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Research Article

GENE EXPRESSIONS ASSOCIATED WİTH NEUROTRANSMITTER METABOLISM IN CHILDREN WITH ATTENTION DEFICIT HYPERACTIVITY DISORDER

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ABSTRACT

Objective: To investigate the expression levels of genes (*SLC6A3, SLC6A4, SLC1A2, SLC18A2, MAOA, COMT, GLYAT, GRM5, DRD4, TPH1, and ADRA2C*) associated with attention deficit hyperactivity disorder (ADHD) by pre and post-treatment with methylphenidate to see if they may serve as biomarkers in the etiopathogenesis of diseases.

Materials and Methods: Thirty-five ADHD-diagnosed children and 38 healthy controls were included and divided three groups as control, pre-treatment and post-treatment group. Following total RNA isolation from participants' peripheral blood samples, cDNA was synthesized via reverse transcription and used for qPCR analyses.

Results: Elevated *SLC6A3* and decreased *SLC6A4, SLC1A2, SLC18A2, ADRA2C, MAOA, COMT, GLYAT, DRD4* and *TPH1* genes expression levels of childiren with ADHD were detected ($p<0.01$). In post-treatment group, while *SLC6A3* and *COMT* expression levels decreased, the expression levels of *SLC6A4, SLC1A2, SLC18A2, ADRA2C, MAOA, GLYAT, GRM5* and *TPH1* significantly increased ($p<0.01$). Highlighted relationships among gene expressions levels of control, pre-treatment, and post-treatment groups were detected. Additionally, cut-off values with diagnostic power for *SLC6A3, SLC6A4, SLC1A2, SLC18A2, ADRA2C, MAOA, COMT, GLYAT, DRD4,* and *TPH1* were detected.

Conclusion: The expression levels of the *SLC6A3, SLC6A4, SLC1A2, SLC18A2, ADRA2C, MAOA, COMT, GLYAT, DRD4,* and *TPH1* genes may play an important role in the formation, prognosis and etiopathogenesis of the disease, and these changes can also be used as biomarkers in the differential diagnosis and development of treatment strategies for the disease.

Keywords: ADHD, methylphenidate, gene expression, qPCR

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INTRODUCTION

Attention Deficit Hyperactivity Disorder (ADHD) is an early-onset, heterogeneous neuropsychiatric disorder whose etiology and pathogenesis are still largely unknown. It is characterized with inattention, hyperactivity, impulsivity and incompatible with age and developmental levels (1). The incidence and prevalence of ADHD were reported as 0.061% and 1.13%, respectively (2). In Turkey, ADHD prevalence and male/female ratio were found to be 13.8% and 2/1, respectively (3). ADHD is multifactorial disorder related with genetic, epigenetic and biological signatures (3). The prefrontal cortex, caudate nucleus, globus pallidus, corpus callosum, and cerebellum volumes decreased in individuals with ADHD (4). Genetic changes may affect cortical functions in the prefrontal area, dopamine (DA) level in the synaptic region, or dopaminergic receptor function. Although the pathophysiology of ADHD states that the change in dopaminergic and noradrenergic pathways is not related to attention and impulse control, studies have focused on catecholaminergic norepinephrine (NE) and DA (5).
Drugs for ADHD are effective on NE and DA release in the prefrontal cortex. In the treatment of ADHD, stimulants drugs such as methylphenidate (MPH) and non-stimulants drugs such as atomoxetine are commonly used. Psychostimulants act by preventing the reuptake of NE and DA from the synapse gap to the presynaptic neuronal space and increasing the release of monoamines into the extraneuronal space (6).

In addition to disclosing the roles of genetic variants in the etiopathogenesis of diseases (7-10), the exploration of both the expression levels of the genes (11-13) and their products (8,14-16) are crucial to understand their function and role in cellular homeostasis, viability and heterogeneous neuropsychiatric disorder. Most studies based on related candidate genes, meta-analyses, genome-wide association studies (GWAS), omics data, transcriptome profiling show that dopaminergic, serotonergic, and glutamatergic signaling, neuronal transmission, neuronal migration, and cell adhesion pathways etc. play a role in the etiology of ADHD (17-22). It is important overrepresentation of candidate genes previously studied in ADHD selected from the gene list provided by the ADHD gene database (http://adhd.psych.ac.cn/index.do) and a comprehensive search for published reviews of ADHD genetic and pharmacogenetic studies (23).Therefore, the genes *solute carrier family 6 member 3 (SLC6A3), solute carrier family 6 member 4 (SLC6A4), solute carrier family 1 member 2 (SLC1A2), solute carrier family 18 member A2 (SLC18A2), monoamine oxidase A (MAOA), catechol-O-methyltransferase (COMT), glycine-N-acyltransferase (GLYAT), glutamate metabotropic receptor 5 (GRM5), dopamine receptor D4 (DRD4), tryptophan hydroxylase 1 (TPH1)* and *adrenoceptor alpha 2C* (*ADRA2C)* that roles in the neurotransmitter pathway were included in the current study. Thus we aim to investigate the role of the mRNA expression profiles of these genes to see if they may serve as biomarkers in the etiopathogenesis of diseases and the possible effect of stimulant drugs such as MPH on the expression of these candidate genes.

MATERIALS AND METHODS

After the study was approved by the Local Ethics Committee (Erciyes University Local Ethic Committee's approval document dated TSD-12-4112 dated 28.07.2017), informed consents were obtained from all of the patients and their parents. The 35 boys between 6-12 years old who applied to the Pre-Interview Policlinic of Erciyes University Faculty of Medicine, Department of Child and Adolescent Mental Health and Diseases and newly diagnosed as ADHD according to DSM IV (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) diagnostic criteria and K-SADS-PL (Schedule for Affective Disorders and Schizophrenia for School-Age Children – Present and Lifetime version) criteria by an experienced psychiatrist and drug-naïve were included as patients group. The Clinical Global Impression-Severity (CGI-S) scale was $\geqslant$4. A dosage range of 0.3 – 1 mg/kg MPH was prescribed depending on the weight and age of patients, prognosis and severity of the diseases by an experienced psychiatrist. 10 ml peripheral blood samples with EDTA was taken from the patient group using MPH (0.3-1 mg/kg) both before and after treatment and from the control group.

One month after the treatment had started, blood samples were taken after the ADHD symptoms had been evaluated. Also 38 boys age matched (6-12 years) withouth any psychiatric or chronic medical diseases were included as a control goup in the study. The blood samples of individuals in the control group were also taken for expression analysis of the targeted genes. The demonstrative example of the study protocol was given in the figure 1.

Blood collection and leukocytes isolation

Blood samples were processed within 24 h of being collected in EDTA tubes. Leukocyte isolation was performed using Erythrocyte buffer. (Qıagen,cat

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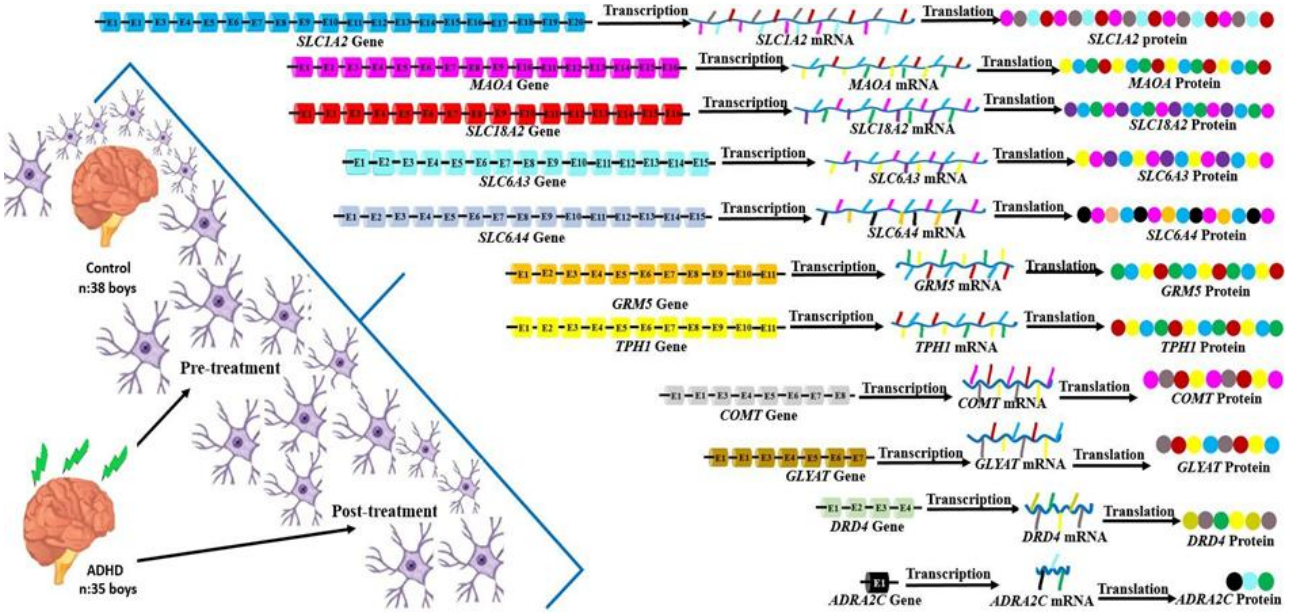

Figure 1. The demonstrative example of the study protocol

no:79217,Germany). Leukocytes were stored at −80 °C in Trizol (QIAzol, cat no:79306, Germany) until RNA extraction.

Table 1. Probe sequences and assay ID used in Real-time PCR

Gene	Assay ID	Primer sequence (5′-3′)
SLC6A3	143278	F;CAGACACCGTGAGCTCTTCA R;CAGGAGCGTGAAGACGTAGAT
SLC6A4	112029	F;AAGTTCAACAACAACTGCTACCAA R;GAAGCTCGTCATGCAGTTCA
SLC1A2	111792	F;CCTGCCAACAGAGGACATC R;GACTGAAGTTCTCATCCTGTCCA
SLC18A2	112188	F;CGAACTTTGGAGTTGGTTTTG R;CCCATGATAGGCATCATTGAC
ADRA2C	145162	F;CAGGAGCTTGGCAGAGAGAT R;GAAGGCAAAGGGGTCTCC
MAOA	113315	F;GGAGGTGGCATTTCAGGAC R;AAAACCAAAACACTAACGCCATA
COMT	112923	F;GTCTTCCTCGACCACTGGAA R;TCACGTTGTCAGCCAGTAGC
GLYAT	118559	F;TGGCCTGATTTTAATACAGTGG R;TTGGTATAGTGATCAAGGTCATCTG
GRM5	100941	F;CCTGCCAACAGAGGACATC R; GACTGAAGTTCTCATCCTGTCCA
DRD4	112907	F;CAGACTCCACCGCAGACC R;GTGATGTGCACCACGAAGAA
TPH1	113010	F;GGACTTATAAAAGCCCTGAAAATCT R; TTCGGGACTCGATATGTAACAG
B.ACT	101125	F;GGCCAGGTCATCACCATT R;GGATGCCACAGGACTCCAT

RNA isolation and cDNA synthesis

After the leukocyte isolation, RNA isolation and cDNA synthesis were performed from individuals in ADHD and control group (1xRBC LysisBuffer, Invitrogen. Leukocytes were taken into 1000 µL of TriPure reagent (Roche Applied Science, Basel, Switzerland) for RNA isolation, and total RNA isolation was performed according to the protocol (24). The quality and quantification of RNA samples were detected with measurement of RNA concentrations and optical density at 260 and 280 nm using Nanodrop 1000 (Thermo Fisher Scientific, Germany). The cDNA was synthesized via the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) following the manufacturer's protocol.

Relative gene expressions of the *SLC6A3, SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT, GRM5, DRD4, TPH1* and *ADRA2C* Genes by Real-Time qPCR

From the obtained cDNA samples, the mRNA expression levels of SLC6A3, SLC6A4, SLC1A2, VMAT2, MAOA, COMT, GLYAT, GRM5, DRD4, TPH1 and ADRA2C genes were determined by Real-Time qPCR method using the Roche LightCycler® 480 instrument II (Roche Diagnostics Ltd., Rotkreuz, Switzerland). Real-Time PCR mixture including 10 µl LightCycler 480 Probes Master, 1 µl RealTime ready Assays, 4µl dH2O was prepared. Temperatures and times set in LightCycler 480 II software were as follows; 1 cycles of preincubation is at 95°C for 10min (Temperature increase-decrease rate (oC/sec):4.4}, 45 cycles of amplification at 95°C for 10sec, at 60°C for 30sec and at 72°C for 1sec {Temperature increase-decrease rate (oC/sec):4.4/2.2 and 4.4, respectively}, 1 cycles of Cooling at 40°C for 30sec {Temperature increase-decrease rate (oC/sec):2.2} in 20µL total reaction volume. Real-Time qPCR was performed using cDNA, UPL probe,

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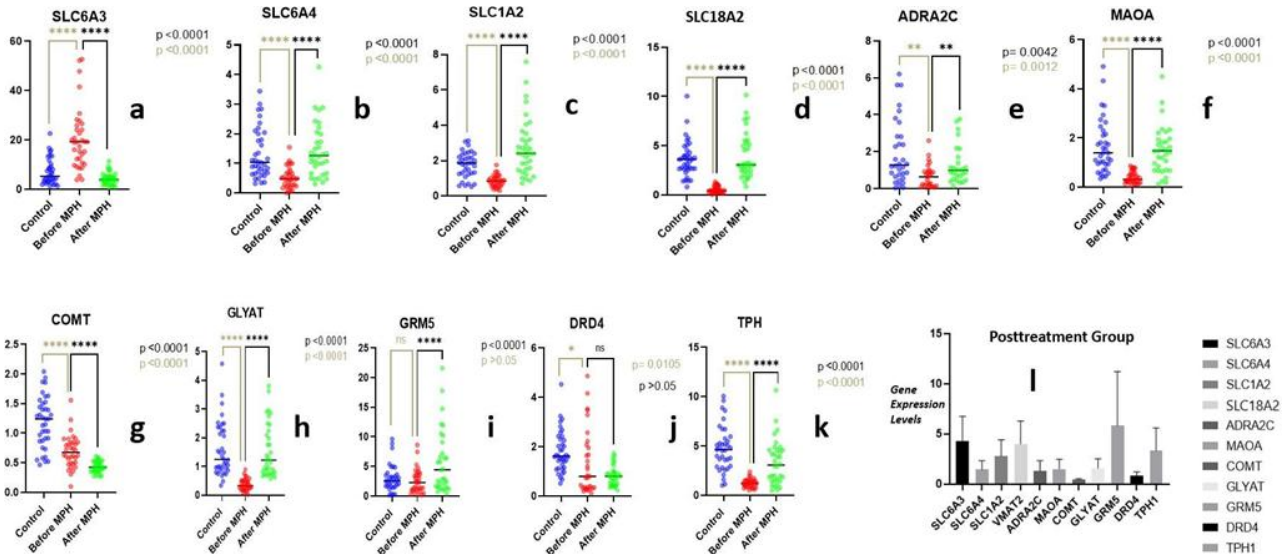

Figure 2. Comparison of groups in terms of expression levels of genes

LightCycler 480 Probes Master mix (Roche Diagnostics, Germany), hydrolysis probes and distilled water. Each sample was run in duplicate, with two negative controls and one calibrator used twice in each run. RealTime ready Catalog (Roche Diagnostic GmbH, Mannheim, Germany) primer-probe kits specific to each gene were used in the study. Analyzes were made with Light Cycler 480 Software (release 1.5.0 SP4) and 2−ΔΔCt levels of the genes were detected.

The Beta-actin mRNA expression level was taken as a reference to normalize *SLC6A3*, *SLC6A4*, *SLC1A2*, *SLC18A2*, *MAOA*, *COMT*, *GLYAT*, *GRM5*, *DRD4*, *TPH1*, and *ADRA2C* gene expression levels. Expression levels of target genes were calculated according to the relative quantification method using the software program of the LightCycler 480 device. The Universal Probe Library (UPL) probe numbers that are specific to the cDNAs of the investigated genes are given in Table 1.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism software (Graphpad Prism Inc., version 9.4.1, California, USA). Outlier values were determined by the route (Q = 1%) method, and all analyses were performed with the cleaned data. According to the distribution of the data deteced by Shapiro-Wilk test, while the Wilcoxon matched-pairs signed rank test or paired t-test was used to compare dependent groups, the Mann-Whitney test or unpaired t-test was used to compare independent groups. The correlation between genes in each group was determined by Spearman's correlation coefficient. Bayesian statistics, based on receiver operating

Table 2. CGI-S, sex and comorbidities of both pre and post-treatment ADHD patients

Patient	Pre-treatment CGI-S	Post-treatment CGI-S
1	5	1
2	4	2
3	5	2
4	6	2
5	5	2
6	5	2
7	5	1
8	6	2
9	5	1
10	5	2
11	5	2
12	5	1
13	5	2
14	6	2
15	5	1
16	5	2
17	5	2
18	5	1
19	6	2
20	6	1
21	5	2
22	5	1
23	5	1
24	5	2
25	5	1
26	4	1
27	4	1
28	6	2
29	6	1
30	6	1
31	5	2
32	6	2
33	5	1
34	6	2
35	5	2

CGI-S: The Clinical Global Impression-Severity scale (1 = normal, not at all ill; 2 = borderline ill; 3 = mildly ill; 4 = moderately ill; 5 = markedly ill; 6 = severely ill; or 7 = among the most extremely ill.)

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Table 3. Positive and negative correlations among SLC6A3, SLC6A4, SLC1A2, SLC18A2, MAOA, COMT, GLYAT, GRM5, DRD4, TP ADRA2C genes expression levels in the groups. *p<0.05, **p<0.001

	Groups	*SLC6A3*	*SLC6A4*	*SLC1A2*	*SLC18A2*	*ADRA2C*	*MAOA*	*COMT*	*GLYAT*	*GRM5*	*DRD4*
Control Group	*SLC6A3*	1	0.47*	0.14	0.30	0.66**	0.08	0.21	0.10	0.06	0.10
	SLC6A4	0.47*	1	0.12	0.68**	0.19	0.43*	0.21	0.46*	-0.11	0.26
	SLC1A2	0.14	0.12	1	0.02	0.07	-0.03	-	-0.14	0.07	0.24
	SLC18A2	0.30	0.68**	0.02	1	-0.06	0.28	-0.03	0.49*	-0.06	0.24
	ADRA2C	0.66**	0.19	0.07	-0.06	1	-0.10	0.08	-0.11	-0.09	-0.25
	MAOA	0.08	0.043*	-0.03	0.28	-0.10	1	0.31	0.28	0.19	0.42*
	COMT	0.21	0.21		-0.03	0.08	0.31	1	0.24	0.18	0.17
	GLYAT	0.10	0.46*	-0.14	0.49*	-0.11	0.28	0.24	1	-0.26	0.16
	GRM5	0.06	-0.11	0.07	-0.06	-0.09	0.19	0.18	-0.26	1	0.23
	DRD4	0.10	0.26	0.24	0.24	-0.25	0.42*	0.17	0.16	0.23	1
	TPH1	0.19	0.39*	0.16	0.43*	-0.22	0.28	-0.05	0.32	0.10	0.29
		SLC6A3	*SLC6A4*	*SLC1A2*	*SLC18A2*	*ADRA2C*	*MAOA*	*COMT*	*GLYAT*	*GRM5*	*DRD4*
Pre-treatment Group	*SLC6A3*	1	0.26	0.31	0.20	0.21	0.15	-0.11	0.19	-0.06	0.01
	SLC6A4	0.26	1	-0.10	0.73**	0.17	0.29	-0.10	0.44*	0.21	0.10
	SLC1A2	0.31	-0.10	1	0.24	-0.15	0.23	0.45*	0.05	0.12	-0.10
	SLC18A2	0.20	0.73**	0.24	1	0.10	0.14	0.08	0.26	0.31	0.02
	ADRA2C	0.21	0.17	-0.15	0.10	1	0.14	-0.18	0.23	0.06	-0.15
	MAOA	0.15	0.29	0.23	0.14	0.14	1	0.27	0.12	0.08	-0.04
	COMT	-0.11	-0.10	0.45*	0.08	-0.18	0.27	1	-	0.13	0.24
	GLYAT	0.19	0.44*	0.05	0.26	0.23	0.12	-	1	0.01	0.17
	GRM5	-0.06	0.21	0.12	0.31	0.06	0.08	0.13	0.01	1	-0.12
	DRD4	0.01	0.10	-0.10	0.02	-0.15	-0.04	0.24	0.17	-0.12	1
	TPH1	-0.02	0.05	0.31	0.35*	-0.23	0.11	0.25	0.05	0.51*	-0.15
		SLC6A3	*SLC6A4*	*SLC1A2*	*SLC18A2*	*ADRA2C*	*MAOA*	*COMT*	*GLYAT*	*GRM5*	*DRD4*
Post-treatment Group	*SLC6A3*	1	0.10	0.09	0.46*	0.18	0.22	0.20	0.20	0.14	0.02
	SLC6A4	0.10	1	0.52*	0.46*	0.34	0.26	-0.15	0.59**	-0.09	-0.06
	SLC1A2	0.09	0.52*	1	0.30	0.18	0.17	-0.12	0.24	0.07	-
	SLC18A2	0.46*	0.46*	0.30	1	0.06	0.35*	-	0.43*	0.33	-0.28
	ADRA2C	0.18	0.34	0.18	0.06	1	0.12	-0.08	0.33	-0.13	0.52*
	MAOA	0.22	0.26	0.17	0.35*	0.12	1	-0.01	0.21	-0.14	0.22
	COMT	0.20	-0.15	-0.12	-	-0.08	-0.01	1	-0.19	0.09	0.25
	GLYAT	0.20	0.59**	0.24	0.43*	0.33	0.21	-0.19	1	-0.02	0.08
	GRM5	0.14	-0.09	0.07	0.33	-0.13	-0.14	0.09	-0.02	1	-0.45*
	DRD4	0.02	-0.06	-	-0.28	0.52*	0.22	0.25	0.08	-0.45*	1
	TPH1	0.14	0.29	0.29	0.40*	0.10	-0.08	-0.14	0.22	0.01	-0.20

characteristic (ROC)-derived cutoff values was performed to determine the diagnostic power of genes in ADHD. The data were given as n, mean, Standard deviation (SD), minimum, maximum, the area under the ROC curve (AUC), Confidence Interval (CI), Cut-off specificity, sensitivity, r and p, The level of significance was set at p<0.05.

RESULTS

All of the individuals in the groups were male. None of the participants have comorbidities. Statistically significant differences were not found between the patients and control group for mean age (8.91± 0.31 vs 9.11± 0.31) and weight, (31.80 ± 1.98 vs 35.18 ± 2.19), respectively (p>0.05). The Clinical Global Impression-Severity (CGI-S) scale was ≥4. In the pre-treatment group, CGI-S of 22 participants was 5, CGI-S of 10 participants was 6 and CGI-S of 3 participants was 4. After the MPH (0.3-1 mg/kg) treatments by an experienced psychiatrist, CGI-S of 15 participants was detected as 1 and CGI-S of 20 participants was detected as 2. The CGI-S, sex and comorbidites of both pre and post-treatment ADHD patients given in the table 2.

A comparison of the groups in terms of expression levels of genes is shown in figure 2. The only expression levels of *SLC6A3* gene was statistically higher in the pre-treatment patient group than the control group (p<0.0001) (Figure 2a). The significantly decreased mRNA levels of *SLC6A4* (p<0.0001) (Figure 2b), *SLC1A2* (p<0.0001) (Figure 2c), *SLC18A2* (p<0.0001) (Figure 2d), *ADRA2C* (p=0.0012) (Figure 2e), *MAOA*(p<0.0001) (Figure 2f), *COMT* (p<0.0001) (Figure 2g), *GLYAT* (p<0.0001) (Figure 2h), *DRD4* (p<0.0105) (Figure 2j) and *TPH1* (p<0.0001) (Figure 2k) were detected in the pre-treatment group compared to the control group. There is no significant differences between the pre-treatment patient and control group for *GRM5* gene expression levels (p>0.05) (Figure 2i). The posttreatment correlation of the all genes were given in the figure 2l.

When the pre-and post-treatment groups were compared, significantly decreased mRNA levels of the *SLC6A3*

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Table 4. Bayesian statistics, based on ROC derived cutoff values of the genes

Genes	AUC (95% CI)	Cut-off	p	Sensitivity%	Specificity%
SLC6A3	0.8723 (0.7911 to 0.9534)	10.47	<0.0001	77.14	76.47
SLC6A4	0.8351 (0.7451 to 0.9252)	0.675	<0.0001	76.47	76.32
SLC1A2	0.8571 (0.7630 to 0.9513)	1.149	<0.0001	81.82	80
SLC18A2	0.9934 (0.9796 to 1.000)	1.24	<0.0001	96.97	97.3
ADRA2C	0.7419 (0.6164 to 0.8674)	0.863	0.0015	62.96	62.5
MAOA	0.9363 (0.8842 to 0.9883)	0.672	<0.0001	82.35	83.33
COMT	0.8274 (0.7302 to 0.9246)	0.857	<0.0001	79.41	78.95
GLYAT	0.9705 (0.9360 to 1.000)	0.675	<0.0001	93.94	94.59
GRM5	0.5843 (0.4429 to 0.7257)	2.424	0.2429	56.25	57.58
DRD4	0.6791 (0.5383 to 0.8200)	1.44	0.0109	64.52	65.79
TPH1	0.9615 (0.9140 to 1.000)	1.838	<0.0001	90.91	91.89

AUC: Area under curve, CI: Confidence interval

(p<0.0001) (Figure 2a) and *COMT* (p<0.0001) (Figure 2g) were detected in the post-treatment group. Although the *DRD4* gene expression level was lower in the post-treatment group than pre-treatment group, the difference was not significant (p>0.05) (Figure 2j). Conversly, expression levels of *SLC6A4* (p<0.0001) (Figure 2b), *SLC1A2* (p<0.0001) (Figure 2c), *SLC18A2* (p<0.0001) (Figure 2d), *ADRA2C* (p=0.0042) (Figure 2e), *MAOA* (p<0.0001) (Figure 2f), *GLYAT* (p<0.0001) (Figure 2h), *GRM5* (p<0.0001) (Figure 2i) and *TPH1* (p<0.0001) (Figure 2k) genes significantly increased in the post-treatment group compared to the pre-treatment group (Figure 2). Spearman correlation analysis that shows relationship the expression levels of the related genes in the control, pre-treatment, and post-treatment groups were given in the table 3.

In the control group, significant positive correlations between the *SLC6A3* gene and both of the *SLC6A4* and *ADRA2C* genes (r=0.47, p=0.0053; r=0.66, p=0.000058, respectively), between the *SLC6A4* gene and all of the *SLC18A2, MAOA, GLYAT,* and *TPH1* genes (r=0.68, p=0.000003; r=0.43, p=0.0089; r=0.46, p=0.0037; r=0.39, p=0.0189, respectively), between the *SLC18A2* gene and both of the *GLYAT* and *TPH1* genes, respectively (r=0.49, p=0.0023; r=0.43, p=0.009, respectively), and between the *MAOA* gene and the DRD4 gene (r=0.42, p=0.011) were detected. Conversly, the relation between the *COMT, SLC1A2, GRM5* genes and other genes were not significant (Table 2).

When the pre-treatment group to be taken into consideration, significant positive correlations between the *SLC6A4* and both of *SLC18A2* and *GLYAT* genes (r=0.73, p<0.001; r=0.44, p<0.05, respectively), between the *SLC1A2* gene and *COMT* gene (r=0.45, p<0.05), between the *SLC18A2* gene and *TPH1* gene(r=0.35, p<0.05), and between the *GRM5* gene and the *TPH1* gene (r=0.51, p<0.05) "were detected. Conversly, the relation between the *SLC6A3, ADRA2C, MAOA, DRD4* genes and other genes were not significant (Table 2).

When the post-treatment group to be considered, significant positive correlations between the *SLC6A3* gene and *SLC18A2* genes (r=0.46, p<0.05), between the *SLC6A4* gene and all of the *SLC1A2, SLC18A2* and *GLYAT* genes (r=0.52, p<0.05; r=0.46, p<0.05; r=0.59, p<0.001, respectively), between the *SLC18A2* gene and all of the *MAOA, GLYAT* and *TPH1* genes (r=0.35, p<0.05; r=0.43, p<0.05; r=0.40, p<0.05, respectively), and between the *ADRA2C* gene and *DRD4* gene (r=0.52, p<0.05) were detected. Conversly, the relation between the *SLC1A2, COMT, GRM5* genes and other genes were not significant (Table 2).
Bayesian statistics, based on receiver operating characteristic (ROC)-derived cutoff values, which allows diagnostic power of the genes were given in tables 4 and figure 3.

DISCUSSION

As a neurodevelopmental disorder, ADHD can lead to functional impairment that primarily manifests in childhood and has effects over an individual's lifespan. Individuals with ADHD may show the different level of impairment, and inheritance patterns can be complex (25).

MPH is a drug commonly used to treat ADHD. MPH inhibits the reuptake of DA and NE, increasing the level of

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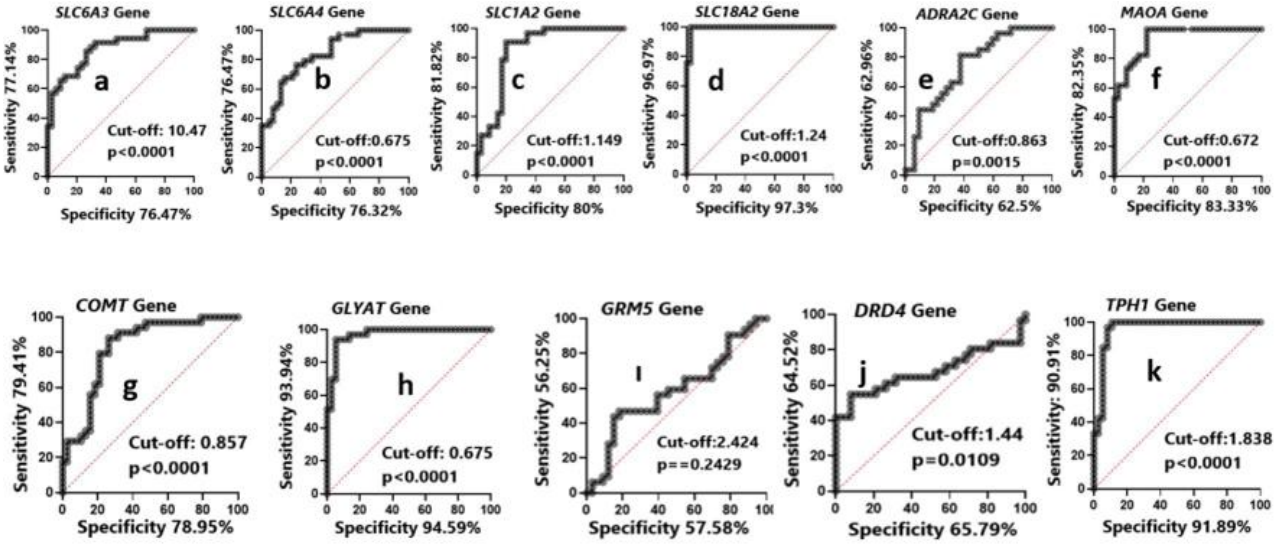

Figure3. ROC derived cutoff values, specificity and sensitivity of the genes

these neurotransmitters in the synaptic cleft. DA is associated with reward and motivation systems, while NE plays an important role in focusing attention. MPH increases central dopamine and norepinephrine activity and may reduce distractibility and impulsivity in individuals with ADHD (5,26).

Many meta-analyses on ADHD sensitivity and MPH response do not show consistent results (Boncivini et al., 2016). Also, pharmacological studies on candidate genes indicate that *SLC6A3* as a key molecular target in drugs containing MPH and atomoxetine for ADHD (27). Grünblatt et al. showed that the expression level of the *SLC6A3* gene in patients is higher than in healthy individuals (28). Because of the high levels of *SLC6A3* in the internal globus pallidus caused a decrease in dopamine levels, it was concluded that neuronal circuits, effective in initiating behavior, are affected, and thus impulsive behaviors emerge (29). In our study, it was observed that the expression level of the *SLC6A3* gene before treatment increased almost two-fold ($p \leq 0.001$) in patients compared to the control group. But after MPH treatments, the reduced expression level of *SLC6A3* was observed ($p \leq 0.05$). This shows that this drug act by regulating the expression of this gene. When the CGI-S to be taken into consideration, CGI-S of 22 participants was 5, CGI-S of 10 participants was 6 and CGI-S of 3 participants was 4 in the pre-treatment group. After the MPH (0.3-1 mg/kg) treatments, CGI-S of 15 participants was detected as 1 and CGI-S of 20 participants was detected as 2. Thus it may be said that, the MPH act by regulating the expression levels of those genes.

The *SLC6A4* gene provides the reuptake of serotonin from the synaptic gap on the presynaptic membrane. Therefore, *SLC6A4* concentration in the membrane is one of the most important factors determining the amount of synaptic serotonin. Also, H3 serotonylation has an important role in coordinating placental transcription at the intersection of maternal physiology and offspring brain development (30). While Grünblatt et al. showed that there was no diffirences between the adult control and patient groups with ADHD in the *SLC6A4* gene expression levels (29). Sener et al. detected significant differences between healthy childeren and age matched patients group with autism spectrum disorders in terms of *SLC6A4* gene expression levels (31). The detection of lower expression in the patient group suggests a deficiency in serotonin reuptake. Allelic variants in the serotonin transporter gene (*SLC6A4*), lower transcriptional efficiency, changes in serotonin concentration in various brain regions, and differences in *SLC6A4* mRNA expression have been associated with the development of ADHD (32). In the current study, the expression level of the *SLC6A4* gene was lower the patient group than control before MPH treatment, but after MPH treatment the expression level of the *SLC6A4* gene increased three times ($p \leq 0.001$) in patient group and reached the expression level of the control group.

SLC1A2 as a a brain-specific gene, encodes a glutamate transporter with high affinity in astroglial cells, is related the occuring of individuals with a high degree of brain disorder. It is known that the expression levels of *SLC1A2* changes in the glutamatergic system in the brain, especially in psychiatric disorders. The loss of *SLC1A2* expression in cases with seizures may has some possible epileptogenic role (33,34). Decreases in the expression level of this gene have been observed in many human and animal depression models (35). The dysregulation of *SLC1A2* causes amyotrophic lateral sclerosis, Alzheimer's

disease and epilepsy, as well as psychiatric disorders such as depression and autism(36). In this study, while the expression level of the *SLC1A2* gene was lower the patient goroup than control before MPH treatment (p≤0.001), Conversely after MPH treatment the expression level of the *SLC1A2* gene significantly increased (p≤0.05) in patient group.

The *SLC18A2* gene has a very important role in the storage and synaptic release of all monoamines, including serotonin. The changes in *SLC18A2* level are associated with depression, bipolar disorder, schizophrenia, Parkinson's disease (37). Tourette syndrome, alcohol addiction, ADHD symptoms in children and cognitive consequences after traumatic brain injuries in adults (38-40). In mouse models lacking *SLC18A2*, DA intake and release into vesicles were reduced by more than 80% and pathophysiologically, dopaminergic adrenergic, cellular oxidative stress, alterations in alpha-synuclein accumulation, and behaviorally decreased mobility, increased depressive mood and sleep disturbances were observed (41-44). It was reported that over-expression of *SLC18A2* resulted an increase in the uptake and relase of DA into vesicles (100%, 80%, respectively), increased mobility, anxiety and decreased depressive behaviors. High *SLC6A3* and low *SLC18A2* levels will theoretically result in cytosolic DA accumulation and minimal DA release. Also, low cytosolic DA concentration and high extracellular DA would arise from low *SLC6A3* and high *SLC18A2* levels (45). Our current study showed that the expression level of the *SLC18A2* gene was lower in the patient goroup than control before MPH treatment (p≤0.001). But after the MPH treatment, the significantly elevated expression level of the *SLC18A2* gene was detected in patient group (p≤0.001).

ADRA2C plays a role in the regulation of NE release from sympathetic nerves and drenergic neurons in the central nervous system. Noradrenergic neurons play a role in modulating wakefulness, regulation of visual attention, learning, and memory (46). Cho et al. showed a connection between the *ADRA2C* (GT) repeat polymorphism and ADHD (47). Even though Barr et al. were unable to establish a link between the same polymorphism and ADHD, they stated that other stronger SNPs in the *ADRA2C gene* are related to ADHD and should be examined (48). In the current study we detected the two fold higher *ADRA2C* expression level in control than patients group before MPH treatment (p≤0.001). After the MPH treatment, significantly elevated *ADRA2C* gene expression levels were found in patient group (p≤0.05).

MAOA roles in breaking down monoamine neurotransmitters such as DA, 5-hydroxytryptamine and NE. *MAOA* enzyme level is known to affect human behavior and characteristics (49). It has been reported that *MAOA* polymorphisms are associated with the hyperactive/impulsive ADHD type and the development of borderline personality disorder (50). Weder et al. showed a correlation between exposure to moderately traumatic conditions during childhood, low *MAOA* gene expression, and the risk of aggressive behavioral problems (51). In the current study, the six times higher expression level of the *MAOA* gene in control group than patient group was detected before MPH treatment (p≤0.001). After the MPH treatment significantly elevated *MAO* gene expression levels of patient group, which reached those of the control group were detected.(p≤0.001).

COMT plays a role in the inactivation of catecholamines, including DA (52) and has been seen as a focal point in studies including expression analyses, SNP and protein studies (53). Chen et al.,was not found significant difference in *COMT* expression for age and disease parameters. Although the Val158Met and the alterations in the 3 'end region are important risk factors for schizophrenia, the presence of these SNPs does not have a significant effect on mRNA expression. Researchers believe that the functional effect of *COMT* has more complex genetic bases because they cannot explain differences in protein studies and enzyme activities with mRNA expression (54). In another ADHD study, a general decrease in the surface area of the total premotor cortex was observed in males (55). Our study showed the significantly lower *COMT* expression level in patient group than control before MPH treatment (p≤0.001). But after MPH treatment, a significant increase of *COMT* gene expression level in patient group was observed (p≤0.001). Glycine-N-acyltransferase, which is responsible for metabolizing some metabolites, encoded by *GLYAT* gene in cells. Drugs are primarily metabolized to Acyl-CoA intermediates. The glycine-N-acyltransferase enzyme catalyzes the combination of mitochondrial Acyl-CoAs with glycine (56). Studies about the *GLYAT* gene on drug metabolism have been carried out, but they have not been focused on individuals with ADHD. Thus we think that the our current study has great importance. According to the our findings, the expression levels of *GLYAT* gene in patient group were lower from half of the expression leveles of the control group before the MPH treatment (p≤0.02). After the MPH treatment, the expression levels of the *GLYAT* gene significantly increased in patient group and reached the expression levels of the control grou(p≤0.01).

Glutamate is the main stimulating neurotransmitter in the brain and plays a role in several ADHD-related processes, such as brain development, modulation of neuronal activity, bidirectional regulation of dopamine signaling, synaptic flexibility, memory formation and learning (57). *GRM5* appears to be critical for inhibitory learning mechanisms because impaired receptor function causes inappropriate retention of deterrent memories that can lead to anxiety disorders (58). Deletion in the CNV region of *GRM5*, one of the glutamate metabotropic receptor genes, has been associated with the presence of comorbid anxiety (59). The lower expression levels of *GRM5 gene* were observed in patients with autism (60). In the current study, the expression level of *GRM5* gene was found to be lower in the patient group compared to the control group before MPH treatment, but this decrease was not statistically significant ($p>0.05$). After the MPH treatment, significantly increased expression level of *GRM5* gene in patient group were seen compared to pretreatment ($p \leq 0.001$).

DRD4 is one of the dopaminergic system genes and DA receptors most associated with ADHD. Grünnblatt et al., found the higher *DRD4* and *DRD5* gene expression levels in ADHD patients compared with controls (28). Our study compatible the literature and significantly higher expression levels of *DRD4* gene were detected in patient gourp compared the control group ($p<0.05$) befor MPH treatment. After MPH treatment, there was a decrease in the expression level of the *DRD4* gene in the post-treatment group compared to the pre-treatment group, but it was not statistically significant ($p>0.05$).

TPH1 encodes a rate-limiting enzyme in the biosynthesis of the monoamine neurotransmitter serotonin. It was reported that *TPH1* and *TPH2* polymorphisms are associated with ADHD. According to a study, while no difference was found for the expression levels of *TPH1* gene between children with ADHD and healthy individuals (19), another study reported that the higher expression levels of *TPH1* gene in patient group with ADHD than the control group (28). According to the our results, significantly lower *TPH1* gene expression levels in patients were found than control group before the MPH treatment ($p\leq0.01$). Hovewer after MPH treatment elevated expression levels of *TPH1* gene was detected in post-treatment group that reached the control group and significantly higher than pre-treatment group ($p\leq0.01$).

Why the expression levels of the genes mentioned above differ between children with ADHD and the control group may be caused from variants in the regulatory regions of genes as well as epigenetic regulatory mechanisms such as DNA methylation, histone modifications and micro-RNAs etc. Therefore, post-transcriptional regulators activate and inactivate the translation of mRNA in some cases. Although methylations generally has a silencing effect by suppressing the transcription of the gene, methylation of a regulatory region can sometimes lead to an increase in the gene product (61). In the current study, the expression levels of genes (*SLC6A3, SLC6A4, SLC1A2, SLC18A2, MAOA, COMT, GLYAT, DRD4* and *TPH1*), which are called candidate genes in the literature, differed between ADHD patients and the control group. The *SLC6A3* gene expression level was found to be higher in children with ADHD compared to controls and this elevation was reduced and reached the expression level of the control group by medical treatments with MPH ($p\leq0.01$). Expression levels of *SLC6A4, SLC1A2, SLC18A2, MAOA, COMT, GLYAT, GRM5* and *TPH1* genes were found to be lower in children with ADHD compared to controls, and this decrease was increased with medical treatments of MPH and reached the expression level of the control group ($p\leq0.01$). Variations and changes in the expression levels of the several common and specific genes may play an important role in the formation, prognosis and etiopathogenesis of diseases, and these changes can also be used as biomarkers in the differential diagnosis of diseases and the development of treatment strategies for them.

Bayesian statistics, ROC-derived cutoff values analysis results showed that the expression levels of genes, except the *GRM5* gene, have diagnostic power in ADHD. Especially the expression levels of *SLC18A2* (cut-off:1.24, 96.97% sensitivity and 97.3% specificity), *GLYAT* (cut-off:0.675, 93.94% sensitivity and 94.59% specificity) and *TPH1* (cut-off:1.838, 90.91% sensitivity and 91.89% specificity) genes in blood can be an important candidate diagnostic marker with higher (over 90%) specificity and sensitivity for diagnostic purpose in the etiopathogenesis of diseases.

Our study suggests a potential association between those candidate *SLC6A3, SLC6A4, SLC1A2, SLC18A2, ADRA2C, MAOA, COMT, GLYAT, DRD4,* and *TPH1* genes' expressions and ADHD. Additionally MPH, drug commonly used to treat ADHD, has possible effect on the expression of these candidate genes. It may be said that the expression levels of those genes may play an important role in the formation, prognosis and etiopathogenesis of the disease and can also be used as biomarkers in the differential diagnosis and development of treatment strategies for the disease. However, our study has certain limitations, including a small sample size, the inclusion of only male children, and the lack of control over

environmental factors. Given these limitations, validation studies with larger sample sizes and in different populations are needed to confirm our findings. To gain more definitive insights on this topic, additional studies with large cohorts should be conducted.

CONCLUSION

The findings of our study highlight the SLC6A3, SLC6A4, SLC1A2, SLC18A2, ADRA2C, MAOA, COMT, GLYAT, DRD4, and TPH1 genes affected by MPH treatment and the differences in the expression levels of these genes contribute to a better understanding of the molecular mechanisms in ADHD. In this context, the genes in question can be considered as potential biomarkers that may affect clinical outcomes with the support of further research.

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Authorship contributions
HA and YE developed the theory and concept of the study. YO and MD designed the study. MD, NG, SO, and MED investigated and supervised the findings of this work. HA wrote the manuscript with support from IOS, NG, SO, MED, YO, and MD. RE, IOS and NG performed the statistical analysis. MD, HA and SO verified the analytical methods. HA, RE, IOS, NG, and YE made literature searches. MED, SO and HA performed clinical studies. SO analyzed and interpreted the patient data. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

Data availibity statement
The data and material can be available from the corresponding author.

Declaration of competing interest
No conflict of interest was declared by the authors.

Ethics
The study received approval from the Local Ethics Committee of Erciyes University, as outlined in the approval document dated 05.06.2012, with reference number 2012/366.

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