

Appendix 1: Study design and patients

The diagnosis of heroin addicts was done according to the substance abuse and dependence in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. All the controls didn't have any serious psychological. None of the participants in our study had serious mental illness. All the subjects met the criteria for heroin dependence. All the participants checked for any bleeding disorder, low platelet count (<100k), or taking medications that interfere with blood clotting, such as aspirin, non-steroid anti-inflammatory agents or warfarin, anemia, evidence of an immune deficiency, such as HIV infection or cancer, taking a medication that affects the immune response within the past month, including oral, intravenous, or injectable steroids. Our control subjects of 80 healthy men aged (23 to 55 years) were selected from the general Iranian population who reported no history of OUD. All the proceedings and clinical research were performed by the ethical standards and amendments of the Declaration of Helsinki. All participants signed the consent form and were informed about the goal and course of this study. A complete protocol (80 patients) includes 2 blood samples obtained under steady-state conditions, one before methadone administration (Heroin Addict) and one 3 months later (under MMT). Patients who were in MMT for at least 3 months, were included if classifiable in 1 of the 2 following groups, based on their compliance with MMT and their daily dose of methadone. good compliance (GC, n=58, methadone dose 40-80 mg/d) or poor compliance (PC, n=22, methadone dose \geq 120 mg/d).

For patients comprised of compliance with treatment with methadone, response to treatment is defined by a non-consumption of heroin, an absence of complaints of withdrawal symptoms, and a steady and regular frequentation of the therapeutic program. Non-consumption is based on self-declaration of the patient confirmed by the absence of urine samples positive for opiates, over at least 3 months, with urine checking at least once a week (tests randomly performed over 5 days of the week). Non-responding to treatment is defined as receiving doses of methadone outside of the previously mentioned range at the time of inclusion and having an adverse outcome of worsening withdrawal symptoms after methadone administration. A higher score on the Subjective Opiate Withdrawal Scale (SOWS) was recorded using a structured flowsheet tool and clinician documentation. In addition, symptoms attributed to withdrawal from physician and nursing documentation.

Appendix 2. Demographic Characteristics (N=80)

VARIABLE	POOR COMPLIANCE (N=22)	GOOD COMPLIANCE (N=58)	TOTAL (N=80)
	Mean±SD	Mean±SD	Mean±SD
AGE GROUP (YEARS)	26±5	41±6	37±9
AGE OF ONSET OF HEROIN USE (YEARS)	17±3	29±5	25±7
DURATION ON HEROIN	9±4	13±4	12±4
METHADONE DOSE RANGE (MG/DAY)	109±13	70±14	81±22
DOSE OF HEROIN (G)	3.6±0.7	1.6±0.8	2.1±1.2
YEARS OF EDUCATION	14.25±4.82	13.32±3.97	13.58±4.21
	N (%)	N (%)	N (%)
CIVIL STATUS SINGLE	68.18%	37.9%	46.2%
MARRIED	9.09%	32.7%	27.5%
DIVORCED	18.18%	25.86%	23.75
WIDOW	0%	3.44%	2.5%
EMPLOYED	68.18%	55.17%	58.75%
POSITIVE FAMILY HISTORY OF ADDICTION	68%	17.2%	31.2%
THE SUBJECTIVE OPIATE WITHDRAWAL SCALE (SOWS) SCORE CATEGORY			
0-10	0%	71.3%	71.3%
10-20	10%	1.3%	11.3%
20-30	17.5%	0%	17.5%

Appendix 3. Primers Used in Real-time PCR

Gene	Genbank Accession Number	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Amplico n size (bp)
<i>DRD1</i>	NM_000794.3	TGTTTCCTGTCGCTGCTCATCC	TCTGACACAGCCAAGGAGATGAC	128
<i>DRD2</i>	NM_016574.3	ATCAGCATCGACAGGTACACAGC	TCGTTCGGTCTGCGTTATTGAG	161
<i>DRD3</i>	NM_000796.3	ACATGCCTACTATGCCCTCTCCTAC	ATTCCAGACTCCACCTGTCACCTC	208
<i>DRD4</i>	NM_000796.3	TTCGTCTACTCCGAGGTCCA	CGCACAGGTTGAAGATGGAG	112
<i>DRD5</i>	NM_000798.4	CCATCCTCATCTCCTTCATTCC	AGTCACAGTCTCTGCATTCACG	153
<i>COMT</i>	NM_000754.3	CTGGAGGCCATTGACACCTA	GGTTGATCTCGATGGTGATGAG	202
<i>OPRM1</i>	NM_000914.4	CTCCATGATCACGGCCATCA	GTAGATGTTGGTGGCAGTCTTCAT	130
<i>OPRD1</i>	NM_000911.4	AGGCCAAGCTGATCAACATCT	TTGGTCACCGTGTCACAGTA	151
<i>OPRK1</i>	NM_000912.5	ACCAAAGTCAGGGAAGACGTC	ATCAGGGTGTAGCAGACGATG	152
<i>RAB22A</i>	NM_020673.3	CGATGTAAGAGAAGTCATGGAGAGAG	AGGTTGGCGTCAGTGGATGG	150
<i>DNM1L</i>	NM_001278466.1	GACTTTTGGGCGAACCTAGAATCTG	CAGGACGAGGACCAGTAGCATTTC	98
<i>BDNF</i>	NM_170734.4	CTTTGGTTGCATGAAGGCTG	CCATTCACGCTCTCCAGAGT	108
<i>β-actin</i>	NM_001101.3	AGACGCAGGATGGCATGGG	GAGACCTCAACACCCCAGCC	161

Appendix 4. Real-time PCR analysis gene set of heroin addiction.

A-Mann–Whitney U test					
Gene	Median	Median	Z	P-Value	Foldchange

	Heroin Addicted (n=80)	Control (n=80)	(Wilcoxon W)	(2-tailed)	
<i>OPRM1</i>	0.4000	0.1300	- 6.703	0.0016	-9.37
<i>DNM1L</i>	1.680	0.3850	- 6.177	<0.0001	3.72
<i>RAB22A</i>	2.265	1.122	- 4.539	<0.0001	3.22

B-Unpaired T-Test Results							
Gene	Group Mean (n=80)		means ± SEM	95% confidence interval		P-value (2-tailed)	Foldchange
	Controls	Heroin Addicted		Down	Up		
<i>DRD1</i>	0.9975	9.552	8.554 ± 1.543	5.508	11.60	<0.0001	9.54
<i>DRD5</i>	1.003	0.2191	-0.7841 ± 0.1326	-1.046	-0.5222	<0.0001	-4.47
<i>DRD2</i>	1.003	0.04025	-0.9628 ± 0.1490	-1.257	-0.6686	<0.0001	-27.24
<i>DRD3</i>	1.003	6.931	5.927 ± 0.7816	4.383	7.471	<0.0001	6.92
<i>DRD4</i>	1.002	0.8788	-0.1230 ± 0.1783	-0.4751	0.2291	0.0017	-1.13
<i>COMT</i>	1.011	6.125	5.115 ± 0.8623	3.411	6.818	<0.0001	6.12
<i>OPRD1</i>	1.062	0.1441	-0.9181 ± 0.1237	-1.162	-0.6739	<0.0001	-7.09
<i>OPRK1</i>	1.001	0.1656	-0.8349 ± 0.1574	-1.146	-0.5239	<0.0001	-6.06
<i>BDNF</i>	0.9995	0.09263	-0.9069 ± 0.3215	-1.542	-0.2720	0.0054	-10.7

Appendix 5. Real-time PCR analysis gene set of MMT.

A-One-Way ANOVA							
1.GC-MMT (n=58)	Gene	Mean Difference	SE of diff.	95% confidence interval		Adjusted P-value	Foldchange
2.PC-MMT (n=22)				Down	Up		
1	<i>DRD1</i>	0.1000	0.2496	-0.4636	0.6636	0.9034	-1.11
2		0.3195	0.4052	-0.5955	1.235	0.6769	-1.47
1	<i>DRD5</i>	-1.735	0.4872	-2.835	-0.6348	0.001	2.73
2		0.6195	0.7911	-1.167	2.406	0.6805	-2.64
1	<i>DRD4</i>	0.2366	0.1893	-0.1910	0.6641	0.3812	-1.31
2		0.04364	0.3074	-0.6505	0.7378	0.9873	-1.04
1	<i>COMT</i>	0.8269	0.3072	0.1332	1.521	0.0157	-5.85
2		-0.2982	0.4988	-1.425	0.8282	0.7983	1.29
1	<i>OPRD1</i>	-1.735	0.4068	-2.654	-0.8161	<0.0001	2.73
2		-1.547	0.6606	-3.038	-0.05516	0.0405	2.54
1	<i>OPRK1</i>	-1.733	0.5545	-2.986	-0.4812	0.0042	2.73
2		-2.920	0.9004	-4.953	-0.8863	0.0029	3.91
1	<i>BDNF</i>	-3.710	0.9875	-5.940	-1.480	0.0005	4.71
2		-0.2982	1.603	-3.919	3.322	0.9783	1.29
1	<i>OPRM1</i>	-4.274	0.5979	-5.624	-2.924	<0.0001	5.27
2		-0.1682	0.9708	-2.360	2.024	0.9811	1.17

B-One-Way ANOVA -Kruskal-Wallis H test							
1.GC-MMT (n=58)	Gene	Median (MMT)	Std. Error of Mean (MMT)	95% confidence interval (MMT)		Adjusted P-value	Foldchange
2.PC-MMT (n=22)				Lower	Upper		
1	<i>DNM1L</i>	0.04000	0.01440	0.05652	0.1142	<0.0001	-11.7
2		2.665	2.320	0.4746	10.13	0.0266	5.3
1	<i>DRD2</i>	2.855	0.8164	3.830	7.100	<0.0001	5.46
2		1.130	0.3584	0.6915	2.182	0.7770	1.43
1	<i>DRD3</i>	0.1100	0.02661	0.1207	0.2273	<0.0001	-5.85
2		0.7100	0.2693	0.5754	1.695	>0.9999	1.13
1	<i>RAB22A</i>	0.22	0.1869	0.2327	0.9814	0.0012	-1.64
2		0.56	0.2161	0.3133	1.212	>0.9999	-1.31

Appendix 6: Correlation of Gene Expression with Methadone Dose in MMT

A-Pearson Correlation Values for Gene Expression Methadone Dose-Response Patterns					One-Way ANOVA Post Hoc Tests		
Gene Expression (delta CT MMT) (n=80)	Correlation Coefficient (r)	95% confidence interval		P-Value (Two-Tailed)	Multiple Comparison p-Value		
		Lower	Upper		40-59.9 (n=10)	60-89.9 (n=45)	90 to above (n=25)
<i>BDNF</i>	-0.010	-0.229	0.210	0.928	ns	ns	ns
<i>DRD1</i>	-0.061	-0.277	0.161	0.59	ns	ns	ns
<i>DRD4</i>	-0.198	-0.401	0.022	0.078	ns	ns	ns
<i>OPRK1</i>	-0.120	-0.331	0.102	0.288	ns	ns	ns
<i>OPRM1</i>	0.540**	0.363	0.679	<0.0001	ns	ns	<0.0001
<i>RAB22A</i>	-0.614**	-0.735	-0.456	<0.0001	ns	ns	<0.0001

B-Spearman Correlation Values for Gene Expression Methadone Dose-Response Patterns					One-Way ANOVA Post Hoc Tests		
Gene Expression (delta CT MMT) (n=80)	Correlation Coefficient (r)	95% confidence interval		P-Value (Two-Tailed)	Multiple Comparison p-Value		
		Lower	Upper		40-59.9 (n=10)	60-89.9 (n=45)	90 to above (n=25)
<i>COMT</i>	-0.596**	-0.725	-0.428	<0.0001	ns	ns	<0.0001
<i>DNM1L</i>	-0.761**	-0.842	-0.646	<0.0001	0.0001	0.0001	<0.0001
<i>DRD2</i>	0.531**	0.347	0.676	<0.0001	ns	ns	<0.0001
<i>DRD3</i>	-0.666**	-0.776	-0.518	<0.0001	ns	ns	<0.0001
<i>DRD5</i>	0.619**	0.462	0.738	<0.0001	ns	ns	<0.0001
<i>OPRD1</i>	-0.061	-0.283	0.167	0.590	ns	ns	ns

****. Correlation is significant at the 0.0001 level (2-tailed)

Appendix 7: The binary logistic regression analysis for each gene expression and non-compliance to methadone

Model Feature	Intercept (β0)	DNM1L (β1)	Intercept (β0)	DRD2 (β1)	Intercept (β0)	DRD3 (β1)	Intercept (β0)	COMT (β1)	Intercept (β0)	RAB22A (β1)
Parameter Estimate	4.340	-9.730	-5.007	1.461	2.702	-4.614	4.694	-4.031	0.01161	0.7387
Standard Error	1.073	3.798	1.377	0.3266	0.5232	1.160	1.144	1.041	0.3807	0.3039
95% Confidence Interval	2.689 to ???	??? to -3.880	-8.517 to -2.819	0.9340 to 2.265	1.774 to 3.855	-7.133 to -2.554	2.960 to 7.792	-6.696 to -2.398	-0.7544 to 0.7507	0.2481 to 1.452
Odds Ratio	76.72	5.950e-005	0.006694	4.310	14.91	0.009917	109.3	0.01775	1.012	2.093
95% CI for Odds Ratio	14.71 to ???	??? to 0.02064	0.0002000 to 0.05966	2.545 to 9.634	5.896 to 47.25	0.0007979 to 0.07780	19.31 to 2420	0.001236 to 0.09087	0.4703 to 2.118	1.282 to 4.270
Significance (P value)	<0.0001	0.0104	0.0003	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.9757	0.0151
P value Summary	****	*	***	****	****	****	****	***	ns	*
Model Accuracy (% Correct)	-	93.75	-	97.50	-	82.50	-	96.25	-	72.50
Area Under ROC Curve	Combined	0.9851	Combined	0.9639	Combined	0.8844	Combined	0.9600	Combined	0.7563
Model Fit (Hosmer-Lemeshow)	Combined	0.8906	Combined	0.3601	Combined	0.6563	Combined	0.3105	Combined	0.3190

Test P value)										
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Appendix 8. The Least Significant Difference (LSD) test to pairwise comparisons between SOWS score and Gene expressions

Gene	Comparison	Mean Difference	Std. Error	p-value	95% CI	Note
<i>DNMIL</i>	0-10 vs 10-20	7.33	0.90	<0.001	(5.54 to 9.13)	Significant increase
	0-10 vs 20-30	9.08	0.75	<0.001	(7.59 to 10.57)	Significant increase
	10-20 vs 20-30	1.75	1.07	0.107	(-0.39 to 3.88)	Not significant
<i>OPRM1</i>	0-10 vs 10-20	-3.30	0.89	<0.001	(-5.07 to -1.53)	Significant decrease
	0-10 vs 20-30	-4.62	0.74	<0.001	(-6.09 to -3.15)	Significant decrease
<i>RAB22A</i>	0-10 vs 10-20	4.18	0.73	<0.001	(2.72 to 5.63)	Significant increase
	0-10 vs 20-30	4.29	0.61	<0.001	(3.08 to 5.50)	Significant increase
<i>COMT</i>	0-10 vs 10-20	6.32	0.75	<0.001	(4.83 to 7.81)	Significant increase
	0-10 vs 20-30	6.62	0.62	<0.001	(5.38 to 7.86)	Significant increase
<i>DRD2</i>	0-10 vs 10-20	-5.99	0.74	<0.001	(-7.47 to -4.51)	Significant decrease
	0-10 vs 20-30	-7.06	0.62	<0.001	(-8.28 to -5.83)	Significant decrease

*Note: CI = Confidence Interval. The mean difference is significant at the 0.05 level.

Appendix 9. ANOVA Effect Sizes for differential gene expression in pairwise comparisons with SOWS score group means

Gene	Eta-squared (η^2)	Epsilon-squared (ϵ^2)	Omega-squared (ω^2) Fixed-effect	Omega-squared (ω^2) Random-effect
<i>DNMIL</i>	0.707 (95% CI 0.587 to 0.773)	0.700 (95% CI 0.576 to 0.767)	0.697 (95% CI 0.573 to 0.765)	0.535 (95% CI 0.402 to 0.619)
<i>OPRM1</i>	0.377 (95% CI 0.201 to 0.502)	0.361 (95% CI 0.180 to 0.489)	0.358 (95% CI 0.178 to 0.486)	0.218 (95% CI 0.098 to 0.321)
<i>DRD3</i>	0.885 (95% CI 0.833 to 0.911)	0.882 (95% CI 0.829 to 0.909)	0.881 (95% CI 0.827 to 0.908)	0.787 (95% CI 0.705 to 0.831)

Note: Eta-squared and Omega-squared values represent the proportion of the total variance that is attributable to the gene. Values closer to 1 indicate a larger effect size. Confidence intervals (CI) provide a range in which the true effect size is likely to fall with 95% certainty.

Appendix 10. Summary of Statistical Analyses for Gene Expression by Heroin Dose

Gene	ANOVA F (df1, df2)	ANOVA Sig.	Effect Size (Cohen's d)	Eta-squared (η^2)	Eta-squared 95% CI	Omega-squared (ω^2)
<i>DNMIL</i>	19.433 (2, 77)	< 0.001	-1.895	0.335	[0.161, 0.465]	0.187
<i>OPRM1</i>	11.418 (2, 77)	< 0.001	1.467	0.229	[0.073, 0.365]	0.115
<i>OPRD1</i>	4.756 (2, 77)	0.011	-0.023	0.110	[0.006, 0.236]	0.045

<i>OPRK1</i>	3.228 (2, 77)	0.045	0.128	0.077	[0, 0.194]	0.027
<i>RAB22A</i>	23.701 (2, 77)	< 0.001	-1.495	0.381	[0.204, 0.506]	0.221
<i>COMT</i>	12.869 (2, 77)	< 0.001	-2.116	0.251	[0.090, 0.386]	0.129
<i>BDNF</i>	1.264 (2, 77)	0.288	0.284	0.032	[0, 0.123]	0.003
<i>DRD1</i>	0.601 (2, 77)	0.551	-0.200	0.015	[0, 0.087]	-0.005
<i>DRD2</i>	9.979 (2, 77)	< 0.001	2.285	0.206	[0.057, 0.342]	0.101
<i>DRD3</i>	15.250 (2, 77)	< 0.001	-2.690	0.284	[0.116, 0.418]	0.151
<i>DRD4</i>	1.833 (2, 77)	0.167	-0.276	0.045	[0, 0.146]	0.010
<i>DRD5</i>	13.046 (2, 77)	< 0.001	2.590	0.253	[0.092, 0.389]	0.131

Note: The ANOVA significance (Sig.) indicates the probability that the observed differences between groups occurred by chance. Cohen's d provides a standardized measure of effect size. Eta-squared (η^2) and Omega-squared (ω^2) represent the proportion of variance explained by group differences. The 95% confidence interval for eta-squared is provided to show the precision of the effect size estimate. Negative effect sizes indicate a decrease in gene expression with increased heroin dose.

Appendix 11. Summary of Statistical Analyses for Gene Expression by Age of Onset of Heroin Use

Gene	ANOVA (Between Groups)	Mann-Whitney U	Effect Size (Cohen's d)	Effect Size 95% CI	Significance
<i>DNM1L</i>	F(2, 77) = 19.433	147.000	-1.895	[-2.464, -1.317]	***
<i>OPRM1</i>	F(2, 77) = 11.418	200.000	1.467	[0.921, 2.005]	***
<i>OPRD1</i>	F(2, 77) = 4.756	627.000	-0.023	[-0.514, 0.468]	*
<i>OPRK1</i>	F(2, 77) = 3.228	575.000	0.128	[-0.363, 0.619]	
<i>RAB22A</i>	F(2, 77) = 23.701	191.000	-1.495	[-2.035, -0.947]	***
<i>COMT</i>	F(2, 77) = 12.869	118.000	-2.116	[-2.704, -1.519]	***
<i>BDNF</i>	F(2, 77) = 1.264	445.000	0.284	[-0.210, 0.776]	
<i>DRD1</i>	F(2, 77) = 0.601	576.500	-0.200	[-0.691, 0.293]	
<i>DRD2</i>	F(2, 77) = 9.979	93.000	2.285	[1.673, 2.888]	***
<i>DRD3</i>	F(2, 77) = 15.250	136.000	-2.690	[-3.332, -2.038]	***
<i>DRD4</i>	F(2, 77) = 1.833	578.500	-0.276	[-0.768, 0.217]	
<i>DRD5</i>	F(2, 77) = 13.046	52.000	2.590	[1.948, 3.222]	***

$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Note: The effect size is based on Cohen's d, with the 95% confidence interval listed for each gene. The significance column indicates the p -value level for the ANOVA between groups comparison.