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CLINICAL STUDY

# The plasma level of glutamic acid decarboxylase 65 (GAD65) increased in severely autistic Iranian children

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**ABSTRACT**
INTRODUCTION: Autism spectrum disorder (ASD) is a neurodevelopmental disorder. The major etiological mechanism lies in glutamatergic/GABAergic imbalance. The aim of this study was to evaluate the plasma levels of glutamic acid decarboxylase 65 (GAD65) protein in mildly and severely autistic patients, and also to compare plasma GAD65 concentration in mild and severe autism.
METHOD: In total, 62 autistic patients (aged 6–9 years) and 17 age-matched neurotypically healthy controls were included in the study. The diagnosis, as well as the level of autism, was assessed by applying the Gilliam Autism Rating Scale. Plasma GAD65 protein level was determined using an enzyme-linked immunosorbent assay (ELISA) kit for GAD65.
RESULTS: Our findings showed no remarkable alteration in plasma GAD65 concentration in patients with mild autism as compared to healthy subjects, while patients with severe autism showed an increased plasma level of GAD65 as compared to healthy controls and mildly autistic patients.
CONCLUSION: Our findings suggest the level of plasma GAD65 to be considered a potential diagnostic biomarker for the severity of autism *(Fig. 2, Ref. 40)*. Text in PDF *www.elis.sk*
KEY WORDS: autism, GAD65, GABA, plasma level, biomarker.

## Introduction

Autism/autism spectrum disorder (ASD) is a neurodevelopmental disorder resulting from abnormalities in brain development. Individuals diagnosed with autism demonstrate a wide range of psychological and behavioral abnormalities including impairment in social interaction, difficulties in verbal and nonverbal social communications, as well as restricted, repetitive patterns of behavior, interests or activities, and resistance to change (1–3).

Symptoms of autism usually appear around the age of three. Autism can occur in a mild or severe form (4). The symptoms of mild autism include strong verbal skills and little behavioral activity. In contrast, severe autism involves people who need intensive medication treatments, as well as cognition and speech therapy strategies. It is to be noted that a person may not be accurately placed in one of these categories, and also the severity of autism can increase or decrease throughout an individual´s life (5, 6).

To develop new prevention and treatment strategies, much research has been done into the understanding of the etiology of autism. Yet, this disorder is still not fully known. Based on the proposed theories, genetic, environmental, and immunological factors may have a crucial role in the pathogenesis of autism (2). Environmental factors such as parental age, job and family, stress, exposure to chemicals and teratogens (thalidomide, valproate, etc.), as well as viral infections during pregnancy are factors associated with autism (7, 8).

The interaction between the immune sand nervous systems begins at the early stages of embryonic development, while the balanced immune system is necessary for the brain to develop successfully (9). It is possible that abnormal activity of the immune system during the sensitive stages of neurodevelopment may lead to the development of autism (10). Research is underway on a wide range of chemical systems, which often results in reporting conflicting outcomes.

One of the recurring themes revealed by neurochemical studies of autistic brains is the impairment of neurotransmitters, including serotonin, dopamine, glutamate, and gamma-aminobutyric acid (GABA) systems (11). Studies suggest that impaired inhibitory neurotransmission and subsequent imbalance in stimulation/inhibition in the developing brain may be a major event in the development of neurodegenerative diseases such as epilepsy and autism (12–14).

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Glutamic acid decarboxylase (GAD) is an enzyme that catalyzes the conversion of glutamate to GABA, and thus plays the key role in in the synthesis of GABA. There are two GAD isoforms with distinct localizations and functions, GAD65 and GAD67, which are named based on their respective molecular weight. GAD65 (glutamic acid decarboxylase isoform 65) is involved in neurotransmission, while GAD67 is involved in the GABA shunt (15). GAD65 alteration has been reported in the plasma of patients with various neurological disorders including chronic cerebellar ataxia, drug-resistant epilepsy, anxiety disorder, and stiff-person syndrome (16–18).

Considering the important role of GAD65 in GABA synthesis and its synaptic transmission, as well as the fact that glutamatergic/GABAergic imbalance is a major etiological mechanism of autism, the aim of this study was to evaluate the plasma levels of GAD65 protein in autistic patients, and also to compare plasma GAD65 concentrations in mild and severe autism.

## Methods

### *Ethical approvement*

The study protocol followed the ethical guidelines of the Declaration of Helsinki (WMA 2013) and confirmed by the ethical committee of Iran University of Medical Sciences (IR.IUMS.REC.1399.613). After a complete explanation of the research process, written consents were obtained from parents, tutors or caregivers of autistic children and controls participating in the study.

### *Participants*

Candidates were children aged between 6 and 9 years. Sixty-two autistic patients (8 females and 54 males) from the psychiatric clinic at the Iran University of Medical Sciences enrolled to contribute to this study by being part of the autistic cohort. The control group included 7 females and 9 males (n = 17). The control subjects were examined for physical and mental health in the pediatric clinic at the Iran University of Medical Sciences and had no physical, neurological, or psychological disorders.

The diagnosis, as well as level of autism, was assessed by the Gilliam Autism Rating Scale (19). Four domains associated with the autism disorder (stereotyped behavior, communication, social interaction, and developmental disturbances) were assessed by the parent-report instrument (GARS test). The total score of the instrument clarified the probability of autism and the degree of its severity. The GARS instruments were filled in by the children's parents and the scores were manually measured.

### *GAD plasma assessment*

Blood samples were collected between 8 to 10 a.m. and were allowed to clot for 30 min at room temperature in sterile plastic tubes. The samples were centrifuged at 4 °C for 15 min at 1,800 rpm and clear plasma was separated and stored at –80 °C until use.

The concentration of GAD65 in plasma was measured using enzyme-linked immunosorbent assay (ELISA) kit for GAD65 (Bioassay Technology laboratory) by following the manufacturer's instructions. A standard curve for the GAD protein was created by plotting the mean absorbance (y-axis) against the protein concentration (x-axis). According to the absorbance value of samples, the protein concentrations were calculated by linear regression of the standard curve.

### *Statistical analysis*

The data were analyzed statistically with SPSS 22.0 software. All data were given as mean ± S.E. and analyzed by nonparametric Kruskal-Wallis test followed by the Mann-Whitney *post hoc* test. The significance was established when the values were lower than 0.05.

## Results

### *Plasma GAD concentration*

Thirty-seven patients with mild autisms and 31 patients with severe autism were diagnosed according to GARS scores. The

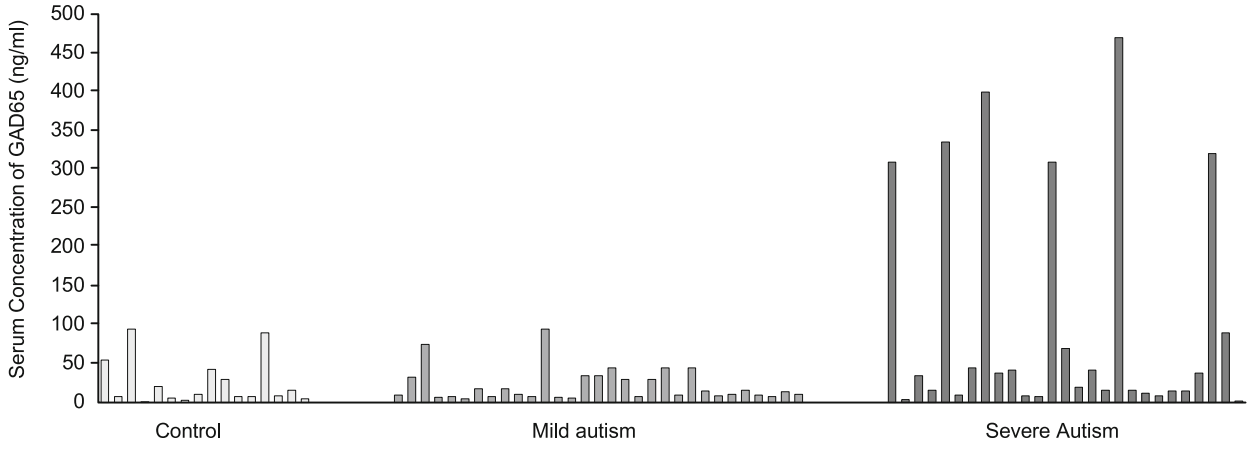


**Fig. 1. GAD65 protein level in the plasma of the control, mild autism and severe autism groups.**

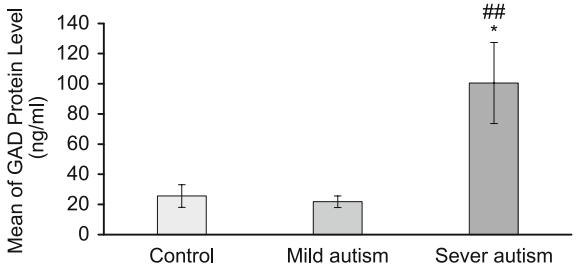


**Fig. 2. Results of the mean plasma concentration of GAD65. Significant increase in GAD65 has been shown in the severe autism group as compared to the control and mild autism groups. The values are means ± SEM. ## p < 0.01 (vs control); * p < 0.05 (vs mild autism).**

plasma level of GAD65 protein was assessed in three groups of subjects (mild autism, severe autism, and control). The plasma level of GAD65 protein is illustrated individually in Figure1.

In addition, the mean level of GAD65 (ng/ml) is illustrated in Figure 2. The mean concentration of GAD65 (ng/ml) was 30.62 ±8.43 in the control subjects, 39.31±9.3 in the mildly autistic patients, and 114.26±30.39 in the severe autism group.

The mean ± S.E.M of GAD65 level significantly increased in the severe autism group as compared to the group with mild autism and control group ($p < 0.05$ and $p < 0.01$, respectively).

**Discussion**

The main purpose of our study was to evaluate the association between alteration of plasma GAD65 protein and autism severity. Our findings showed no remarkable alteration in plasma GAD65 concentration in patients with mild autism, while patients with severe autism showed an increased plasma level of GAD65 as compared to healthy controls and mildly autistic patients.

GAD65 is one of two enzymes that in presynaptic GABAergic neurons catalyze the formation of the neurotransmitter gamma-aminobutyric acid (GABA) from glutamate. GABA is the main neuro-inhibitor in the central nervous system that is released by the GABAergic interneurons during inhibitory neurotransmission and has a crucial role during the early development of the nervous system (13, 20). GABA and glutamate are derived from glutamine and are also able to convert into each other in the glutamine-glutamate/GABA cycle. Therefore, alterations in one of these neurotransmitters can affect the other one (21). Several studies have revealed an imbalance in glutamatergic/GABAergic neurotransmission in neurological disorders such as epilepsy and ASD, which may be is the reason why a significant percentage of autistic patients have comorbid disorders such as epilepsy (22–24).

In a recent study on children with different spectrums of autism, the plasma levels of glutamate, glutamine, and GABA, together with their relative ratios, were measured. The findings revealed an imbalance between GABAergic and glutamatergic modes of neurotransmission in ASD and suggested that an increased plasma level of GABA can be potentially used as an early diagnostic biomarker for ASD. No significant association was found between the autism severity and evaluated biomarkers, i.e., glutamate, GABA, and glutamine (25). Further, the high level of GABA, as well as low levels of glutamate/GABA, in the autistic patients as compared to control subjects have been reported (26).

A similar study on 52 Saudi patients with autism, ranging from 3 to 12 years of age (mean age 7.0±2.34 years), demonstrated a significantly higher level of plasma GABA in patients with autism than in controls. Moreover, in this study, severely autistic patients have shown higher plasma GABA levels as compared to mildly autistic patients (27). Our finding was in line with this study as we found the level of GAD65 to be associated with the severity of autism, since, in the severe autism group, a significant increase in plasma GAD65 was observed in comparison with the mild autism group. Also, in another investigation, the plasma levels of GABA were assessed in 5–15-year-old autistic patients. The data from this study have shown elevated plasma GABA levels in younger autistic patients, and interestingly, it was found that plasma GABA levels tended to decrease with age (28).

In line with previous studies by others, our findings indicated an increase in the plasma level of GAD65 in patients with severe autism. The enhancement of GAD65 which was followed by GABA elevation is thought to be due to the increase in glutamate activity, which is the substrate of glutamate decarboxylase (GAD). GABA and glutamate chemical pathways comprise a set of monolith and interconverted processes. Glutamate is converted into GABA by GAD enzymes. Since glutamate and GABA are constantly converted into each other in the glutamate-GABA metabolic cycle, the changes in one can affect the other (29). This evidence can be supported through the prior reports indicating glutamate elevations in autistic patients (30).

It is noteworthy that despite these finding in support of plasma GABA level elevation in autistic patients, many studies indicate a deficiency in GABA and GAD (both GAD65 and GAD67 isoforms) in different areas of the brain such as cerebellum or cortico-striatal circuitry in association with brain abnormalities (31–34). Indeed, using magnetic resonance spectroscopy studies, a diminution in GABA has been detected in somatosensory, motor, visual and auditory cortices, as well as in the peri-sylvian area of the left hemisphere, which leads to abnormal processing of information and development of complications observed in autism (35, 36).

The plasma GABA level might represent the total or local amounts of GABA in the brain, however, the correlation between plasma, CSF and/or brain GABA level is unclear. Few studies have measured plasma, CSF and brain GABA simultaneously under different pathophysiological conditions to clarify their correlations in different neurological disorders. Nevertheless, so far, no clear association has been found between plasma GABA level and brain and/or CSF (37–40). Our findings might suggest that high plasma level of GAD65 predicted the low level of GAD65, as well as GABA production in the brain of autistic patients. Further studies are needed to clarify this probable relationship.

In conclusion: Our data indicated a notable association between plasma GAD65 level and severity of autism. Based on this

finding, we suggested that the plasma level of GAD65 might be considered as potential biomarker to diagnose autism severity as well as assess treatment efficacy of newly developed strategies of therapy.

**Learning points:**

- The footprint of GABA signaling pathway has been indicated in several neurological disorders containing autism.
- GAD65 (glutamic acid decarboxylase isoform 65) is one of the critical enzymes in the GABA-synthesizing pathway.
- The plasma level of GAD65 has the potential of being considered as a biomarker to determine severity of autism.
- Determination of the severity of autism might help to administer the most effective treatments.

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