

## The Glutamic Acid Decarboxylase (GAD1) Gene Polymorphisms (Rs 3749034 and Rs 11542313) and Their Association to Attention- Deficit\ Hyperactivity Disorder (ADHD)

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### Abstract:

**Objectives:** Attention-deficit hyperactivity disorder (ADHD) is a common childhood-onset neurodevelopmental disorder and may persist into adulthood. ADHD is a complex and heterogeneous disorder with a strong heritability estimation averaging 75% in children. Recent studies recommended a role for the  $\gamma$ -aminobutyric acid (GABA) on ADHD etiology due to behavioral disinhibition caused by inappropriate modulation of glutamatergic and GABAergic signaling. The glutamic acid decarboxylase (GAD1) gene encodes a key enzyme of GABA biosynthesis.

**Aim of the Study:** The study aims to explore the potential association between attention deficit \hyperactivity disorder (ADHD) and glutamic acid decarboxylase (GAD1) gene polymorphisms (rs3749034 and rs11542313).

**Methods:** The present study was done on 20 ADHD families (ADHD children and their biological parents) and 30 healthy children served as control group and were matched by age and gender with ADHD children.

GAD1 SNPs (rs3749034 and rs11542313) were evaluated by Real-time polymerase chain reaction.

**Results:** Most of our children were boys (85%) in school age ( $9.4 \pm 2.6$  years). Regarding the rs3749034 SNP, having the GG allele caused the highest risk for the child to have ADHD (OR=29.3), followed by the AG allele (OR=5.6). There was statistically significant increase in the percentage of GC (rs3749034/rs11542313) haplotype in ADHD group as compared to the control group (P value <0.001\*). Family based association analyses for each GAD1 SNP and haplotypes were carried out. The rs11542313C allele was overtransmitted from parents to ADHD probands. No significant transmission from rs3749034 or haplotypes from rs3749034/rs11542313 were observed (P=0.799 and P=0.821, respectively)

**Conclusions:** Our results suggest that the GAD1 gene might be associated with susceptibility to ADHD.

**Key Words:** ADHD; GABA; GAD1; susceptibility; association

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### I. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental psychiatric disorder characterized by persistent symptoms of inattention, hyperactivity and impulsivity with a prevalence of about 5%.<sup>1</sup> The ADHD etiology is multifactorial, i.e., social, environmental, neurobiological, and genetic factors have been recommended to be relevant.<sup>2</sup> Although traditionally considered a disorder of childhood, mounting evidence suggests that ADHD may persist into adulthood. While the developmental course and neurobiological basis of ADHD have not yet been completely recognized, an imbalance between inhibitory/excitatory neurotransmitters is believed to have a significant role in the pathophysiology of ADHD.<sup>1</sup> The  $\gamma$ -amino butyric acid (GABA) is considered the main inhibitory neurotransmitter of the mammalian central nervous system.<sup>3</sup> The study of GABAergic system in ADHD has been limited. Recent studies recommended a role for GABA on ADHD hyperactive/impulsive symptoms owing to behavioral disinhibition caused by inappropriate modulation of GABAergic and glutamatergic signaling. Short intercortical inhibition (SICI), which is modulated by (GABA)A-mediated inhibition at the level of the primary motor cortex, was reported to be reduced in children with ADHD and associated with ADHD symptom severity and motor skills.<sup>4</sup> Glutamic acid decarboxylase (GAD) is the main enzyme in GABA synthesis in inhibitory interneurons. GAD67 is an enzyme that converts glutamate to GABA. It is one of the two major isoforms of GAD. GAD67 is encoded by GAD1 gene located at chromosome 2q31.<sup>5</sup>

## II. Subjects And Methods

Subjects recruited to this study are divided into three groups. The first group is the ADHD group that included 20 ADHD children (17 male and 3 female) diagnosed according to the diagnostic and statistical manual of mental disorder, fourth edition (DSM-IV) criteria. The second Group is the Parents group. This group included the biological parents of ADHD children (40 subjects). The third Group is the Control group including 30 healthy children (24 male and 6 female) age and gender matched with ADHD children and served as control group. ADHD children had been recruited from the child and adolescent outpatient clinic of Tanta Psychiatry and Neurology Center- Tanta University.

**ADHD group and control group were subjected to:** complete history taking and thorough clinical examination. A validated Arabic version <sup>6</sup> of the Mini International Neuropsychiatric Interview for kids (mini-kid) <sup>7</sup> was used to assess psychiatric disorders in all participants. The Arabic translation <sup>8</sup> of the Stanford-Binet Intelligence quotient (I.Q) fourth edition <sup>9</sup> was used to assess the IQ of the children to determine the general level of intelligence and to exclude mental retardation in all subjects.

An Informed consent (approved by The Ethics Committee of Tanta University) was obtained from all participants in this research.

### Laboratory investigations:

All the three groups in this study were subjected to isolation of Genomic DNA from peripheral blood samples of all studied subjects using GeneJET Whole Blood Genomic DNA Purification Mini Kit #K0781.

GAD1 SNPs (rs3749034 and rs11542313) were evaluated using TaqMan allelic discrimination assay technique (Real-time polymerase chain reaction).

### SNP Selection and Genotyping

Two SNPs were selected for this study. The first is rs3749034, which is located at the 5' untranslated region (UTR). The second SNP is rs11542313 located at exon 3. This variant is a synonymous codon for histidine<sup>10</sup> recently merged into rs769404.

The selection of these SNPs based on the previous association studies with psychiatric disorders.<sup>10,11,12</sup>

### Sample collection:

Prior to the collection of samples, an Informed consent (approved by The Ethics Committee of Tanta University) was obtained from all participants in this research.

2ml of venous blood were collected from all subjects included in this study by sterile venipuncture from the cubital vein, and were collected into EDTA containing tubes, for genotyping of (GAD1) gene polymorphisms (rs3749034 and rs11542313) and stored at -80°C till the time of DNA extraction.

### DNA Extraction.

Genomic DNA was extracted from frozen EDTA treated blood sample using GeneJET Whole Blood Genomic DNA Purification Mini Kit #K0781 supplied from ThermoFisher Scientific.

### SNP Genotyping of (GAD1) gene polymorphisms (rs3749034 and rs11542313).

**Technique:** TaqMan<sup>®</sup> Allelic Discrimination (AD) assay

#### Master Mix:

Using TaqMan<sup>®</sup> Genotyping Master Mix (Catalog No. 4371353).

#### PROBES

Probe sequence labeled with VIC and FAM dyes was as follow:

**rs3749034** (VIC/FAM)

CTGGAGGTGACGCCGGCAGATTAC[A/G]CCTGTGTCAGGGCCGAGCCGAGCGGAT

**rs11542313** (VIC/FAM)

ACGATACCTGGTGC GGCGTGGCCCA[C/T]GGATGCACCAGAAA ACTGGGGCTCA

Primers and probes were supplied from ThermoFisher Scientific.

### Thermal cycling conditions:

The (GAD1) gene polymorphisms (rs3749034 and rs11542313) were genotyped using the 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

The reaction mix included a mixture of 10ul master mix, 1.25 (probe and primers) with 3.75 ul of DNAase free water, 5 ul (0.1 ug/ul) of genomic DNA template and for negative control reaction 5ul of DNA free water was added. The cycle conditions were set as follows: initial denaturation step at 95°C for 10 min, 45 cycles of denaturation at 94°C for 15s, annealing temperature at 50°C for 60s and extension at 72°C for 2 min and a final extension step at 72°C for 1 min.

**Statistical analysis of the data**

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level.

**Chi-square test** was used for categorical variables, to compare between different groups.

**Fisher’s Exact or Monte Carlo correction** used for correction of chi-square when more than 20% of the cells have expected count less than 5. **Student t-test** used for normally distributed quantitative variables, to compare between two studied groups. **Odds ratio (OR)** used to calculate the ratio of the odds and 95% Confidence Interval of an event occurring in one risk group to the odds of it occurring in the non-risk group.

**III. Results**

The present study was done on 20 ADHD families (ADHD children and their biological parents) and 30 healthy children served as control group. There were no statistically significant differences between ADHD group and control group as regard to gender (P value 0.7), age (P value 0.4) and IQ (P value 0.1)(Table 1).

As regard to GAD1 polymorphism rs3749034, there was statistically significant increase in the percentage of GG genotype in ADHD group as compared to the control group (P value <0.001\*) with increased probability for occurrence of ADHD in GG genotype. Also there was statistically significant increase in the percentage of G allele in ADHD group as compared to the control group (P value <0.001\*)(table 2, figure 1, 2).

There was no statistically significant difference between ADHD group and control group as regard to GAD1 polymorphism rs11542313 (table 2, figure 3, 4).

It was found that there was statistically significant increase in the percentage of GC (rs3749034/rs11542313) haplotype in ADHD group as compared to the control group (P value <0.001\*) (table 3).

Family based analyses for GAD1 polymorphisms (rs3749034 and rs11542313) and haplotypes revealed that the rs11542313C allele was observed to be over-transmitted from the parents to ADHD probands (table 4, figure 6).

There was no statistically significant differences between transmitted and untransmitted alleles as regard to alleles frequencies of GAD1 polymorphism rs3749034 (P value 0.799) and haplotypes frequencies of GAD1 polymorphisms (rs3749034 and rs11542313) (P value 0.821)(table 5, figure 7).

**Table no 1:** Comparison between children with ADHD and control children regarding demographic data, and IQ

Variable	Children with ADHD (n=20)	Control Children (n=30)	Statistic	p Value
Age	9.4 ± 2.5	9.9 ± 2.2	t= 0.8	0.4
Sex (Female %)	15	20	x2 = 0.3	0.7
IQ	86.2 ± 9.4	89.7 ± 5.6	t= 1.5	0.1

**Table no 2:** The genotype frequencies of GAD1 polymorphisms rs3749034 and rs11542313 between ADHD group and control group

Variant	ADHD Child		Control		x <sup>2</sup>	p	OR	95% C. I.
	No.	%	No.	%				
<b>rs3749034</b>								
AA	2	10.0	18	60.0			Reference	
AG	5	25.0	8	26.7	3.8	0.08	5.6	0.9 – 35.4
GG	13	65.0	4	13.3	16.8*	<0.001*	29.3*	4.6 – 184.4
A allele	7	35.0	26	86.7			Reference	
Not A allele	13	65.0	4	13.3	14.3*	<0.001*	12.1*	2.9 – 48.8
Not G allele	2	10.0	18	60.0			Reference	
G allele	18	90.0	12	40.0	12.5*	<0.001*	13.5*	2.6 – 69.1
<b>rs11542313</b>								
TT	7	35.0	15	50.0			Reference	
CT	9	45.0	9	30.0	1.4	0.2	2.1	0.6 – 7.8
CC	4	20.0	6	20.0	0.2	0.7	1.4	0.3 – 6.7
T allele	16	80.0	24	80.0			Reference	
Not T allele	4	20.0	6	20.0	0.0	1.0	1.0	0.6 – 7.8
Not C allele	7	35.0	15	50.0			Reference	
C allele	13	65.0	15	50.0	1.1	0.3	1.9	0.6 – 5.9

OR: Odds ratio CI: Confidence interval\*: Statistically significant at p ≤ 0.05

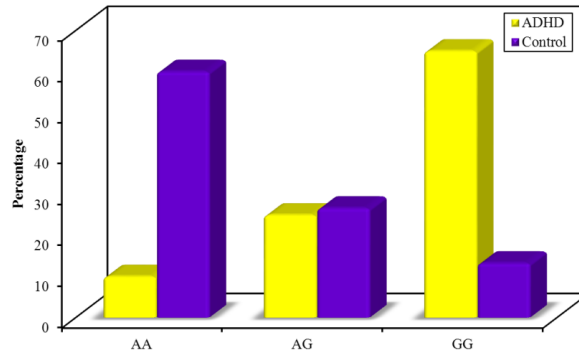


Figure (1): The genotype frequencies of GAD1 polymorphism rs3749034 in ADHD group and control group

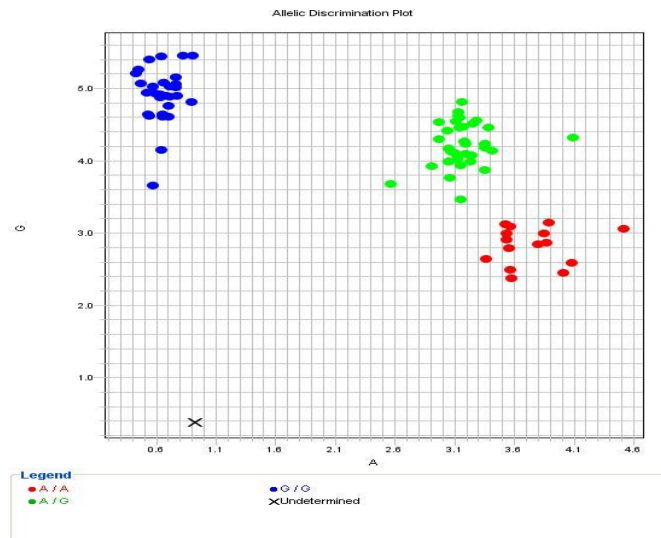


Figure (2): Allelic discrimination plot for GAD1 polymorphism rs3749034

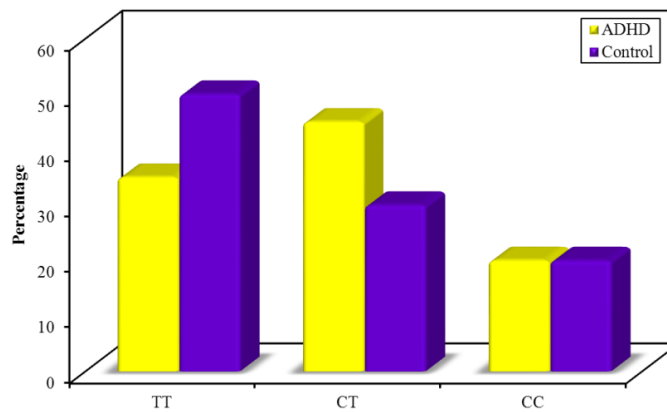


Figure (3): The genotype frequencies of GAD1 polymorphism rs11542313 in ADHD group and control group.

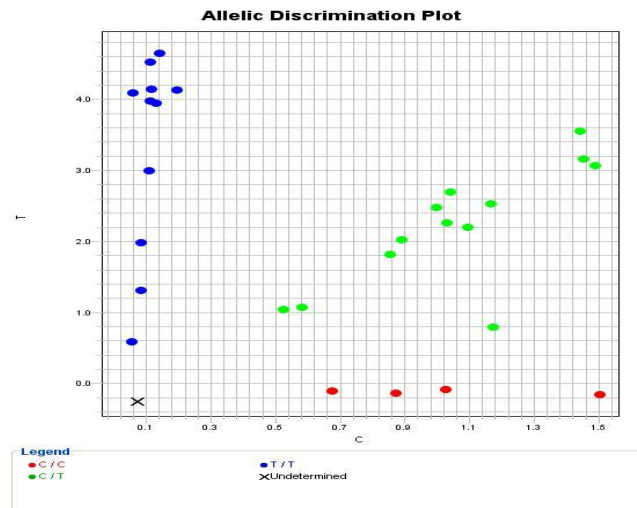


Figure (4): Allelic discrimination plot for GAD1 polymorphism rs11542313

Table (3): Comparison between ADHD group and control group according to haplotype frequencies of GAD1 polymorphisms rs3749034 and rs11542313

Haplotype rs3749034(A/G) /rs11542313(C/T)	ADHD group (n= 40)		Control (n= 60)		$\chi^2$	P
	No.	%	No.	%		
AC	2	5.0	11	18.3	3.722	0.052
AT	7	17.5	33	55.0	14.063*	<0.001*
GC	15	37.5	10	16.7	5.556*	<0.001*
GT	16	40.0	6	10.0	1.904	0.168

$\chi^2$ : Chi square test

p: p value for comparing between the studied groups

\*: Statistically significant at  $p \leq 0.05$

Table (4): Comparison between transmitted and untransmitted alleles from parents group to ADHD group

	Transmitted(n= 40)	Untransmitted(n= 40)	$\chi^2$	P
<b>rs1 374903</b>				
A	10 (0.25)	11 (0.28)	0.065	0.799
G	30 (0.75)	29 (0.72)		
<b>rs11542313</b>				
C	17 (0.42)	14 (0.35)	0.474	0.491
T	23 (0.58)	26 (0.65)		

$\chi^2$ : Chi square test

p: p value for comparing between the studied groups

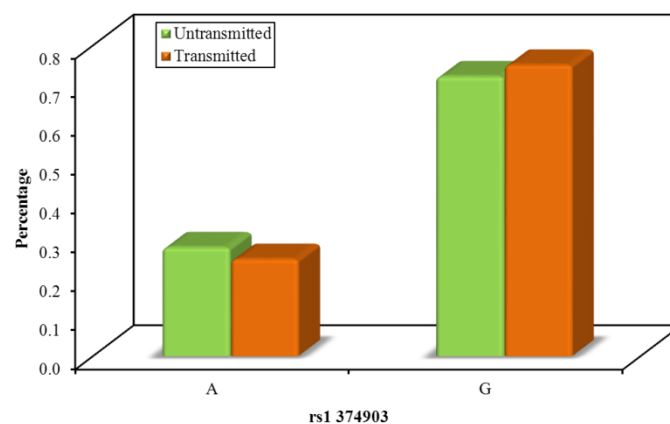


Figure (5): Comparison between transmitted and untransmitted alleles from parents group to ADHD group.

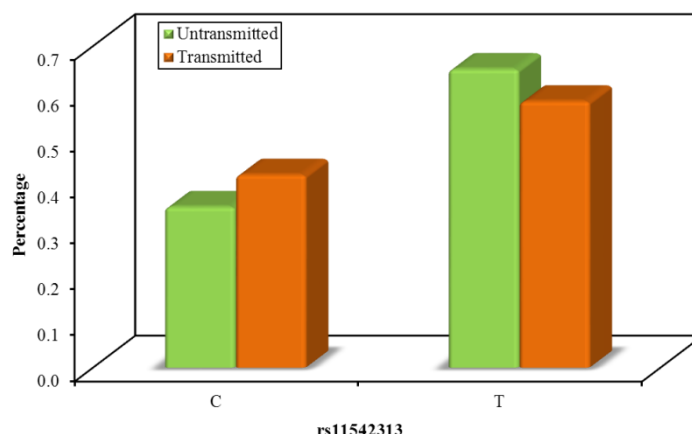


Figure (6): Comparison between transmitted and untransmitted alleles from parents group to ADHD group.

Table (5): Comparison between transmitted and Untransmitted haplotypes from parents group to ADHD group

Haplotype rs3749034(A/G) /rs11542313(C/T)	Transmitted (n= 40)	Untransmitted (n= 40)	$\chi^2$	<sup>MC</sup> p
AC	4 (0.10)	5 (0.12)	1.163	0.821
AT	6 (0.15)	6 (0.15)		
GC	13 (0.32)	9 (0.23)		
GT	17 (0.43)	20 (0.5)		

$\chi^2$ : Chi square test  
 MC: Monte Carlo  
 p: p value for comparing between the studied groups

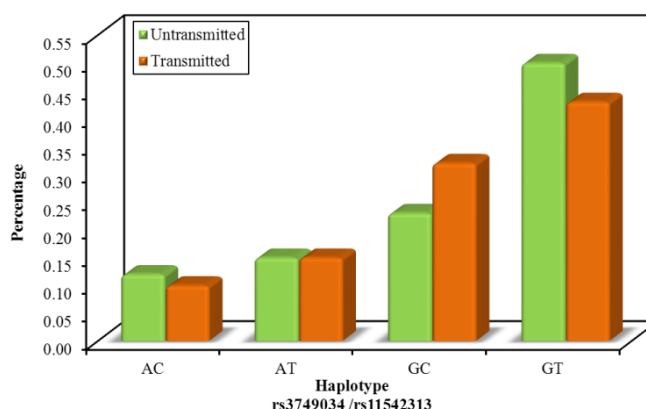


Figure (7): Comparison between transmitted and untransmitted haplotypes from parents group to ADHD group.

#### IV. Discussion

Our study provides evidence for an association between (GAD1) gene polymorphisms (rs3749034 and rs11542313) and ADHD susceptibility. Despite that SNPs rs3749034 and rs11542313 are reported to be mapped in the promoter region and in exon 3, respectively, LD between them was statistically significant ( $D'=-0.42$ ,  $R^2=0.12$ ,  $P=0.01^*$ ). Bruxel et al. reported that, LD between the same SNPs was strong ( $D'=0.98$ ,  $R^2=0.16$ ,  $P<0.001$ ). This result is in agreement with data reported by the Ensembl database (<http://www.ensembl.org>).<sup>5</sup>

As regard to GAD1 polymorphism rs3749034, there was statistically significant increase in the percentage of GG genotype in ADHD group as compared to the control group ( $P$  value  $<0.001^*$ ) with increased probability for occurrence of ADHD in GG genotype (OR 29.250<sup>\*</sup>) with 95% C.I (4.639 – 184.4). Also there was statistically significant increase in the percentage of G allele in ADHD group as compared to the control group ( $P$  value  $<0.001^*$ ).

These results were coordinated with Straub et al. They reported that SNPs of GAD1 gene have been associated with mRNA levels of the GAD67 isoform. The G allele at rs3749034 was reported to be associated with reduced transcript levels in the hippocampal and prefrontal regions of schizophrenic postmortem brains. Also they concluded that change of G to A allele at rs3749034 creates two extra putative transcription factor (TF) binding sites - namely ATP1a1 regulatory element binding factor 6 (AREB6) and myoblast determining factor (MYOD).<sup>12</sup>

In the present study it was found that there was no statistically significant difference between ADHD group and control group as regard to GAD1 polymorphism rs11542313. We revealed that there was an increase in the percentage of C allele of GAD1 polymorphism rs11542313 in ADHD group as compared to the control group but it was statistically non-significant (P value 0.449), (OR 1.373) and 95% C.I (0.604 – 3.121). These negative results may be attributed to small sample size as we also found that the rs11542313C allele was over-transmitted from parents to ADHD probands.

Marengo S et al evaluated the genetic modulation of GABA levels by the proton magnetic resonance spectroscopy (MRS) in healthy volunteers. This study showed significant low GABA/ creatine levels in rs11542313C allele carriers (P = 0.001) and a tendency for interaction between this SNP and COMT rs4680 (Val158Met) (P = 0.05).<sup>10</sup> Similar results were reported by Nackley AG et al who found that in the case of rs11542313 (a synonymous coding SNP), there was changes in the efficiency of protein production resulting from alteration of the mRNA secondary structure.<sup>13</sup>

In this study, it was found that there was statistically significant difference between ADHD group and control group as regard to the GC (rs3749034/rs11542313) haplotype (P value <0.001\*). This was in agreement with Bruxel EM et al who reported that the GC (rs3749034/rs11542313) haplotype had the highest hyperactive/impulsive mean scores in patients with ADHD (P value = 0.03).<sup>5</sup>

In the current study, family based analyses for GAD1 polymorphisms (rs3749034 and rs11542313) and haplotypes revealed that the C allele of rs11542313 was over-transmitted from the parents to ADHD probands. We reported that the frequencies of the transmitted C allele in ADHD child group were (42.0%). The frequencies of untransmitted C allele were (35.0%). These results were statistically non-significant (P value 0.491) and this may be attributed to small sample size.

The present study showed that there were no statistically significant differences between transmitted and untransmitted alleles as regard the alleles frequencies of GAD1 polymorphism rs3749034 (P value 0.799) and haplotypes frequencies of GAD1 polymorphisms (rs3749034 and rs11542313) (P value 0.821). These results were coordinated with Bruxel EM et al study, in which the rs11542313C allele was significantly over-transmitted from parents to ADHD probands (P=0.02). No significant transmission from rs3749034 or haplotypes from rs3749034/rs11542313 were observed (P=0.27 and P=0.08, respectively).<sup>5</sup>

The results of the present study suggest that GAD1 gene, a gene significant for the production of a key enzyme in the biosynthesis of GABA, is associated with ADHD susceptibility.

GABAergic interneurons control the amount of the inhibitory and the excitatory inputs in the neurons of cortical networks, mediating the precise gating information through specific signaling pathways.

It has been concluded that severe deficits in inhibitory function may lead to a persistent imbalance between inhibition and excitation, resulting in cognitive impairments as observed in autism and schizophrenia.<sup>14</sup>

It is reasonable to suggest that an abnormal GABA action can result in pathological changes in the neuronal activities and may contribute to neurodevelopmental disorders such as ADHD. This is consistent with some reviews done by Castellanos FX and Tannock R & Kieling C et al who demonstrate that ADHD subjects have smaller brains as compared to controls.<sup>15,16</sup>

Edden RAE et al revealed that reduced GABA levels have been observed in children with attention deficit/hyperactivity disorder (ADHD).<sup>17</sup>

Bollmann S et al reported that ADHD patients show different levels of GABA+ in the subcortical voxel (centered on the basal ganglia) which revealed to be altered with development. ADHD children also showed increased Gln levels in the subcortical voxel, however this alteration normalized in adults. These imbalances in neurotransmitter levels are linked to symptom scores and provide new insight in the development and the pathophysiology of ADHD.<sup>1</sup>

Another study demonstrated that reduced GABA synthesis in the prefrontal cortex of an animal model increases the locomotor activity but not the attention in the 5-choice serial reaction time task (5-CSRTT).<sup>18</sup>

Caprioli D et al explained that high impulsivity on the 5-choice serial reaction time task (5-CSRTT) is accompanied by a marked decrease in the gray-matter density and related to a reduced expression of dendrite spine markers and both GAD enzyme isoforms.<sup>19</sup>

Also, the GAD67 protein encoded by the GAD1 gene in humans has been implicated in ADHD.<sup>5</sup>

Moreover, Tritsch et al reported that GABA has been implicated in the dopaminergic neurotransmission in the striatum.<sup>20</sup>

The study of Smith KM revealed that GABAergic neuron involvement in neuropsychiatric disorders that exhibit impaired executive function and impulse control such as schizophrenia, bipolar disorder, ADHD and Tourette syndrome. This same study also proved that this reduced GABA dosage result in increase in locomotion and a hyperactive phenotype.<sup>21</sup>

This evidence was in agreement of Naaijen J et al study which implicating GABA signaling and imbalances in excitatory/inhibitory tone in ADHD.<sup>22</sup>

Silveri MM et al study showed a role for GABA in impulsivity and response inhibition.<sup>3</sup>

Naaijen J et al reported that Deficits in inhibition can be associated with the fronto-striatal deficits in glutamate and GABA levels, which is consistent with findings in ADHD and ASD showing altered glutamate and GABA signaling.<sup>23</sup>

The present findings should be viewed in the light of certain limitations. First, the present study is a cross-sectional study. The longitudinal approach for investigating the GABA and glutamate genes involvement in ADHD would be more satisfactory because hyperactivity/ impulsivity symptoms tend to decline over time and also GABA and glutamate levels change throughout development. Second, as our sample was recruited from university hospital, our results may not be generalizable to other populations. Third, sample sizes might have been too small for detecting an effect of GAD1 gene polymorphisms on ADHD susceptibility, limiting statistical power. Fourth, only a few SNPs were examined in this study among the various genes implicated in the various ADHD phenotypes. Although it is clear that ADHD is a multifactorial disease and not just one genetic factor causes increase in ADHD risk, we did not study the interaction with the other risk factors. Fifth, ADHD diagnoses were not assessed in parents that would give relevant extra genetic information. Finally, this field is clearly still in its early stages. Thus, results should be considered as preliminary and must be replicated in larger and independent samples.

Up to our knowledge, the present study is the first familial study conducted on the association between ADHD and GAD1 gene polymorphisms in Egypt.

## V. Conclusion

The current study provides genetic evidence that ADHD risk is more likely to be associated with GAD1 polymorphisms (rs3749034 and rs11542313).

In the specific haplotype test, the GC(rs3749034/rs11542313) haplotype was observed to be significantly increased in ADHD group as compared to control group.

Family based tests demonstrated an over transmission of rs11542313C allele from parents to the affected offspring.

Taken all together, the present results suggest that GAD1 gene, a gene responsible for the production of a key enzyme in GABA biosynthesis, might be associated with susceptibility to ADHD but additional research is necessary to clarify these findings.

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