

Expression of Reactive Oxygen Species–Related Transcripts in Egyptian Children With Autism

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ABSTRACT: The molecular basis of the pathophysiological role of oxidative stress in autism is understudied. Herein, we used polymerase chain reaction (PCR) array to analyze transcriptional pattern of 84 oxidative stress genes in peripheral blood mononuclear cell pools isolated from 32 autistic patients (16 mild/moderate and 16 severe) and 16 healthy subjects (each sample is a pool from 4 autistic patients or 4 controls). The PCR array data were further validated by quantitative real-time PCR in 80 autistic children (55 mild/moderate and 25 severe) and 60 healthy subjects. Our data revealed downregulation in *GCLM*, *SOD2*, *NCF2*, *PRNP*, and *PTGS2* transcripts (1.5, 3.8, 1.2, 1.7, and 2.2, respectively; $P < .05$ for all) in autistic group compared with controls. In addition, *TXN* and *FTH1* exhibited 1.4- and 1.7-fold downregulation, respectively, in severe autistic patients when compared with mild/moderate group ($P = .005$ and $.0008$, respectively). This study helps in a better understanding of the underlying biology and related genetic factors of autism, and most importantly, it presents suggested candidate biomarkers for diagnosis and prognosis purposes as well as targets for therapeutic intervention.

KEYWORDS: Autism, gene expression, oxidative stress, neurodevelopmental disorders, PCR array, ROS

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Introduction

Autism is a set of childhood neurodevelopmental disorders that affect young children (<3 years old) with severe manifestations, such as impaired social skills, abnormal verbal and non-verbal communication, and restricted-repetitive behaviors.¹ The fifth edition of *Diagnostic and Statistical Manual of Mental Disorders* (DSM-V) considered the autistic disorder, pervasive developmental disorders not otherwise stated, and the Asperger syndrome as a single disorder with variable severity in the communication impairment and/or the repetitive behaviors domain.² Autism prevalence is on rise over the recent few years (current global incidence rate is 0.15% versus 0.03% prior to 1990).³ Several causes contribute to the development of autism, including immunological (immune dysregulation),⁴ neurological (neuroinflammation and altered neurotransmission),⁵ and environmental factors,⁶ in addition to the genetic background of the host.⁷ Gene variants associated with autism are myriad and regional specific⁸; they can even be perpetuated by some factors such as consanguineous marriage which is highly prevalent in Egypt.⁹ For instance, a study found a specific *BCKDK* gene variant mutation among autistic offspring in some consanguineous families where certain amino acids may relieve symptoms of this rare form of autism.¹⁰ Oxidative stress is the underlying mechanism that links different causes and is the main determinant of autism development and progression.^{11–13}

Oxidative stress refers to a pathologic state arisen from the imbalance between the cellular reactive oxygen species (ROS) and the ability of the cell to detoxify them with the resulting severe damage of all macromolecules (protein, lipid, and DNA) and disruption in several signaling pathways.¹⁴ Endogenous and dietary antioxidants combat oxidative stress through controlling the levels of ROS produced, scavenging excess ROS, and repairing the oxidative damaged biomolecules.¹⁵ The brain is highly exposed to increased oxidative stress due to the presence of excitatory amino acids whose catabolism ends with the production of ROS causing neuronal damage.¹⁴ Thus, increased oxidative stress is a primary risk factor for the pathophysiology of many neuropsychiatric disorders such as the Parkinson disease, the Alzheimer disease, the Huntington disease, and multiple sclerosis.^{16,17} Numerous oxidative stress markers (antioxidant enzymes, lipid peroxidation, and protein/DNA oxidation) were detected in abnormal levels in autistic children, such as protein dityrosine.^{18,19} Several studies have documented reduced levels of glutathione, glutathione peroxidase, methionine, and cysteine besides elevated levels of oxidized glutathione in children with autism.^{12,20} The excreted antioxidants are lower in autistic patients compared with healthy age-matching subjects and upon correlation with severity of the disease, such as superoxide dismutase.^{21,22} Also, ceruloplasmin and transferrin antioxidants show suboptimal levels in serum



of autistic children, leading to abnormal metabolism of toxic and oxidative stress–mediating metal ions.²³ Recent genetic studies have identified variants of some antioxidant enzyme-coding genes that increase the susceptibility to autism. For example, the interaction between glutathione S-transferase P1 and glutathione S-transferase M1 mutated genes contributed to autism risk.²⁴

Autism is complicated by the absence of both specific medical diagnostic tests and definitive drug therapy. Its diagnosis is based mainly on the presence of abnormal behaviors associated with the disease,⁵ and the current therapeutic approaches aim to ameliorate those behavioral deficits.²⁵ Although autism is associated with a high degree of heritability, there is a big research gap in studying the autism-driving genes. Identifying those panels of genes will help in developing reliable biomarkers for early diagnostic and therapeutic purposes. Transcriptome analysis identified new messenger RNA (mRNA) putative markers; for instance, a study developed a panel of 66 genes involved in neurological processes that showed significant dysregulation in autism.²⁶ Similarly, proteome analysis revealed some autism-associated biomarkers, such as urinary kininogen 1.²⁷

However, the transcriptional profile of most antioxidant enzyme families remains understudied in autism. In this study, we sought to uncover the molecular basis of the pathophysiological role of oxidative stress in autism. We investigated the mRNA expression of several oxidative stress-related transcripts, noninvasively, in peripheral blood mononuclear cells (PBMCs) derived from children having autism with varying degrees using pathway-focused polymerase chain reaction (PCR) array. Transcriptional profile of several genes was altered in autistic patients compared with healthy controls. We then validated the altered mRNA abundance of 8 key signaling molecules in a larger number of patients by quantitative real-time polymerase chain reaction (qRT-PCR).

Materials and Methods

Ethical statement

All experiments were approved by the institution ethical review board (medical research ethics committee at National Research Centre, Cairo, Egypt) according to Helsinki Declaration 1975 revised in 2008. Written informed consent was obtained from the caregiver of each child before collecting blood samples. All studied cases (55 mild/moderate and 25 severe autistic patients in addition to 60 controls) were recruited from Autism Disorders Clinic, Medical Research Center of Excellence, and National Research Centre. Exclusion criteria included all subjects with other causes of mental subnormality, delayed language, and all patients receiving antioxidants. The typically developing (TD) controls included 60 age-matched children (3–6 years) sampled from 1 common kindergarten with no abnormal histories of motor, language, or social developmental disorders, as determined according to reports of parents and teachers. The 80 autistic cases included 60 boys (75%) and 20

Table 1. Classification of the autistic children according to the Childhood Autism Rating Scale score.

DEGREE OF AUTISM	NO. OF CASES	%
Mild to moderate	55	68.75
Severe cases	25	31.25

girls (25%) along with frequency-matched controls. In addition, 22 autistic children (27.5%) had consanguineous parents. All patients were subjected to the following. (1) Detailed history taking including 3-generation pedigree analysis, pregnancy history, perinatal history, developmental history, similarly affected family members, and age of onset of presenting manifestation. (2) Clinical diagnosis was based on the criteria for autistic disorder as defined in the DSM-V² and Autism Diagnostic Interview–Revised.²⁸ The Childhood Autism Rating Scale is used for assessing autism severity,²⁹ and it was completed by the caregivers of children with autism. Total scores were calculated for each child with autism (Tables 1 and 2). Criteria of selection for study were defined as Comprehensive Diagnostic Evaluation. This included looking at the child's behavior and development and parents' interview. It also included a hearing screening and neurological examination.

RNA extraction

RNA isolation was performed on freshly collected blood samples (3 mL) following the steps of the single-step method,³⁰ in which we lysed and discarded red blood cells and then mixed the formed pellets with fixed ratios of guanidinium thiocyanate, sodium citrate, 2-mercaptoethanol, chloroform isoamyl alcohol, phenol, and sodium acetate; then, we precipitated, washed, and dissolved the RNA using isopropanol, 70% ethanol, and nuclease-free water, respectively. This was followed by quantification of the recovered RNA using a Thermo Scientific NanoDrop spectrophotometer.

PCR array

Eight hundred nanograms of RNA was reverse transcribed into complementary DNA (cDNA) using the RT² PCR Array First Strand Kit (SABiosciences, Valencia, CA, USA); 102 μ L of the total 111 μ L synthesized cDNA was mixed with 1150 μ L RT² SYBR Green/ROX qPCR master mix (SABiosciences), and nuclease-free water was added to adjust the final volume to 2300 μ L; 20 μ L of the PCR mix was added to each well of the 100-well rounded plate. The PCR array used in this study was the human oxidative stress RT² Profiler PCR Array (SABiosciences). The thermal profile was as follows: initial incubation for 10 minutes at 95°C (AmpliTaq Gold pre-activation) and then at 95°C for 15 seconds and 60°C for 1 minute repeated for 40 cycles. The PCR run was performed on Rotor Gene Real-Time PCR System (Qiagen, Santa Clarita, CA, USA).

Table 2. Comparison between the ADI-R domains according to the severity of autism among the autistic cases.

	DEGREE OF CARS		P VALUE
	MEAN ± SD		
	MILD TO MODERATE	SEVERE	
Social domain	19.5 ± 4.7	21.8 ± 2.2	.05
Verbal domain	11.2 ± 3	9.3 ± 4.5	.4
Nonverbal domain	8.5 ± 2.4	8.5 ± 3.8	.6
Repetitive domain	5.8 ± 2.7	4.8 ± 1.7	.1

Abbreviations: ADI-R, Autism Diagnostic Interview–Revised; CARS, Childhood Autism Rating Scale.

Similar threshold was set for all qRT-PCR runs, and the cycle threshold (C_T) values were exported to a blank data analysis excel sheet to proceed with statistical analysis. The *RPLP0* housekeeping gene (HKG) was selected for normalization of data because it showed the smallest C_T value across different samples. The statistical analysis of PCR array data was performed using the SABiosciences web-based software: (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

We entered the C_T data and the software automatically quantified the mRNA abundance using $2^{(-\Delta\Delta CT)}$ method in which the C_T of the HKG is subtracted from each gene of interest to calculate ΔC_T ; then the mean ΔC_T of all samples in control group was calculated and subtracted from ΔC_T of each experimental sample (mild or severe autism) to calculate the $\Delta\Delta C_T$. The fold change is calculated as $2^{(-\Delta\Delta CT)}$.

Real-time polymerase chain reaction

Two hundred nanograms of RNA was used in the reverse transcription step. A master mix (25 μ L) containing 1 μ L of the synthesized cDNA, 1 μ L of gene-specific PCR primer for human *GCLM*, *SOD2*, *TXN*, *NCF2*, *PRNP*, *PTGS2*, *GPX7*, and *FTH1* (10 μ M) (SABiosciences), 12.5 μ L of RT² SYBR Green/ROX qPCR master mix (SABiosciences), and 10.5 μ L of H₂O was incubated for 10 minutes at 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute in rotor gene Real-Time PCR System (Qiagen). The same threshold was set for all the individual primer-based real-time polymerase chain reaction (RT-PCR) runs, the HKG human *RPLP0* (SABiosciences) was used for normalization, and the expression of each individual gene was calculated by the $2^{(-\Delta\Delta CT)}$ method and expressed as fold change over the mean of control group as described above. This was followed by statistical comparison.

Statistical analysis

The statistical comparison of the data was performed using Prism software version 5. The data were analyzed by the parametric unpaired *t* test or the nonparametric Mann-Whitney *U*

test according to the normal distribution curve. Data were presented as the mean and standard error of the mean unless mentioned. Statistically significant differences between the groups were considered if $P \leq .05$.

Results

Transcriptional profiling of oxidative stress-related genes in PBMCs of autistic children

An initial explorative experiment to identify the most regulated ROS genes was performed using pathway-focused PCR array, including 84 oxidative stress-related genes. The relative gene expression of the 84 transcripts was measured in PBMC RNAs from 16 mild autistic children, 16 severe autistic children, and 16 age-matched healthy children (RNAs from each 4 synonymous subjects were pooled in 1 sample to perform the PCR array experiment; these 32 cases and 16 controls are subsets of the total 80 autistic and 60 normal children enrolled in the study). The selected array had primers for transcripts encoding peroxidases, peroxiredoxins, antioxidants, oxidative stress-responsive proteins, and oxygen transporters in addition to genes involved in superoxide and ROS metabolism (Table 3). Only 13% of the arrayed genes ($n = 11$) showed altered expression in mild/moderate or severe autistic children when compared with the control group (greater or less than 1.5-fold differential regulation associated with statistical cutoff at $P \leq .05$; Table 3). Three oxidative stress responsive genes (*CCL5*, *DHCR24*, and *GCLC*) besides glutathione peroxidase (*GPX7*) showed dysregulation in mild autistic group compared with controls. *CCL5* and *GCLC* were downregulated (1.7- and 1.8-fold downregulation, respectively; Table 4), whereas *DHCR24* and *GPX7* were upregulated (2.5- and 1.5-fold upregulation, respectively; Table 4). We observed a trend of downregulation in most of the differentially regulated transcripts of severe autistic group when compared with controls (*GCLM*, *SOD2*, *TXN*, *NCF2*, *PRNP*, *PTGS2*, and *FTH1*; the mean fold regulation ranges from -1.5 to -4.3 ; Table 4) and upregulation of a single transcript (*GPX7*; 1.6-fold regulation; Table 4). However, none of the 84 genes showed more than 1.5-fold regulation plus statistical significance at $P \leq .05$ between mild and severe autism. Together, these results suggested that, regardless of the severity of the disease, autism amended the antioxidative state in PBMCs.

Quantitative RT-PCR of dysregulated ROS transcripts confirms the data of explorative PCR array

We further validated the PCR array data on a larger cohort size (60 healthy subjects and 80 autistic patients [55 mild cases and 25 severe cases]) using gene-specific qRT-PCR assay. We selected the 8 genes that showed differential regulation in severe autistic patients compared with controls (*GCLM*, *SOD2*, *TXN*, *NCF2*, *PRNP*, *PTGS2*, *GPX7*, and *FTH1*) for the

Table 3. Differential gene expression of oxidative stress genes by RT-PCR array.

GENE ID	FOLD REGULATION (MILD AND MODERATE) ^a	FOLD REGULATION (SEVERE) ^a	GENE ID	FOLD REGULATION (MILD AND MODERATE) ^a	FOLD REGULATION (SEVERE) ^a
Glutathione peroxidases			<i>BNIP3</i>	1.0619	1.0473
<i>GPX1</i>	-1.2687	-1.0497	<i>MPV17</i>	-1.0668	-1.0668
<i>GPX2</i>	-1.1355	-1.8618	Oxidative stress-responsive genes		
<i>GPX3</i>	-1.162	-1.5619	<i>TXN</i>	-1.47	-2.1735
<i>GPX4</i>	-1.4224	-1.0817	<i>KRT1</i>	-1.6424	-1.4373
<i>GPX5</i>	1.0041	1.3379	<i>LPO</i>	-2.2076	-2.2605
<i>GPX6</i>	1.633	-1.1674	<i>TPO</i>	-1.0122	-2.7895
<i>GPX7</i>	1.4515	1.5511	<i>MPO</i>	-2.2423	-1.3947
<i>GSTP1</i>	-1.2709	-1.0093	<i>GCLM</i>	-1.5324	-1.651
<i>GSTZ1</i>	-1.3021	-1.2255	<i>MSRA</i>	-1.4658	-1.632
Peroxiredoxins			<i>CAT</i>	-1.5692	-1.4012
<i>PRDX1</i>	1.1467	-2.555	<i>CCL5</i>	-1.7112	-1.5087
<i>PRDX2</i>	1.0749	1.2924	<i>DUSP1</i>	-1.5404	-1.8361
<i>PRDX3</i>	-1.2491	-1.8965	<i>HMOX1</i>	1.0151	-1.0595
<i>PRDX4</i>	1.3195	-1.2628	<i>TXNRD2</i>	1.3558	-2.7959
<i>PRDX5</i>	-1.2262	-1.217	<i>ATOX1</i>	1.0347	1.014
<i>PRDX6</i>	-1.4389	-1.3629	<i>HSPA1A</i>	1.1076	-1.8877
Other peroxidases and antioxidants			<i>TXNRD1</i>	-1.2333	-2.2089
<i>CYBB</i>	-1.4273	-1.5227	<i>DHCR24</i>	2.4566	1.1355
<i>MGST3</i>	-1.3457	-1.2658	<i>NQO1</i>	1.065	-1.4743
<i>PTGS1</i>	-1.1534	-1.162	<i>NUDT1</i>	-1.1348	1.007
<i>PTGS2</i>	-2.0837	-4.2673	<i>TTN</i>	1.127	-1.1728
<i>PXDN</i>	11.0171	5.5277	<i>GCLC</i>	-1.7994	-2.2191
<i>ALB</i>	2.9862	3.6723	<i>GSS</i>	-1.0187	1.2058
Superoxide dismutases			<i>APOE</i>	2.3349	2.2089
<i>SOD1</i>	-1.276	-1.1121	<i>OXR1</i>	-1.1218	-2.0046
<i>SOD2</i>	-1.3964	-2.4852	<i>OXSRI</i>	-1.3605	-1.6702
<i>SOD3</i>	-2.7023	-1.6663	<i>FOXMI</i>	2.0777	2.3403
Other genes involved in superoxide metabolism			<i>PDLIM1</i>	1.2226	1.0968
<i>NCF1</i>	-1.5819	-2.1287	<i>PNKP</i>	-1.0246	-2.0753
<i>NCF2</i>	-1.8758	-1.8704	<i>EPX</i>	1.2072	-1.2198
<i>NOS2</i>	1.0975	-2.9214	<i>PRNP</i>	-1.7151	-1.9816
<i>NOX4</i>	4.542	1.3379	<i>FTH1</i>	-1.5538	-1.454
<i>NOX5</i>	1.7573	1.3379	<i>RNF7</i>	-1.2782	-1.3348
<i>PREX1</i>	-1.4931	-1.6702	<i>SCARA3</i>	-1.8628	-3.539
<i>UCP2</i>	1.2433	1.1892	<i>VIMP</i>	-1.1264	-1.5192

GENE ID	FOLD REGULATION (MILD AND MODERATE) ^a	FOLD REGULATION (SEVERE) ^a	GENE ID	FOLD REGULATION (MILD AND MODERATE) ^a	FOLD REGULATION (SEVERE) ^a
<i>DUOX1</i>	1.7901	-1.1303	<i>SEPP1</i>	-1.2376	-1.6058
<i>DUOX2</i>	1.0662	-1.2658	<i>GSR</i>	-1.0413	-1.4473
<i>MT3</i>	1.6606	1.9274	<i>SIRT2</i>	-1.0081	-1.3787
<i>CCS</i>	-7.8173	1.146	<i>SQSTM1</i>	-1.5755	-1.6857
<i>ALOX12</i>	1.2205	1.2775	<i>SRXN1</i>	-1.6663	-3.0596
<i>GTF2I</i>	-1.5764	-1.6283	<i>STK25</i>	-1.0041	-1.2058
Other genes involved in ROS metabolism			<i>MBL2</i>	-1.2534	-1.2255
<i>AOX1</i>	-2.6558	-1.4175	Oxygen transporters		
<i>SFTPD</i>	-1.5502	-1.7818	<i>CYGB</i>	-1.6682	-3.8906
<i>EPHX2</i>	1.0099	-1.0595	<i>MB</i>	2.5257	1.1355

Abbreviations: PBMCs; peripheral blood mononuclear cells; ROS, reactive oxygen species; RT-PCR, real-time polymerase chain reaction.

A negative sign indicates reduced expression in the autistic patients relative to the normal ones.

^aFold regulation was calculated as the ratio of expression of each gene measured in RNA isolated from PBMCs of severe and mild/moderate autistic children relative to nonautistic healthy children (n = 4 per group, and each RNA sample is a pool of 4 samples).

Table 4. Differential regulation of oxidative stress–related genes exhibiting statistically significant differences in autistic patients compared with healthy controls.

GENE SYMBOL	GENE DESCRIPTION	UNIGENE	REFSEQ	DIFFERENTIAL EXPRESSION MILD AND MODERATE/C	P VALUE
<i>CCL5</i>	Chemokine (C-C motif) ligand 5	Hs.514821	NM_002985	-1.7112	.02
<i>GCLC</i>	Glutamate-cysteine ligase, catalytic subunit	Hs.654465	NM_001498	-1.7994	.03
<i>DHCR24</i>	24-dehydrocholesterol reductase	Hs.498727	NM_014762	2.4566	.04
<i>GPX7</i>	Glutathione peroxidase 7	Hs.43728	NM_015696	1.4515	.05
GENE SYMBOL	GENE DESCRIPTION	UNIGENE	REFSEQ	DIFFERENTIAL EXPRESSION SEVERE/C	P VALUE
<i>GCLM</i>	Glutamate-cysteine ligase, modifier subunit	Hs.315562	NM_002061	-1.651	.01
<i>GPX7</i>	Glutathione peroxidase 7	Hs.43728	NM_015696	1.5511	.01
<i>SOD2</i>	Superoxide dismutase 2, mitochondrial	Hs.487046	NM_000636	-2.4852	.01
<i>TXN</i>	Thioredoxin	Hs.435136	NM_003329	-2.1735	.03
<i>NCF2</i>	Neutrophil cytosolic factor 2	Hs.587558	NM_000433	-1.8704	.04
<i>PRNP</i>	Prion protein	Hs.610285	NM_183079	-1.9816	.04
<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Hs.196384	NM_000963	-4.2673	.05
<i>FTH1</i>	Ferritin, heavy polypeptide 1	Hs.712676	NM_002032	-1.454	.05

Differential expression of the most regulated oxidative stress–related genes (fold regulation ≥ 1.5 [upregulation/downregulation] with $P \leq .05$) in mild/moderate (Table 4) or severe (Table 4) autistic patients compared with healthy controls (C). A negative sign refers to downregulation in patients relative to the healthy ones. Genes are arranged in a descending order according to their significant P value.

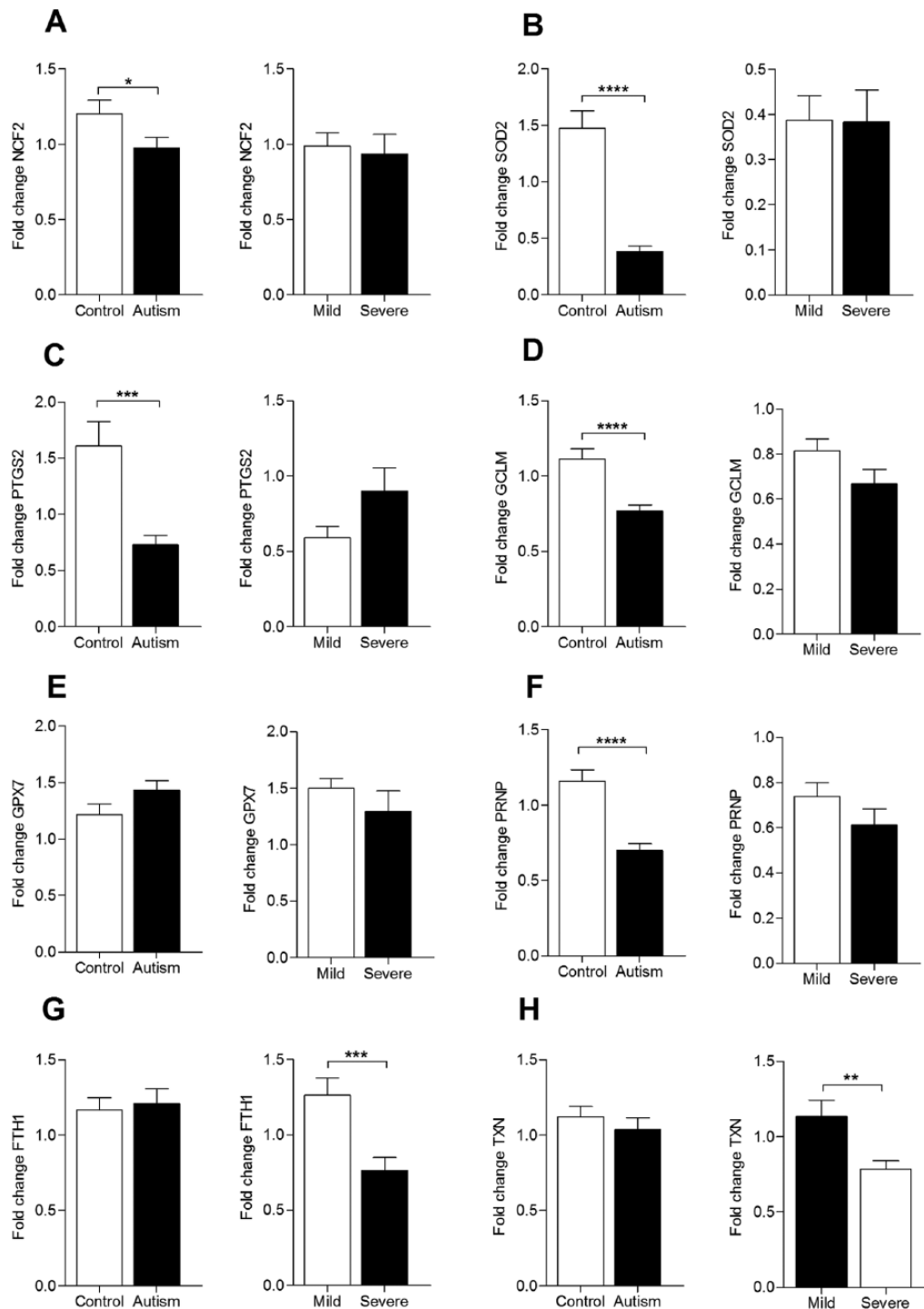


Figure 1. The expression pattern of 8 oxidative stress–related transcripts in autism patients and healthy controls. Quantitative real-time polymerase chain reaction was performed to measure the messenger RNA expression of (A) *NCF2*, (B) *SOD2*, (C) *PTGS2*, (D) *GCLM*, (E) *GPX7*, (F) *PRNP*, (G) *FTH1*, and (H) *TXN* in peripheral blood mononuclear cells of healthy controls ($n = 60$) and patients with mild/moderate autism ($n = 55$) and with severe autism ($n = 25$). The housekeeping gene *RPLP0* was used for data normalization. Data are presented as fold change relative to the average of controls (* $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$).

validation experiment. First, we compared the transcriptional profile of the above 8 genes between autistic patients and controls. The data presented in Figure 1 showed downregulation in 5 transcripts (*GCLM*, *SOD2*, *NCF2*, *PRNP*, and *PTGS2* [1.5, 3.8, 1.2, 1.7, and 2.2, respectively; $P < .05$ for all]) and similar regulation of 3 transcripts (*FTH1*, *TXN*, and *GPX7*) in autistic

patients relative to healthy counterparts. Second, to test for a biomarker for severe autistic presentation, we compared the transcriptional profile of the 8 genes between mild/moderate and severe autistic patients. We noticed a dramatic downregulation of *FTH1* (1.7-fold, $P = .0008$; Figure 1) and *TXN* (1.4-fold, $P = .005$; Figure 1) in severe autistic patients. However,

mRNA abundance of the other transcripts was comparable between the 2 autistic groups.

Discussion

Oxidative stress is implicated in the pathophysiology of many neurological disorders. However, little is known about the linkage between oxidative stress and the occurrence or the progress of autism. The present data showed that autistic patients exhibited transcriptional dysregulation of 5 oxidative stress–regulating genes (*GCLM*, *SOD2*, *NCF2*, *PRNP*, and *PTGS2*) when compared with healthy subjects. Moreover, *FTH1* and *TXN* genes showed altered regulation in different stages of autism; their mRNA abundance was less in severe autistic cases than in mild ones.

Studying the transcriptional profile of brain is limited by the highly invasive brain tissues and the poor quality of RNA recovered from neurological tissues. Therefore, there is a great demand to use alternate noninvasive tissue such as peripheral blood to characterize the neural transcriptome.³¹ The transcriptional profile of PBMCs has been widely studied in several neurological disorders, such as neurofibromatosis type I, tuberous sclerosis type II, and Down syndrome.^{32,33} X-box-binding protein 1 was initially recognized as a genetic risk factor for bipolar disorder based on some gene expression studies performed on lymphoblastoid cell lines derived from 2 discordant twins.³⁴ In autism, studies performed on cell lines derived from autistic children showed that monozygotic twins with autism have differential gene expression in correlation with the disease severity and language impairment.³⁵ The role of peripheral blood cells in understanding transcriptional dysregulation in autism was further confirmed in TD children who showed altered expression of dopamine- and serotonin-related genes in lymphoblastic cells.³⁶

We focused on investigating the antioxidative milieu in autistic children. Brain tissues are rich in redox-active metals and fatty unsaturated lipids that may serve as substrates for lipid peroxidation³⁷; in addition, they show high metabolic rate and oxygen consumption through the mitochondrial respiratory chain³⁸; moreover, they have deficit in antioxidative stress with the consequent altered gene expression and cell apoptosis.¹³ In this study, the elaboration of redox strategies in peripheral cells may have significant relevance in this neuropsychiatric disease.

GPX7 is a member of glutathione peroxidase family that can neutralize H_2O_2 in the absence of glutathione.³⁹ Several studies showed reduced blood levels of glutathione peroxidase in autistic children,⁴⁰ but, however, some studies reported augmented activity level in autism.⁴¹ Our initial explorative experiment showed significant upregulation of *GPX7* gene expression in both mild and severe autistic cases relative to TD controls, which may represent an adaptive mechanism to a probable glutathione deficiency that may ensue from *GCLM* gene downregulation. Although experimental⁴² and clinical⁴³ studies suggested a protective role of *GPX7* against oxidative stress, the

data of the validation experiment showed similar *GPX7* expression in the recruited cohort of autistic children and controls—a finding that requires further confirmation on larger number of cases. Generally, the discrepancies in results obtained from different studies may be attributed to the highly heterogeneous cause of autism. Such heterogeneity is most evident in *SOD* activities in autism.

The blood level of *SOD* in autistic patients did not reach consensus. Some studies showed increased *SOD* activities in erythrocytes,^{41,44} whereas other studies reported unchanged activities of erythrocyte *SOD*.⁴⁵ The currently observed downregulation of *SOD2* transcript in PBMCs of autistic children agrees with the previous literatures that reported reduced levels of *SOD2* protein in the temporal lobe of brain⁴⁶ and plasma⁴⁵ as well as reduced *SOD* activity in erythrocytes of autistic children.⁴⁷ Unlike *SOD1* (CuZn*SOD*) which is mainly located in cytoplasm, *SOD2* is the mitochondrial isoform of *SOD* which uses manganese to scavenge the superoxide radicals produced by mitochondria—the main source of cellular ROS. The downregulation of *SOD2* may explain the mitochondrial dysfunction implicated in autism.⁴⁸

Ferritin is the main iron-storing protein that protects the cell from the toxic effect of iron and from the free radicals leading to oxidative stress.⁴⁹ Ferritin consists of multisubunits of heavy and light chains. *FTH1* gene encodes the heavy chain of ferritin. Because variation in the composition of ferritin subunit highly affects the rates of iron storage and release, our finding of the low mRNA expression of *FTH1* in severe autistic patients may present a possible explanation for the reduced levels of serum ferritin and iron in autistic children that was reported in the literature.^{50–52} *FTH1* also delivers iron to many organs including brain,⁵³ so the reported *FTH1* downregulation may imply iron deficiency in brains of the studied cases. Because iron is essential for brain development,⁵⁴ the current finding suggests a possible role of *FTH1* gene downregulation in pathophysiology of autism. On clinical basis, severe autistic children in our study experienced a range of sleep fragmentation, where the exact mechanism could be due to low serum ferritin. This agreed with a previous study by Youssef et al.⁵⁵

PRNP gene codes for the cellular isoform of the prion protein (PrP^C). PrP^C is expressed by neurons along with other cells where it plays a prominent antioxidant role.⁵⁶ Experimental animal studies revealed that PrP^C knockout mice are highly vulnerable to the toxic effect of oxidative stress agents.⁵⁷ So, the reported downregulation of *PRNP* gene may explain the defective antioxidant status in autism. Because the prion protein plays vital roles in central nervous system development, neuroprotection, and synaptic plasticity,^{58,59} we suggest that the current *PRNP* gene downregulation may underlie the incidence or progression of autism. However, reports describing the regulation of PrP^C in autism are scarce or even absent, and our study may be the first to depict the downregulation of PrP^C-encoding gene (*PRNP*) in autism.

Similarly, our study is the first to report the dysregulation in *NCF2* gene transcription. *NCF2* gene encodes neutrophil cytosolic factor 2, a subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase—the enzyme that produces a burst of superoxide in neutrophil phagosome using NADPH and oxygen. Autistic children exhibited mitochondrial dysfunction associated with low oxidative burst and reduced NADH oxidase level⁶⁰ and enzymatic activity.⁶¹ The present findings confirm the reduced *NCF2* expression in autism, thus highlighting the mitochondrial dysfunction in this neuropsychiatric disease. The combined downregulation of *SOD2* and *NCF2* transcripts may cause mitochondrial dysfunction in our cohort of autistic children.

GCLM gene encodes the modulatory subunit of glutamate-cysteine ligase (*GCL*)—the enzyme that catalyzes the first rate-limiting step of glutathione synthesis.⁶² The present finding displays a downregulation of *GCLM* transcript in the autistic children and supports the findings of Gu et al⁶³ who noticed reduced *GCL* activity in autistic patients and attributed this to the reduced *GCLM* protein expression in their cerebellum tissues. Both findings may explain the glutathione deficiency in children with autism. We also suppose that *GCLM* gene downregulation and the probable subsequent diminished abundance of glutathione may underlie the development of autism as they may weaken the detoxification of xenobiotics that interfere with early postnatal neurodevelopment.^{64,65}

PTGS2 gene encodes cyclooxygenase 2 (*COX-2*).⁶⁶ is a key enzyme in the pathway of prostaglandin synthesis with 2 existing isoforms *COX-1* and *COX-2*. Cyclooxygenase 2 and oxidative stress play in a vicious cycle where the *COX-2* expression is induced under oxidative stress condition⁶⁷ with the consequent production of prostaglandin E₂ that in turn leads to ultimate production of ROS.⁶⁸ Several studies demonstrated elevated levels of plasma prostaglandin E₂ in autistic patients,^{68,69} which in excess leads to neuroinflammation.^{70,71} Unexpectedly, we found diminished mRNA expression of *PTGS2* in autistic children. Regulation of gene expression at the transcriptional level is not always associated with similar pattern of protein expression.^{72,73} Accordingly, *PTGS2* protein might be regulated in autism at either posttranscriptional or posttranslational levels. An alternative explanation is that PBMCs may not properly reflect the *PTGS2* regulation in nervous tissues. Therefore, tissue-specific studies at the protein level are warranted to confirm the current downregulation of *PTGS2* expression in autism.

TXN gene encodes thioredoxin (*TRX*) which functions as an antioxidant with a redox-regulating activity.⁷⁴ Children with autism exhibited elevated levels of serum *TRX* in correlation with the disease severity.^{75,76} Our data of the similar mRNA abundance of *TXN* gene between autistic children and controls as well as reduced levels of *TXN* transcript in severe autistic children suggest that the elevated protein level of *TRX* observed by the other studies is perhaps due to regulation at posttranscriptional level. In addition, we reported for the first time that the level of *TXN* varies with the severity of autism.

In summary, our data possibly expose the link between oxidative stress and autism at the molecular level and present possible novel noninvasive biomarker candidates—a finding that is believed to be so important for autism where definitive diagnostic tests and intervention monitoring tools are not available. Further studies are required on a large sample size to validate the current findings at the protein level to investigate the consequence of the altered transcriptional profile.

Author Contributions

NAM diagnosed the enrolled autistic children, designed the study and revised the paper; **SASG** and **MFM** critically revised the paper; **MKI**, **RMD**, **NGBED**, **THA** and **MH** performed the molecular biology experiments; **MKI** analyzed the data and wrote the paper; and finally **MKEA** designed the molecular experiments, analyzed the data and revised the paper.

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