sup>n</sup> & ntv <sup>p</sup>
10	NUTM2D	2	5, 5	mv, mv

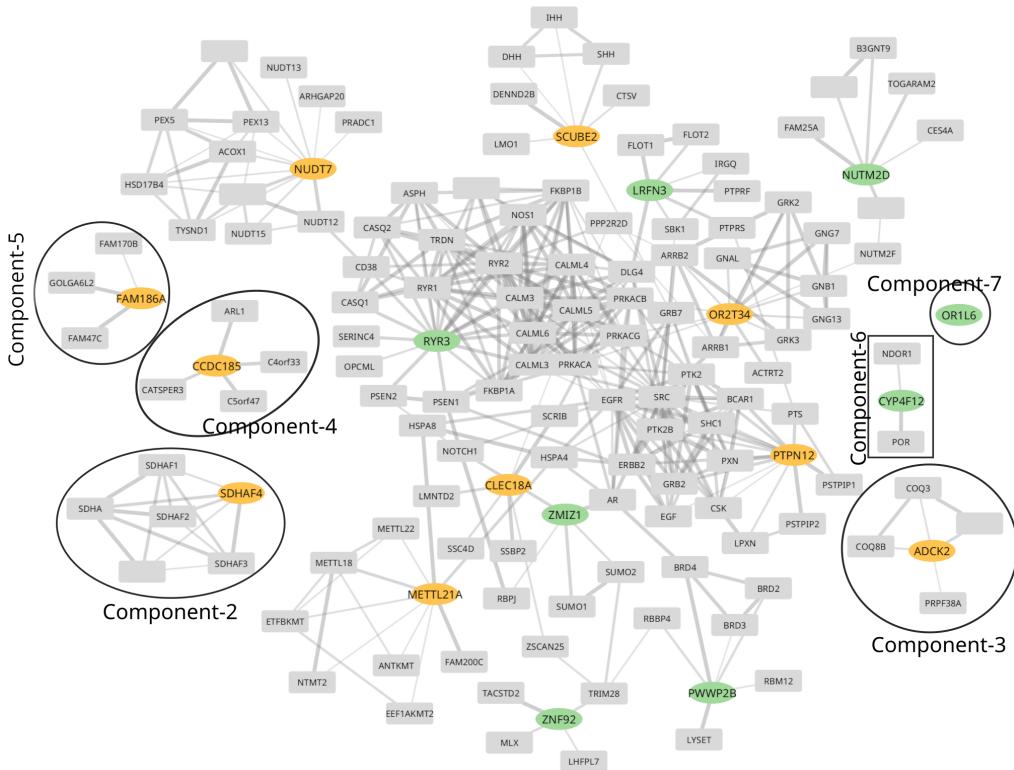
<sup>a</sup> Chromosome; <sup>b</sup> number of variants; <sup>c</sup> alternate allele counts in cases for each variant; <sup>d</sup> variants' consequence; <sup>e</sup> frameshift variant; <sup>f</sup> missense variant; <sup>g</sup> inframe deletion; <sup>h</sup> protein altering variant; <sup>k</sup> inframe insertion; <sup>m</sup> stop gained; <sup>n</sup> splice donor variant; <sup>p</sup> nmd transcript variant

So far, among the genes/proteins listed in Table 4, none has been directly associated with addiction. However, some of these have been known to be associated with functions relevant to OAD. For example, *LRFN3* (Leucine Rich Repeat And Fibronectin Type III Domain Containing 3), also known as *SALM4* (Synaptic Adhesion-Like Molecule 4), is part of the *LRFN* family, which is involved in synaptic adhesion and regulation of excitatory synapses [41]. *SALMs*, including *LRFN3/SALM4*, play important roles in synaptic development and plasticity [42], which are critical processes in learning, memory, and behavior—all relevant to addiction. Similarly, *RYR3* (Ryanodine Receptor 3) is part of the ryanodine receptor family, which functions as intracellular calcium channel that releases calcium from the endoplasmic reticulum into the cytoplasm [43,44]. Calcium signaling is critical for various cellular processes, including neuronal activity, synaptic plasticity, and neurotransmitter release [45]. *ZMIZ1* (Zinc Finger MIZ-Type Containing 1) is a transcriptional coactivator that interacts with the androgen receptor and other transcription factors [46], and the androgen receptor has been implicated in reward-related behaviors, including aggression and stress responses, which can influence addiction [47]. In summary, the genes listed in Table 4 can potentially influence addictive behaviors in direct or indirect ways and in individual- or cohort-specific manners. This led us to further analyze the interaction networks of these genes and investigate their collective behaviors and plausible linkage to opioid addiction.

### 2.3. PPI Network Analysis of Genes of Interest

To gain insight into the functions involving the proteins in GOI, we built a protein-protein interaction (PPI) network based on the STRING database [48] as detailed in section 4.4. We observe a total of seven connected components. Connected components 2 through 7 are small and shown in Figure 1; all other genes form a large connected component, marked as component-1. Component-1 consists of six genes listed in Table 3 and five genes harboring multiple variants listed in Table 4. This indicates that genes in component-1 are functionally interdependent, and any perturbation to this

network of PPIs can potentially be associated with altered function and, thereby, some phenotype. To further investigate this hypothesis of perturbation of the PPI network and association with OAD, we performed a functional enrichment analysis of the genes in the network.



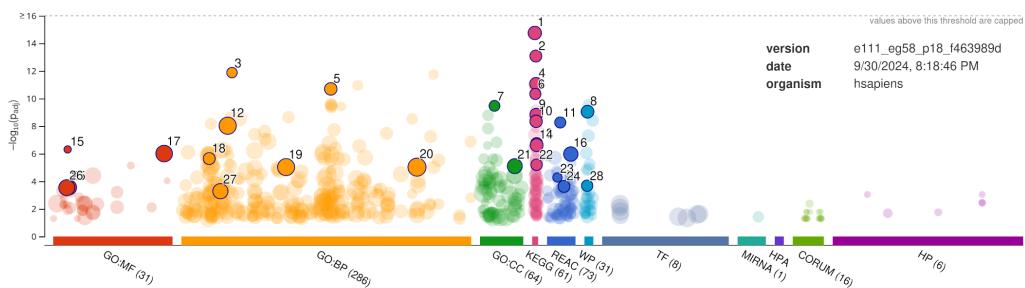
**Figure 1.** Protein-Protein interaction (PPI) network of high confidence (normalized interaction score  $> 0.8$ , details in section 4.4) PPIs subset from STRING database. The genes harboring a single variant with a very high alternate allele count exclusively (Table 3) in cases are shown with orange oval nodes, while genes harboring multiple variants with a high alternate allele count exclusively in cases (Table 4) are shown in green oval nodes/genes. All other gray nodes are either immediate neighbors (directly interacted by PPIs) of green or orange nodes or nodes having at least two neighbors in the list of selected nodes/genes. Finally, the subnetwork is created using selected nodes and all the edges.

#### 2.4. Gene Ontology and Functional Enrichment Analysis

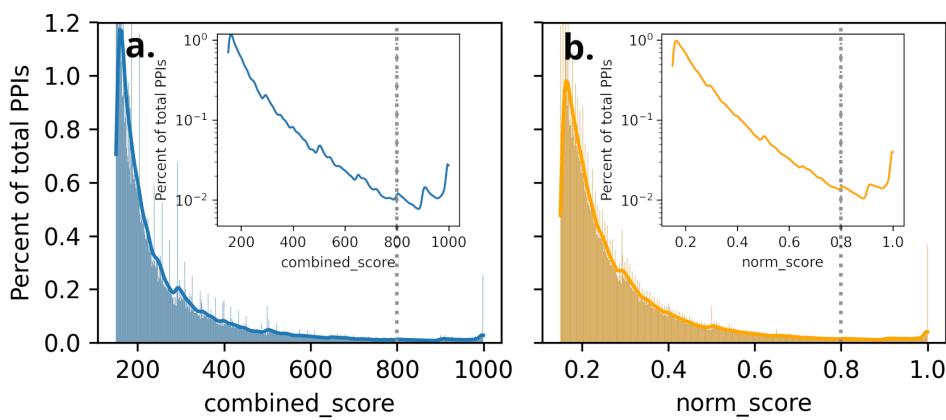
The functional enrichment analysis was performed using the g:Profiler's webservice[49], and its results are summarized in Figure 3 and Table 5.

**Table 5.** Description of annotated terms shown in Figure 2. Terms relevant to addiction are shown with underlined-text.

ID	Source	Term ID	Term name	$P_{adj}(\text{query})$
1	KEGG	KEGG:04020	<u>Calcium signaling pathway</u>	$1.780 \times 10^{-15}$
2	KEGG	KEGG:04713	<u>Circadian entrainment</u>	$8.371 \times 10^{-14}$
3	GO:BP	GO:0014808	release of sequestered calcium ion into cytosol by sarcoplasmic reticulum	$1.334 \times 10^{-12}$
4	KEGG	KEGG:04728	<u>Dopaminergic synapse</u>	$8.377 \times 10^{-12}$
5	GO:BP	GO:0051208	sequestering of calcium ion	$2.017 \times 10^{-11}$
6	KEGG	KEGG:04340	Hedgehog signaling pathway	$4.704 \times 10^{-11}$
7	GO:CC	GO:0033017	sarcoplasmic reticulum membrane	$3.405 \times 10^{-10}$
8	WP	WP:WP3929	Chemokine signaling pathway	$9.043 \times 10^{-10}$
9	KEGG	KEGG:04921	Oxytocin signaling pathway	$1.489 \times 10^{-9}$
10	KEGG	KEGG:04915	Estrogen signaling pathway	$4.481 \times 10^{-9}$
11	REAC	REAC:R-HSA-5578775	Ion homeostasis	$5.472 \times 10^{-9}$
12	GO:BP	GO:0010646	regulation of cell communication	$9.459 \times 10^{-9}$
13	KEGG	KEGG:05032	<u>Morphine addiction</u>	$1.816 \times 10^{-7}$
14	KEGG	KEGG:05034	<u>Alcoholism</u>	$2.403 \times 10^{-7}$
15	GO:MF	GO:0005219	ryanodine-sensitive calcium-release channel activity	$4.909 \times 10^{-7}$
16	REAC	REAC:R-HSA-9006934	Signaling by Receptor Tyrosine Kinases	$1.062 \times 10^{-6}$
17	GO:MF	GO:0140096	catalytic activity, acting on a protein	$9.842 \times 10^{-7}$
18	GO:BP	GO:0006942	regulation of striated muscle contraction	$2.257 \times 10^{-6}$
19	GO:BP	GO:0036211	protein modification process	$9.419 \times 10^{-6}$
20	GO:BP	GO:1901564	organonitrogen compound metabolic process	$9.464 \times 10^{-6}$
21	GO:CC	GO:0098797	plasma membrane protein complex	$8.009 \times 10^{-6}$
22	KEGG	KEGG:05031	<u>Amphetamine addiction</u>	$6.351 \times 10^{-6}$
23	REAC	REAC:R-HSA-180292	GAB1 signalosome	$5.328 \times 10^{-5}$
24	REAC	REAC:R-HSA-111885	<u>Opioid Signalling</u>	$2.349 \times 10^{-4}$
25	GO:MF	GO:0005509	calcium ion binding	$2.788 \times 10^{-4}$
26	GO:MF	GO:0005102	signaling receptor binding	$2.920 \times 10^{-4}$
27	GO:BP	GO:0009725	response to hormone	$5.256 \times 10^{-4}$
28	WP	WP:WP3680	Physico chemical features and toxicity associated pathways	$2.095 \times 10^{-4}$



**Figure 2.** Summary of Gene Ontology and functional enrichment of Genes in PPIs network of GOIs performed using g:Profile webserver. Highly significant (considering the adjusted p-value), but non-redundant terms in the enrichment are annotated. The description of annotation labels to terms is provided in Table 5.



**Figure 3.** The distribution of percent of total PPIs as a function of scores: (a) *combined\_score* and (b) Normalized score(*norm\_score*) listed in STRING database for humans. The kernel density of the percent of PPIs is shown in a logarithmic scale to highlight distributions in low percent regions (in inset) within respective plots. The thresholds in this work are marked with dashed-vertical lines in both cases.

From the enrichment analysis, the calcium transport, regulation and binding, enzyme binding, transmembrane transporter binding, and signaling receptor binding emerge as the high-confidence molecular function from gene ontology. While, signaling and amino-acid modification as the top biological function. The GO:cellular-component is the membrane and channels, which host most of the signaling and transmembrane proteins to carry the signaling in cells.

The KEGG pathway analysis shows enrichment in Calcium signaling pathways, circadian entrainment, dopaminergic synapses, morphine addiction and alcoholism (Figure 2 and Table 5). This further supports that the variants of GOIs are impairing or abolishing the function of the proteins, which in turn compromises the protein's normal function in the PPI network since these proteins are involved in crucial reward pathways and addiction. Therefore, the association of these genetic variants to OAD risk is highly plausible, and further study is needed to confirm this.

### 3. Discussion

Opioid addiction disorder (OAD) is a complex phenotype with significant heritability estimated to be between 40% and 60%. The heritability raises the genetic susceptibility of individuals carrying the associated variants in their genome. In the past, several candidate gene studies and genome-wide association studies have implicated the association of variants on genes to OAD. Though, they have rarely been replicated in other studies. It has been pointed out that this situation can be due to the following limitations of the studies: (a) the cohort sizes used in these studies are relatively small

compared to those used for substance use addiction, (b) the DSM definitions have been used to label controls, which may not be appropriate and thus diluting the association results, and (c) the ethnicity of populations of cohorts varied in different studies, which brings another variability in the study. Another limitation of some of the previous studies is that the controls were not exposed to substances of interest. Such controls could potentially carry genetic variants, making them predisposed to OAD, but they have never been exposed to drugs.

To address the abovementioned deficiency, We performed the genome-wide association study of a cohort of 23 samples, including 12 cases and 11 controls, where all controls were given opioid-based pain killers. In our study, we could find a weak association for two variants, one on *BDNF* and the other on *OPRM1*, when our focus was on variants only with coding consequences. Upon extending the search to variants with no direct coding consequence, we identified variants of *DRD3*, *KCNG2*, and *NRXN3* genes with significant association with OAD. Interestingly, these genes have been predicted to have an association with OAD. This indicates that these variants may be altering the expression of the protein and thereby indirectly affecting the phenotype OAD. Next, we expanded our search space for the variants to the entire genome. However, genome-wide search yields many variants. Thus, only variants with coding consequences are prioritized in association analysis. We chose only variants with alternate alleles in case subjects and predicted them to have an association with OAD with significance. Several variants of the same gene protein may have a cumulative effect on the gene's function, thus altering its biological function. We used co-occurrence analysis to test this hypothesis. Our co-occurrence analysis identified *LRFN3*, *RYR3*, *ZMIZ1*, and *OR1L6* as genes harboring multiple variants exclusively in cases. From the genes with predicted association to OAD with significance (genes of interest/GOI), a network of protein-protein interaction is extracted based on PPIs listed in the STRING database for humans. The network is further expanded by including genes directly interacting with two or more genes in the GOI list. Then, the pathway and functional enrichment of the genes in the network are done to find the pathways with significant enrichment for the genes and their association with the OAD phenotype. We observe considerable enrichment for terms like calcium signaling pathway, circadian entrainment, dopaminergic synapse, oxytocin signaling pathway, estrogen signaling pathway, morphine addiction, alcoholism, and opioid signaling, which are closely related to OAD or addiction in general.

#### 4. Materials and Methods

##### 4.1. Sample Collection

Local Institutional Review Board approval was obtained before subjects recruitment and enrollment, and written informed consent was obtained from all eligible participants. Eligible subjects were those over 18 who had undergone treatment for a small surgery in the past and provided informed consent. Subjects were excluded if they had an active oral lesion, had a history of opioid abuse before treatment of their injury, or had a history of opioid use within one year of treatment for their injury. Subjects who provided informed consent were subsequently asked to provide a saliva sample for DNA analysis. Twenty subjects were recruited and enrolled between November 2022 and June 2023. Twelve subjects were persistent opioid users at the time of enrollment and saliva sample collection, whereas the non-persistent opioid users were only eight. To make the set balanced, we collected additional samples from researchers from the corresponding author (E.A.) department who, in the past, have been sedated for small surgery but are not persistent users. Thus, the number of cases of non-persistent users is eleven, very similar to the number of cases of persistent users.

Age, gender, and race demographics were recorded at the enrollment visit, in addition to details of their orthopedic injuries. The average age of all enrolled participants was 66 years, with a minimum age of 36 and a maximum age of 84. There were 8 females and 3 males in the non-persistent opioid user group versus 7 females and 5 males in the persistent opioid user group.

#### 4.2. DNA Sequencing and Genotyping

The samples' DNA was extracted using a modified Zymo Quick-DNA Microprep Plus Kit protocol. Libraries were constructed using 500 ng of extracted DNA with the Tecan Revelo DNA-Seq Enz kit on the Tecan MagicPrep NGS system. The libraries were quantified for concentration with the Invitrogen 1x dsDNA HS assay kit on the Invitrogen Qubit 4 Fluorometer. Library size was quantified using Agilent High Sensitivity D1000 assay on the 4150 TapeStation system. Libraries were normalized to 3 nm, pooled, and sequenced on an Illumina Novaseq 6000 sequencer using an S4 flow cell and 2x150 sequencing chemistry at Clemson University Center for Human Genetics.

Raw reads were filtered for low-quality and short reads, then aligned to human reference genome version GRCh38 to produce alignment files using the GPU-accelerated *fq2bam* module in the NVIDIA Clara Parabricks suite. Base quality recalibration was performed using GPU-accelerated Genome Analysis Tool Kit [50] BQSR module from Parabricks suite and known variant information from Mills and 1000 Genomes Gold Standard Indel dataset and dbSNP v138 dataset. Variant calling was performed on the recalibrated alignment files using GPU-accelerated GATK's *haplotypecaller* module from the Parabricks suite and GRCh38 reference genome. Individual sample GVCFs were combined, indexed and joint-called using the Short Variant Discovery workflow from GATK's Best Practices.[51] Joint-called variants were hard-filtered using gold standard default values recommended by the Broad Institute [52]. After that, the effects for variants are predicted using the Variant Effect Predictor (VEP) version 111 [38] compared to standard human genome assembly GRCh38.

#### 4.3. Variants Filtering, Effects Prediction and Association Analysis

A subset of all variants found after variant calling is extracted into a vcf file using the bcftools module in SAMtools [53] for analyzing the association of a subset of genes listed in Table 1. The subset .vcf file is indexed and compressed using bcftools [53]. The variant effects are predicted using Ensembl Variant Effect (VEP) version 111 [38] with reference to human genome assembly GRCh38. We used PLINK v1.90b7.2 [39] to test the significance of the genotype-phenotype association.

#### 4.4. PPIs Network Construction and Analysis

The Protein-Protein Interactions data for humans is downloaded from STRING database [48]. Considering the large number of human PPIs listed in STRING and varying degrees of confidence in PPIs, we started filtering in only high-confidence PPIs based on absolute *combined\_score*, including only interactions with *combined\_score* greater than a threshold.

However, we noticed that filtering based on absolute *combined\_score* excluded all the interactions of proteins that had no partner with *combined\_score* greater than the chosen threshold. However, such filtering excluded some of the GOIs altogether. To avoid such stringent absolute *combined\_score* based filtering, we used normalization of *combined\_score*, carried as follows. The *combined\_score* is normalized by the max *combined\_score* for the gene among all its interacting genes. Such normalization is carried out to ensure that high-confidence PPIs can be included in a subnetwork featuring only high-confidence interactions Figure 3. The percentage of PPIs keeps decreasing as a function of *combined\_score* and reaches a minimum of around 800; after that, it starts increasing, implying there are high-confidence PPIs pairs with scores greater than 800; based on this, we choose *combined\_score*=800 as the threshold for unnormalized scores (Figure 3). We observe a similar trend for normalized scores as well, and around *norm\_score*=0.8, again the percent PPIs start increasing; thus, we choose 0.8 as a threshold for the *norm\_score* to find the filtered subset of the STRING database consisting of 19,622 nodes and 204,572 edges (after removing self-loop and duplicate edges) for further analysis using Cytoscape-v3.10.2 [54].

We start with creating a subnetwork of PPIs for GOIs. Initially, the genes listed in Table 3 or Table 4 and any immediate neighbor to these are also selected. Further, any node within a distance of one and has at least two neighbors in the selected nodes set is also considered part of the subnetwork to extend the network of PPIs of GOIs. Finally, the subnetwork is created using selected nodes and all the edges. The created subnetwork consisted of 146 nodes and 390 edges, as shown in Figure 1.

#### 4.5. Gene Ontology and Functional Enrichment

The g:Profiler [49] carries the enrichment of the genes/proteins list provided as input against the data sources: Gene ontology (molecular function, biological process and cellular component), biological pathways (KEGG, Reactome and WikiPathways), regulatory motifs in DNA (TRANSFAC, miRTarBase), protein databases (Human Protein Atlas and CORUM), and human phenotype ontology (HP). We used the list of genes present in the connected component-1 of the network for the enrichment analysis. The gene ids are: Gn148400, Gn132535, Gn139549, Gn198838, Gn157322, Gn145687, Gn089250, Gn105426, Gn103254, Gn140368, Gn172824, Gn109971, Gn166444, Gn110031, Gn129007, Gn133835, Gn137486, Gn203740, Gn188612, Gn141480, Gn141738, Gn160691, Gn237172, Gn175470, Gn050820, Gn139197, Gn153485, Gn167378, Gn152229, Gn221886, Gn206069, Gn088832, Gn120899, Gn213965, Gn204256, Gn132589, Gn197037, Gn177885, Gn116030, Gn221874, Gn176533, Gn067365, Gn214562, Gn119782, Gn178372, Gn189350, Gn198363, Gn078369, Gn169717, Gn165059, Gn137312, Gn169083, Gn244462, Gn197122, Gn162521, Gn184292, Gn142875, Gn203791, Gn143318, Gn143801, Gn198626, Gn142949, Gn196218, Gn139160, Gn166321, Gn180900, Gn168214, Gn188322, Gn188100, Gn169398, Gn166407, Gn108175, Gn141404, Gn186439, Gn146757, Gn185522, Gn183310, Gn183715, Gn080815, Gn184716, Gn100077, Gn178363, Gn173020, Gn175356, Gn072062, Gn171806, Gn171813, Gn169925, Gn169885, Gn170606, Gn164690, Gn163501, Gn162928, Gn161533, Gn160014, Gn156521, Gn150787, Gn146700, Gn146648, Gn144401, Gn141736, Gn140876, Gn089159, Gn138798, Gn141867, Gn118729, Gn137727, Gn136943, Gn136159, Gn135617, Gn130950, Gn130726, Gn127947, Gn127588, Gn108788, Gn126243, Gn112874, Gn004468, Gn103653, where prefix Gn is used in listing here in place of ENSG00000 for brevity. The enrichment query parameters are as follows: *version* e111\_eg58\_p18\_f463989d; *date* 9/30/2024, 8:18:46 PM; *organism* hsapiens; *query length* 122; *all results* false; *ordered* false; *no ieas* false; *sources* GO:MF, GO:CC, GO:BP, KEGG, REAC, TF, MIRNA, HPA, CORUM, HP, WP; *multiquery* false; *numeric ns* ENTREZGENE\_ACC; *domain scope* annotated; *measure underrepresentation* false; *significance threshold method* g\_SCS; *user threshold* 0.05; *no evidences* false; *highlight results* true. The results are summarized and discussed in the result section.

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**Institutional Review Board Statement:** The study of twenty patients from Prisma Health Upstate clinic was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Prisma Health Upstate clinic (protocol code Pro00084425 approval date November 8, 2019).

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#### Abbreviations

The following abbreviations are used in this manuscript:

DSM-IV	Diagnostic and Statistical Manual of Mental Disorders 4th edition
GWAS	Genome-Wide Association Study
DSM	Diagnostic and Statistical Manual of Mental Disorders
OAD	Opioid Addiction Disorder
GOI	Genes of Interest
SNP	Single Nucleotide Polymorphism
PPI	Protein-Protein Interaction
GO	Gene Ontology

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