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Neurotransmitter and amino acid levels in Nigerian children with autism spectrum disorders

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Abstract*:* Autism Spectrum Disorder (ASD), often referred to as Autism, is a clinically heterogeneous neurodevelopmental disorder with core-defining features of impaired socialization, impaired verbal and nonverbal communication, and restricted and repetitive patterns of behaviour. The disorder is presently diagnosed behaviourally, however the search for possible biomarkers that could aid earlier diagnosis have been on the increase. The present study aims to investigate plasma amino acid levels as a potential biomarker in ASD screening. Plasma levels of 20 amino acids (AA) (including neurotransmitters-GABA and glutamate) of autistic individuals and typical age and sex matched control, were determined using reversed-phase high performance liquid chromatography (RP-HPLC). All statistical analyses (independent t-test, spearman correlation, cohen's d effect size) were done using IBM SPSS version 20. A total of 31 plasma samples (18 Autism cases and 13 age-sex matched controls) were analyzed with mean ages 8.44±4.87 for cases and 8.15±4.88 for controls. No significant intergroup difference was observed in the individual amino acid levels with the exception of glutamate ($t = 5.472$, $df = 2.324$, $p = 0.000063$), glutamine ($t = 8.342$, $df = 14.780$, $p = 0.000001$) , GABA ($t = 6.601$, $df = 24.593$, $p = 0.000001$), tryptophan ($t = 3.568$, $df = 16.472$, $p = 0.002$) and cysteine ($t = 4.000$, $df = 13.762$, $p = 0.001$).The amino acid profile and the glutamate - GABA levels can serve as biochemical markers for ASD, and can thus be utilized as screening for earlier diagnosis of the disorder.

Keywords: Autism Spectrum Disorder (ASD), Biomarkers, Neurotransmitters, GABA, Glutamate, Diagnosis.

Introduction

Autism Spectrum Disorder (ASD), often referred to as Autism, is a clinically heterogeneous neurodevelopmental disorder with affected children exhibiting marked behavioural phenotypes deviant from the norm (i.e. of their age-matched peers)[1].The core-defining features are impaired socialization, impaired verbal and nonverbal communication, and restricted and repetitive patterns of behaviour[2] and more recently, impaired social/ communication deficits and restricted interests/repetitive behaviours[3]. These behavioural phenotypes are often detected between eighteen months to three years of age and persist to adulthood. Different levels of severity in the cognitive ability of affected children are observed, ranging from near mental retardation to geniuses[4]. The disorder is often detected before the age of three years more often in males than female, with a male-female ratio of occurrence of 4: 1[5] and affected children usually have no visible physical abnormalities (except when there are comorbid conditions). Presently, diagnosis of the disorder is typically behavioural through the administration of standardized interviews and questionnaire, sometimes with direct observations (Autism Diagnostic Interview (ADI) and the Autism Diagnostic Observation Schedule (ADOS) which are designed based on the diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM) (published in revisions by the American Psychiatric Association [APA]) and the International Statistical Classification of Diseases & Related Health Problems (ICD), also published in revisions by the World Health Organization (WHO). Currently there is no known cure for Autism.

Autism is believed to have a 1% prevalence Worldwide[6], with different rates of occurrences observed in different countries. In a clinic based population study in Enugu, south-eastern Nigeria, prevalence of Autism Spectrum Disorders had been noted to be 0.8% of the total population of children that attended the clinic over a one year period[7] and 11.4% among children with intel-

lectual disability[8]. A study in Egypt and Tunisia documented the prevalence of children with ASD among children with developmental disorders to be 33.6% and 11.5% respectively[9].

Despite great efforts to move forward and clarify the precise mechanisms underlying the pathophysiology of Autism, its pathophysiological mechanisms still remains largely unknown[10]. However several hypotheses have been postulated. One of such hypothesis is the neurotransmitter pathophysiology of Autism. Increasing number of literature have implicated elevated levels of neurotransmitters and related amino acids in autistic individuals compared to age and sex-matched controls [11,12,13,14, 15]. This is often correlated with dysfunctional neuronal connectivity (hyper and hypo connectivity) in regions of the brain involved in cognitive and social functions[16,17,18,19].Glutamate and Gamma Aminobutyric Acid (GABA) are the main excitatory and inhibitory neurotransmitters in the human brain and both have important roles during early development of the nervous system and during synapses[20]. At the developing stage, GABA acts as an excitatory neurotransmitter, modulating neuronal migration and a wide range of functions that lead to the correct formation of neuronal circuits while in the mature adult brain its function is mainly inhibitory, generating synchronous rhythms of cortical assemblies (adequate synapses), allowing for time-precise communication between neurons and cerebral regions/centers [21,22]. This is of particular importance as Autism has been shown to be a disorder of impaired functional integration (modulated by neuronal connectivity), a factor responsible for characteristic features observed[21] such as hyperactivity and epileptic seizures. Cumulative evidence indicates that dysfunctional excitatory and inhibitory synaptic activities underlie several of the characteristics of Autism[23].

Moreover, the GABAergic and glutamatergic neuronal system, highly implicated in genetic studies of Autism as a result of mutations in genes coding for GABA and glutamate receptors, have been postulated to be responsible for the observed differential neurotransmitter levels in autistic individuals[12]. Also, given that the GABAergic and glutamatergicneuronal pathway appear to be convergent nodes of genetics, epigenetics and probably environmental factors that may cause Autism, the GABA and glutamate receptors may form important targets for pharmacological interventions. Studies on amino acid (AA) profile/levels of autistic individuals have become common[12,24]. Since AA exert various influences on one another in the process of synthesis/degradation, any changes in plasma neurotransmitter levels could be attributed to changes in other amino acids[12]. This is because most essential amino acids are mostly precursors for the formation/production of some neurotransmitters. For instance, glutamine which is an essential AA, is the precursor for glutamate and tryptophanis the precursor for serotonin. To the best of the authors' knowledge there are no studies of the amino acid and neurotransmitter levels of individuals with Autism in Nigeria. The present study is a comparative description/analysis of plasma levels of 18 essential amino acid and two neurotransmitters (GABA and glutamate) in individuals with Autism and their sex and age-matched controls.

Materials and methods

Ethical Approval

Ethical approval to carry out the research was obtained from Lagos University Teaching Hospital (LUTH) Health Research and Ethics Committee (ADM/DCST/HREC/954), Idi-Araba, Lagos and the Ethics Review Committee, Federal Neuro Psychiatric Hospital (FNPHY/ERC/13/088), Yaba, Lagos.

Experimental Model	Enrolment criteria for control subjects
Diagnosis of Autism	age: ≥3 years
age: ≥3 years	Absence of neurological disorder signed informed consent

Recruitment and Sample Collection

Five millilitres (5ml) of whole blood was collected into EDTA bottles via venipuncture from affected individuals after written informed consents were obtained from their parents and/or guardians. Collected samples were transported in cooling bags from site of collection to the laboratory where they were centrifuged for plasma separation. The plasma samples were decanted, aliquoted and stored at -24^{0}C until analysis. Participants were recruited from the Neuro-paediatric clinic of University of Lagos Teaching Hospital (LUTH) and the Child and Adolescent Unit of the Federal Neuro-Psychiatric Hospital, Oshodi Annex Lagos. While the control subjects were recruited from the Medical Centre, University of Lagos and the Pathology Unit, Orthopaedic Hospital, Igbobi, Lagos and the University of Lagos Health Centre, Akoka, Lagos. All caseshad been previously diagnosed with ASD using the DSM-1V[2].

Plasma Quantification of Amino Acids (AA)

Plasma samples were deproteinized as described by [12,25]. A 300µl aliquot of plasma was homogenized with 450µl of 5% sulfosalicylic acid and centrifuged at 12000rpm in 4^{0}C for 10 minutes. The resulting supernatant was decanted, filter sterilized through 0.22µm membrane filter (Millipore) into 1.5 ml amber vial (Agilent PN 5182-0716). Amino acid concentrations were measured using an automatic HPLC system (1200 series: Agilent Technology Inc., USA) with a reversed-phase (RP-HPLC) method and pre-column derivatization with Ortho-Phthalaldehyde (OPA: Agilent-PN5061-3337)[26,27]. After derivatization, 20µl of derivatized mixture was injected and run through a C-18 column (Agilent ZORBAX Eclipse Plus C-18 4.6×250, 5µm) for 40 minutes at a flow rate of 1.5 ml/min. Eluted amino acids were detected and quantified via fluorescence detector (FLD: Agilent G1321C). All statistical analysis were done

using IBM SPSS version 20. All results are presented as mean±SD. The sample (blood and plasma) preparation and storage was done in the Post Graduate Laboratotory of th department of Cell Biology and Genetics, University of Lagos, while the HPLC analysis of the deproteinized plasma samples was done in the Central Research Laboratory (CRL) of the University of Lagos.

Results

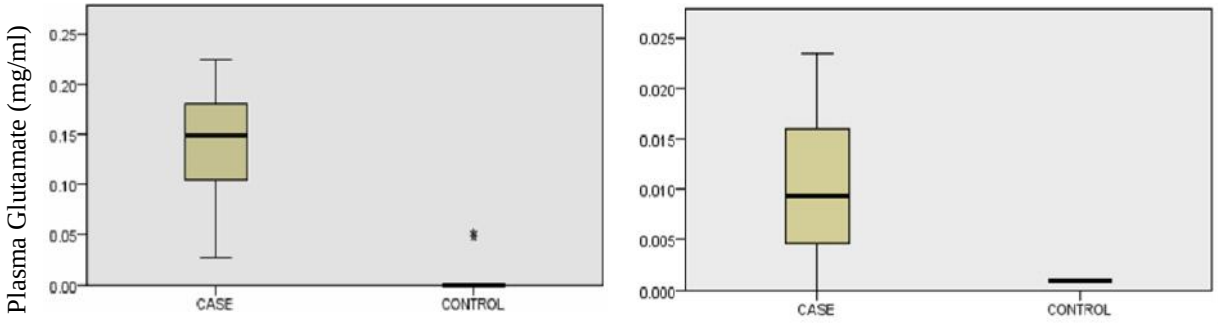
A total of 31plasma samples (18 Autism cases and 13 age-sex matched controls) were analyzed with mean ages 8.44±4.87 for cases and 8.15±4.88 for controls. No significant difference (t = 0.164, df = 29, p = 0.871) was observed in ages of both groups. The result also indicated no significant intergroup difference in the mean concentration levels of individual amino acid analyzed with the exception of glutamate (GLU: t = 5.472, df = 2.324, p = 0.000063), glutamine (GLN: t = 8.342, df = 14.780, p = 0.000001) , GABA (t = 6.601, df = 24.593, p)= 0.000001), tryptophan (TRP: t = 3.568, df = 16.472, p = 0.002) and cysteine (CYS: St = 4.000, df = 13.762, p = 0.001) (Table 1; Fig. 1) from a two-tailed independent t-test, unequal variances assumed. Further analysis with nonparametric Mann-Whitney U test revealed a nonsignificant difference of mean for glutamate (t = 2.000, *Exact* p = 0.052) but significant for the Kruskal-Wallis test (t = 3.868, df = 1, p = 0.049) using an asymptotic 2-sided test (Fig 1a). As expected the plasma levels of glutamate, GABA and glutamine were significantly higher in autistic individuals than controls with mean differences, 9.838µg/ml (95% confidence interval [CI]: 6.008 to 13.667µg/ml), 42.596µg/ml (95% CI: 29.294 to 55.898µg/ml) and 133.562µg/ml (95% CI: 99.392 to 167.732µg/ml) with Cohen's d effect sizes 2.81, 2.66 and 4.33 respectively. A spearman ranked correlation analysis revealed strong positive correlations between the concentrations of the amino acids with significant group difference- GLN-GLU (Spearman's *rho* 0.529 p<0.05), GLN-GABA (0.796 p<0.01), GLN-TRP (0.654 p<0.01), GLN-CYS (0.654 p<0.01), GABA-TRP (0.662 p<0.01) and between GABA-CYS (0.838 p<0.01

Table 1: Plasma Levels of Neurotransmitters and Amino Acids in Autistic Individuals and control subjects

Plasma Amino Acid Concentration(µg/ml)			Independent t-test	
Amino Acids/ Neurotransmitters	Autism Case	Control	*t*	*p-value*
Aspartate	29.446±1.958e-2	20.793±2.854e-2	0.506	0.657
Glutamate	10.743±7.176e-3	0.905±1.626e-4	5.472	0.000063***
Serine	24.958±2.091e-2	28.805±1.754e-2	-0.382	0.718
Glutamine	141.332±5.461e-2	7.769±1.872e-2	8.342	0.000001***
Histidine	40.143±6.838e-2	0.000±0.000	1.017	0.416
Glycine	5.700±8.061e-3	0.000±0.000	1.000	0.500
Threonine	4.859±3.069e-3	30.174±4.084e-2	-1.38	0.238
Arginine	0.000±0.000	0.000±0.000	-[a]	-
Alanine	3.699±6.485e-3	3.030±3.954e-3	0.233	0.825
GABA	62.205±2.152e-2	19.609±1.234e-2	6.601	0.000001***
Tyrosine	8.540±1.619e-2	3.996±3.479e-3	0.923	0.373
Cystine	23.205±1.009e-2	11.603±2.741e-3	4.000	0.001**
Valine	1.777±3.777e-3	6.493±7.599e-3	-1.184	0.308
Methionine	9.625±2.178e-3	9.144±4.049e-3	0.252	0.811
Tryptophan	29.190±1.919e-2	5.158±1.194e-2	3.568	0.002**
Phenylalanine	0.000	19.400	-[a]	-
Isoleucine	10.670	2.840±1.697e-4	-	-
Leucine	15.950	0.000±0.000	-[a]	-
Lysine	63.020	2.955±2.980e-3	-	-
Proline	3.385±9.661e-3	0.113±3.926e-4	1.435	0.169

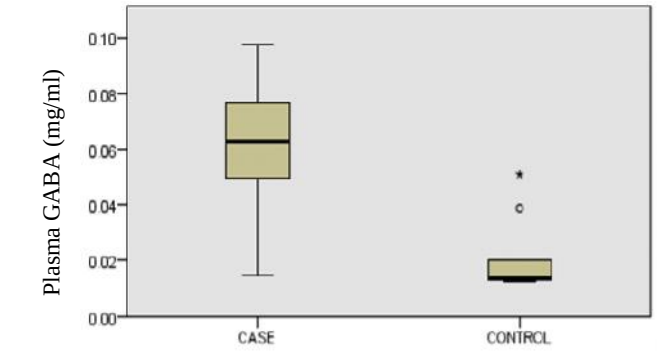
Values are expressed as mean±SD; **p<0.05 ***p<0.00001

a. t cannot be computed because the standard deviation of at least one of the groups is 0.

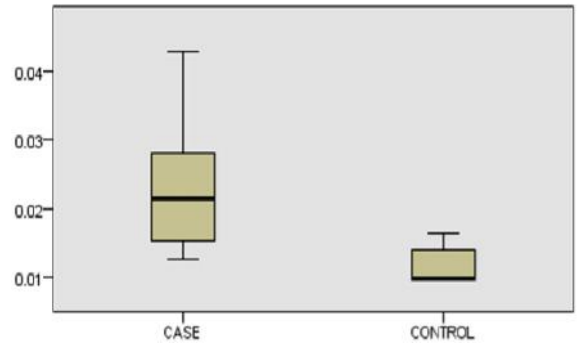


a) Diagnosis

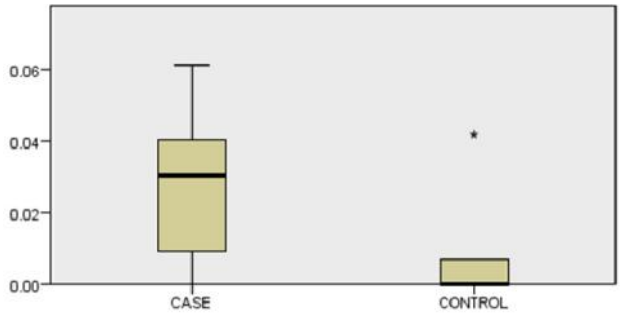
b) Diagnosis



c) Diagnosis



d) Diagnosis



e) Diagnosis

Fig 1: Mean plasma concentrations of Neurotransmitters and Amino Acids with statistically significant levels between autistic individuals and typical age and sex matched controls. a) Glutamate, b) Glutamine, c) GABA, d) Cystine and e) Tryptophan.

Discussion

Observed differential plasma levels of the neurotransmitters and related other amino acids amongst autistic individuals against controls conform with the neurotransmitter pathophysiology of Autism and other reported studies[12,13,14,15,24]. Glutamine (GLN), glutamate (GLU) and γ-amino butyric acid (GABA) are essential amino acids for brain metabolism and function[28].Glutamine had the highest observed mean concentrations of all the analysed amino acids. This is similar to the findings reported[12]where glutamine concentration was higher than 24 out of the 25 amino acids analyzed. This trend is not uncommon as glutamine is believed to have the highest plasma level of all essential amino acids. The strong correlation observed between GLN-GLU, GLN-GABA levels, could be due to the glutamine -Glutamate (GABA) cycle[29]. In order to maintain a rhythm of neuronal synapses/connectivity, the GABA-glutamate levels in the brain are strictly monitored via the glutamine-glutamate cycle through uptake, reuptake mechanisms between postsynaptic, astrocytes and presynaptic neuronal cells respectively [20,23].

It is believed that glutamate does not cross the blood brain barrier (BBB)[30], this might account for it having the least mean concentrations (10.743±7.176e-3) among the variables with significant group difference in this study. Also, its presence extracellularly is deemed to be neurotoxic and its extracellular elevated levels have been implicated in Alzheimer's disease, epilepsy and most neurophysiological disorders [15]. Apart from the 3 main amino acids involved in the excitatory and inhibitory connectivity in the brain, significant elevated levels was also observed in the amino acid –Tryptophan (TRP). This is of no surprise as TRP is the main precursor for serotonin synthesis, the serotonergic neuronal system have also been implicated in Autism pathogenesis[31].

Conclusions and Recommendations

The results reported from the present study supports the neurotransmitter pathophysiology of Autism, thus it is proposed that the neurotransmitter/amino acids plasma levels could serve as biomarkers in the aetiology of Autism. However, it should be noted that several other conditions such as comorbidity (other developmental disorders), pharmacological conditions (use of psychoactive medications) and gender could also influence plasma neurotransmitter and amino acid levels. T he African population is still greatly understudied and underrepresented in Autism research, thus it is recommended that more studies and awareness of Autism research be encouraged in Nigeria and Africa.

Conflict of interest: None
Funding: None

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