World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:15, No:2, 2021

Underivatized Amino Acid Analyses Using Liquid Chromatography-Tandem Mass Spectrometry in Scalp Hair of Children with Autism Spectrum Disorder

Ayat Bani Rashaid, Zain Khasawneh, Mazin Alqhazo, Shreen Nusair, Mohammad El-Khateeb, Mahmoud Bashtawi

***Abstract*—**Autism Spectrum disorder (ASD) is a psychiatric disorder with unknown etiology that mainly affects children in the first three years of life. Alterations of amino acid levels are believed to contribute to ASD. The levels of six essential amino acids (methionine, histidine, valine, leucine, threonine, and phenylalanine), five conditional amino acids (proline, tyrosine, glutamine, cysteine, and cystine), and five non-essential amino acids (asparagine, aspartic acid, alanine, serine, and glutamic acid) in hair samples of children with ASD (n = 25) were analyzed and compared to corresponding levels in healthy age-matched controls (n = 25). The results showed that the levels of methionine, alanine, and asparagine were significantly lower in the hair samples of ASD group compared to those of the control group ($p \leq 0.05$). However, the levels of glutamic acid were significantly higher in the ASD group than the control group ($p \leq 0.05$). The current findings could contribute towards further understanding of ASD etiology and provide specialists with a hair amino acid profile utilized as a biomarker for early diagnosis of ASD. Such biomarkers could participate in future developments of therapies that reduce ASD-related symptoms.

***Keywords*—**Autism spectrum disorder, amino acids, liquid chromatography-tandem mass spectrometry, human hair.

I. Introduction

ASD is a neurodevelopmental disorder that refers to the five disorders: Pervasive development, Autistic, Asperger's, Childhood disintegrate and Rett's [1]. ASD is diagnosed during the early stages of the child's life. Diagnosis mainly depends on the appearance of some symptoms ranged from mild to severe according to standard tests such as diagnostic and statistical manual of mental disorders (DSM test) [1]. The symptoms include impairments of verbal and non-verbal communication skills, social interactions, presence of repetitive and stereotyped behaviors, language delay, avoidance of eye contact, limited range of interests and intellectual disability [2]. Children with ASD habitually consume restricted diets; therefore, they could have nutritional deficiencies of different biomolecules such as amino acids.

Amino acids are the building monomers of proteins and have various physiological functions in the human body as hormones enzymes and others. They are organic compounds containing an amine group (NH_2) and a carboxyl group (COOH) in addition to unique side chains. Amino acids are connected to each other by peptide bonds forming proteins [3]. They are divided into three types: essential amino acids which cannot be synthesized by human body and must be provided by food such as, phenylalanine (Phe), valine (Val), threonine (Thr), tryptophan (Trp), methionine (Met), leucine (Leu), isoleucine (Ile), lysine (Lys), and histidine (His). The second type is conditionally essential amino acids, which are required by humans at certain ages or at certain environmental/health conditions such as, arginine (Arg), glutamine (Gln), glycine (Gly), proline (Pro), serine (Ser), and tyrosine (Tyr). The third type is non-essential amino acids, synthesized by de novo pathways inside the body such as, asparagine (Asn), alanine (Ala), aspartic acid (Asp), citrulline (Cit), cysteine (Cys), and glutamic acid (Glu) [4].

Some amino acids such as Gly and Glu act as neurotransmitters in the central nervous system (CNS). These neurotransmitters are responsible for transmission of signals from one neuron to the target cell [3]. Glu, the most abundant free amino acid in the brain, is important in metabolic processes and plays a major role in catabolic reactions and DNA synthesis [5]. Serotonin is a neurotransmitter mainly composed of Trp [6]. Some amino acids are involved in hormone synthesis, as Tyr, a precursor of adrenaline for mood improvement and neuronal stimulation [7].

Hair is composed of keratin protein [8]. It is built from random arrangement of 21 amino acids, and uses nutrients from the circulating blood in the skin. Generally, the levels of amino acids in hair reflect their levels in blood [8]. The specific etiology of ASD is not clear with no available biochemical informative diagnostic test. However, the concentration of amino acids in human hair could reflect protein-related diseases in the body.

Many studies have explained the importance of studying plasma levels of amino acids in children with ASD [9]-[11]. The plasma levels of Leu and Glu in 27 children with ASD (age range from 3 to 12 years) were assessed using liquid chromatography–mass spectrometry (LC–MS) with propyl chloroformate as a derivatizing agent. The results indicated that five out of the measured 20 amino acids (Gln, Thr, Ser, try, and Leu) were significantly lower, while Glu was higher in children with ASD compared to healthy controls [9]. Another study revealed that the plasma amino acids in

Ayat Bani Rashaid*, Zain Khasawneh, and Mohammad El-Khateeb are with the Department of Chemistry, Faculty of Sciences and Art, Jordan University of Science and Technology, Irbid, Jordan (*e-mail: ahbanirashaid@just.edu.jo).

Mazin Alqhazo is with the Department of Rehabilitation Sciences, Applied Medical Sciences, Jordan University of Science and Technology, Irbid, Jordan

Shreen Nusair is with the Department of Clinical Pharmacy, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan.

Mahmoud Bashtawi is with the Department of Neuroscience, Faculty of Medicine, Jordan University of Science and Technology, Irbid, Jordan.

children with ASD with gluten/casein restricted-diets had significant deficiencies of essential amino acids relative to healthy children on normal diets [10]. A recent meta-analysis of 12 studies (880 participants and 446 incident cases) proved that the Glu blood levels were higher in children with ASD than other participants [11]. Considering these studies, the diagnosis of altered levels of amino acids could help specialists to normalize the body levels of amino acids in a profile for the purpose of early diagnosis.

This is a study that measures the levels of 16 amino acids in the scalp hair of children with ASD using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) without derivatization in an attempt to provide a diagnostic tool for ASD. The selected method is quick and sensitive with a detection limit that reaches femtomole levels. Moreover, hair could indicate history of diseases and medication for longer durations compared to other biological samples such as, blood and urine [12]. Further, in children with ASD, the scalp hair is easier to collect relative to blood and urine.

II. Materials and Methods

A. Materials

16 amino acids Ala, Val, Leu, Pro, Ser, Thr, Asp, Glu, Phe, Tyr, Cys, Met, His, Gln, Asn and Cyt were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl) was used as a hydrolyzing agent, and horse heart myoglobin was also purchased from Sigma-Aldrich. Formic acid, ammonium formate, HPLC grade acetonitrile (used as a mobile phase of LC-MS/MS), acetone and Triton X-100 (used to clean the samples) were purchased from (Barnstead International, Dubuque, IA, USA). The 4-mL glass vials used to evaporate the samples after digestion were from Qorpak (Bridgeville, PA, USA) and polytetrafluoroethylene (PTFE) filters (25 mm × 0.45 µm) were from Bonna-Agela Technologies (Wilmington, DE, USA).

B. Subjects

A total of 25 (20 males and 5 females) children with ASD of 4 to 11 years old (average 7.1 ± 2.3 years) were recruited from the Pediatric Psychiatry Clinic at King Abdullah University Hospital (KAUH), Irbid, Jordan. The age-matched controls were 25 healthy subjects (18 males, and 7 females) with similar diets, no recent history of medication (up to 6 months) and no neurological disorders. Controls were recruited from Irbid, Jordan by advertisement and were medically evaluated at KAUH. Both groups were subdivided into two groups according to age (4-8 and > 8-11 years old). All the children within the ASD group were diagnosed 'moderately autistic' according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) [13] and the Childhood Autism Rating Scale (CARS) [14]. The procedure of sample and data collection was ethically approved by the Institution Review Board (IRB) at KAUH [Approval No. 8/114/2018].

C. Data and Samples Collection

After obtaining the verbal approval of the participants' parents, a consent form was signed by every parent. Specific questionnaires for personal information, medical history, and diets of the participants were filled. Then, 2 g of scalp hair samples (1-2 cm long) were collected per child and stored in properly labelled envelopes with sample ID codes; samples were anonymized for blinded analysis [8], [15], [16].

D. Standard Preparation

Stock solution of 16 amino acids was prepared by dissolving 10 mg of each amino acid powder in 1% HCl (dissolved in distilled water) at room temperature. 100 µl of each stock was mixed in a 10 ml volumetric flask; the volume was completed to 10 ml with HCl to form a mixture of 1000 ppm of each amino acid. Further dilution of each stock to 1000 ppb concentration was prepared by adding 10 µl amino acid stock solution to 10 ml distilled water. Serial dilution was done 11 times to prepare the following standard concentrations: 1000 ppb, 500 ppb, 250 ppb, 125 ppb, 62.5 ppb, 31.25 ppb, 15.63 ppb, 7.81 ppb, 3.91 ppb, 1.95 ppb, 0.98 ppb, and 0.5 ppb. The standards were injected into LC-MS/MS starting from the lowest concentration to the highest one. Calibration curve was constructed using the standards concentrations to calculate the amino acids concentrations in hair samples.

E. Hair Samples' Preparation

To prepare the scalp hair samples for digestion, a visual inspection was performed and any visible particulate matter or connected tissue was scraped away before cleaning. They were cut into small pieces and cleaned to remove any external impurities using 0.1% Triton X-100. Then samples were shacked for 10 minutes and Triton X-100 was discarded. Afterwards, the samples were rinsed three times using deionized water and one-time using acetone. Samples were determined to be clean if no discoloration or particulate matter was observed in the rinsate. Cleaning was continued until these criteria were met. Once the samples were cleansed, they were incubated overnight to dry in an oven at 95 °C [8], [15]. After drying, the samples were ground into powder by Retsch Mixer Mill MM 301 (Retsch GmbH, Haan, Germany) for 15 minutes at 25 °C and 500 s^{-1}.

Three hair powder subsamples (10 mg) and nine myoglobin standard subsamples (12 mg) were transferred into micro insert vessels of the microwave digestion system ETHOS UP (Milestone Inc., Via Fatebenefratelli, Sorisole (BG), Italy). The digestion tubes were cleaned twice by adding 10 ml HCl which were allowed to reflux in the microwave for 20 minutes at 180 °C. A total of 5 ml of 6 M HCl was added to each sample, every 3 micro insert was located in one vessel in SK rotor. Then, 10 ml distilled water was added to all vessels as an external medium for the micro insert. The microwave was programmed to perform a 30-minute run time at 200 °C and 1800 W. The digested samples were filtered using 25 mm x 0.45 µm PTFE filter (Bonna Agela Technologies, Wilmington, DE, USA). HCl was evaporated from samples using a hot

plate at 100 °C until the samples dried. Upon analysis, samples were reconstituted in 1 ml of 1% HCl [8], [15], [16]

F. Instrumentation

Amino acids were analyzed using LC-MS/MS Agilent 1200 series (Agilent Technologies, USA). Agilent zorbax C18 column, 3.0 mmI.D. x 50 mm, 3 µm with a wide range pH from 1 to 12 (Imtakt Corporation, Kyoto, Japan) was used to separate the amino acids. Acetonitrile/formic acid (100/0.1, v/v) and 100 mM ammonium formate were used as mobile phase A and B, respectively. Nitrogen was used as the carrier gas with a flow rate of 700 µl/min. The injection volume was 40 µl. Each subsample was measured three times equaling nine measurements per subject. The mass spectrometer was operated in the electrospray ionization (ESI) positive mode [17]. Table I presents the precursor and product ions of 16 underivatized amino acids.

TABLE I
PRECURSOR AND PRODUCT IONS (M/Z), AND RELATIVE RETENTION TIMES IN SECONDS FOR LC- MS/MS ANALYSIS OF 16 UNDERIVATIZED AMINO ACIDS

Amino acid	Precursor ions (m/z)	Product ions (m/z)	Relative retention time (s)
Phe	166.10	120.10	4.90
Met	150.10	56.10	3.03
His	156.10	110.10	2.13
Val	118.10	72.05	2.51
Leu	132.10	86.15	3.21
Thr	120.10	74.00	2.14
Ser	106.10	60.20	2.08
Gln	147.10	84.10	2.13
Pro	116.10	70.10	2.09
Tyr	182.10	136.00	3.32
Asn	133.10	74.05	2.06
Asp	134.20	74.10	2.12
Ala	90.10	44.10	2.28
Glu	148.10	84.10	2.30
Cys	241.00	151.95	2.15
Cyt	241.037	122.00	2.14

Accuracy is a measure of the closeness of the calculated analyte concentration to the theoretical analyte concentration [7]. Horse heart myoglobin was hydrolyzed for analytical purposes to make sure that all peptide bonds between all amino acids were broken [8], [15], [16] Actual mole contents was calculated for 14 amino acid in this study as Cys and Cit have known sequences of amino acids. Accuracy of the method (expressed as relative error %) was measured by comparing the actual moles of the myoglobin samples to the measured moles in samples. Calibration curves of the 16 amino acids were fitted by linear regression (1/x). Linearity was assed using coefficient correlation (R2) of the external calibration curves. Limit of detection (LOD) is the lowest limit of concentration could be detected but could not be quantified. LOD was calculated by dividing three times the deviation error of the calibration line at the intercept by the slope of the calibration line (3* (SD of intercept/ Slope)) [18]. Limit of Quantitation (LOQ) is the lowest limit of concentration. LOQs were quantified for samples with acceptable accuracies. LOQ could equal LOD or higher [18]. In this study, LOQs equaled approximately 3 times LODs.

G. Statistical Analysis

Statistical analysis was used for instrument control, data acquisition and analysis using the Social Sciences (SPSS) Analyst 1.6.3 software version 21 (IBM, Armonk, NY, USA). Analysis of variance (ANOVA) was applied with a confidence level of 95% to calculate concentrations of amino acids in hair samples, calculate accuracies of the external curves and to calculate Areas under Curve (AUCs). The data were presented as mean ± standard deviation (M ± SD). $p \leq 0.05$ was considered significant.

Receiver Operating Characteristic Curve (ROC) which is a plot of the true positive probability (TPP), also known as sensitivity, against the false positive probability (FPP) at a specific threshold (defined by the analyst, mostly, relative to the lowest concentration) was constructed to determine AUC. Generally, performance increases with larger AUC [19].

III. RESULTS

Table II shows the actual moles of amino acids in myoglobin, the measured moles of amino acids in myoglobin, and the relative error.

TABLE II
MEASURED MOLES AND ACTUAL CALCULATED MOLES OF AMINO ACIDS OF MYOGLOBIN PROTEIN WITH RELATIVE ERROR %.

Amino acid	Measured moles in myoglobin x 10-8	Actual moles in myoglobin x 10-8	Relative error %
Met	1.34	1.14037	17.54
His	4.49	4.18135	7.36
Phe	1.82	2.66086	31.74
Val	2.96	2.66086	11.14
Leu	6.56	6.46208	1.45
Thr	2.36	2.66086	11.36
Ser	2.06	1.90061	8.14
Gln	2.01	2.28073	11.69
Pro	1.74	1.52049	14.25
Tyr	0.719	0.76025	5.36
Asn	0.795	0.76025	4.53
Asp	3.15	3.04098	3.65
Ala	5.78	5.70184	1.30
Glu	4.61	4.94159	6.74
Average	2.89	2.90522	9.73

The relative errors of amino acids were less than 32%, and the average relative error of the 14 amino acids was 9.7%. This indicated that the method was compatible for amino acids analyses.

AUC of Glu, Ala, Asn and Met were 0.702, 0.655, 0.496 and 0.261, respectively. Fig. 1 shows the AUC-ROC curve for Glu, as an example, which displays a very good sensitivity and specificity.

As shown in Table III, the R^2 values of all amino acids were greater than 0.91. LODs were located within the range of 0.3-7.1 ng/ml, and LOQs were in the range of 0.9-21.7 ng/ml. Therefore, this method was considered as a good method for amino acid analysis in hair without derivatization.

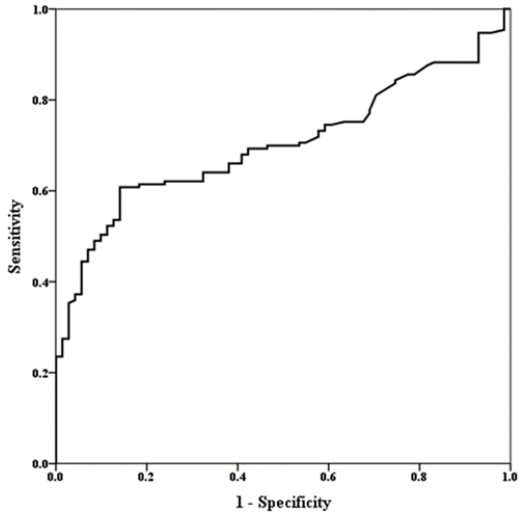

Fig. 1 Receiver operating characteristic curve of Glu

TABLE III
LODs, LOQs, EQUATIONS AND R^2 OF UNDERIVATIZED 16 AMINO ACIDS

Amino acid	LOD (ng/ml)	LOQ (ng/ml)	Coefficient (R^2)	Equation
Met	0.3	1.017	0.9870	y=261 x
His	0.9	2.866	0.9938	y= 335 x
Phe	1.3	4.221	0.9837	y= 2.77e3 x
Val	0.7	2.346	0.9832	y= 1.08e3 x
Leu	0.6	1.972	0.9972	y=1.72e3 x
Thr	2.7	8.263	0.9984	Y= 69.3 x
Ser	2.4	7.402	0.9910	y= 25.4 x
Gln	1.7	5.344	0.9866	y= 293 x
Pro	1.0	3.186	0.9199	y= 4.58e3 x
Tyr	0.9	2.799	0.9789	y= 384 x
Asn	0.9	2.748	9906	y= 41 x
Asp	7.1	21.709	0.9989	y= 48.4 x
Ala	2.2	6.766	0.9952	y= 39.1 x
Glu	0.9	2.876	0.9994	y= 212 x
Cys	1.4	4.487	0.9889	y= 15.9 x
Cyt	0.3	0.963	0.9927	y= 4.13 x

The results showed that the levels of Met, Ala and Asn were significantly lower ($p \leq 0.05$) in hair samples of the ASD group than those of the control group, while the levels of Glu were significantly higher than the controls ($p \leq 0.05$), see Table IV. There was no significant effect of age on the levels of amino acids in both groups.

IV. DISCUSSION

This study is to measure amino acids concentrations in hair samples from children with ASD and compare it to age-matched controls. The outcomes revealed distinct lower levels of the essential amino acids Met in the ASD group hair, as well as two non-essential amino acids (Asn and Ala) than the control group. Glu levels of the ASD group were remarkably higher than the controls. Other amino acids were at normal levels. Partially similar findings were reported by a previous analysis of plasma amino acids from children with ASD relative to controls [20]. Though the children exhibited significant lower levels of plasma β-alanine with higher levels of plasma glutamic acid, three amino acids (Cys, Tyr, and Ser) were significantly lower than controls [20]. The partial differences between both studies could have been due to genetic and environmental factors such as diet. All subjects of the current study were on regular Mediterranean diet. It is known that the type of diet could alter body absorption and metabolism of amino acids.

TABLE IV
MEAN ± STANDARD DEVIATION OF 16 AMINO ACIDS' LEVELS IN HAIR SAMPLES OF CHILDREN WITH ASD AND CONTROLS

Amino Acid		ASD M ± SD (ng/ml)	Control M ± SD (ng/ml)	P-value
Essential	Met	2.13 ± 0.93	5.12 ± 1.02	0.02
	His	2101 ± 737	2190 ± 2121	0.98
	Val	64 ± 56	194 ± 91	0.11
	Leu	33 ± 41	87± 2	0.30
	Thr	4380 ± 1472	6685 ± 883	0.31
	Phe	257.51± 26.92	204± 27.06	0.70
Conditionally essential	Pro	4151 ± 1178	4270 ± 70	0.29
	Tyr	21 ± 42	48 ± 1	
	Cys	4607 ± 4047	4825± 247	0.47
	Gln	1947 ± 641	1850 ± 410	0.72
	Cyt	4446 ± 3622	4825 ± 304	0.56
Non-essential	Asn	5.97 ± 2.62	13.70 ± 1.980	0.005
	Asp	2247 ± 531	1625 ± 318	0.42
	Ala	3571 ± 529	8990 ± 99	0.01
	Ser	4224.29 ± 2300.21	7225.00 ± 1590.99	0.34
	Glu	7679.29 ± 1752.40	3060.00 ± 212.13	0.02

Bold indicates significant values with $p \leq 0.05$

Different results were reported by another study. Children with ASD and Asperger syndrome, their siblings and parents, all had significantly reduced plasma Glu and raised Asp, Ala, Glu, Phe, Tyr and Lys than age-matched controls [21]. The study explained the alteration of the amino acid levels by a potential family background of dysregulated amino acid metabolism, which provided a further evidence for an underlying biochemical basis for the condition and explained the differences between those results and ours. Another important factor that could further explain these differences is the type of samples, which was plasma for the previous studies while it was hair for the present one. This may imply down-regulation of glutamate transport from blood into the hair. Further studies with larger populations are required to correlate amino acids levels in the hair to the plasma of children with ASD.

Another interesting record is the low levels of Met in our study compared to controls and to previous studies. This specific record was in harmony with previous records in plasma of children with ASD [12]. Met has a very important role in metabolism in human body. It contains a methyl group (CH3) that is involved in the process of methylation of DNA and proteins [22]. Met functions in CNS depend on one-carbon metabolism pathway, which is essential for the synthesis of DNA, RNA and neurotransmitters (i.e. serotonin, dopamine and acetylcholine). Vitamin B12 acts as a cofactor

in one-carbon cycle for Met synthesis. Thus, if children with ASD have Met deficiency they also could have vitamin B12 deficiency leading to neurotransmitters dysfunctions and affecting brain development [22], [23]. The currently involved participants could be warned for Met deficiency with potential B12 deficiency to reduce ASD associated symptoms and improving the action of their neurotransmitters.

Glu levels were higher in the hair of children with ASD compared to the controls. This was consistent with previous findings in blood samples from children with ASD [21], [24], [25]. In a recent review, blood Glu levels were stated as a potential ASD biomarker [11]. In fact, Glu is an excitatory neurotransmitter with important roles in the brain development and neuronal signaling. Accumulation of Glu in the blood of children with ASD is potentially neurotoxic causing excitotoxicity, neurodegeneration, and inflammation contributing to the general pathophysiology of ASD [11], [12]. Whatever the process involved, because glutamine is required for the metabolism of enterocytes, the reduction in plasma concentrations could be one of the parameters contributing to gut dysfunction in autism [21].

Ala levels in the current study were significantly lower in the ASD group than the control group. Ala is synthesized in astrocytes and transferred to the GABAergic synapse, where it can be utilized as a metabolic fuel for the GABAergic neurons [26]. Thus, Ala deficiency would lead to abnormal functioning of the CNS and contribute to ASD etiology.

Asn is another excitatory amino acid with roles in the brain development and functioning [27]. This amino acid was significantly reduced in the hair of children with ASD, which is on the contrary of earlier records that indicated significant increases of its plasma levels in ASD children [28]. Nevertheless, the study attributed the raised Asn plasma levels to be due to altered permeability of blood brain barrier that allowed the efflux and influx of Asn from brain to blood and Vice-versa. Repeatedly proposed, such differences are plausible as the deposition of Asn in hair of ASD children would be altered as well.

V. Conclusion

In conclusion, the herein enclosed levels of Met, Ala and Asn in the hair samples of ASD group with Mediterranean diet were significantly lower than the controls whereas, the levels of Glu were significantly higher. Consequently, the hair amino acid profile could be used as a biomarker signature for children with ASD. Further studies with larger sample sizes of coupled blood and hair collection are beneficial to evaluate the significance of amino acid levels as a diagnostic and therapeutic tool for children with ASD. Additionally, researchers could correlate neurotransmitters levels to amino acids levels in the hair of those children.

The present outcomes could help specialists diagnose and treat children with ASD through altering levels of neurotransmitters/amino acids during therapeutic/diet regimes. Also, profiling of amino acids in hair could provide a therapeutic/nutritional tracking tool with a larger time window relative to other biological samples.

Acknowledgments

The authors are grateful to the subjects and their relatives for their participation in the existing study.

Disclosures

The research was funded by the Deanship of Research at Jordan University of Science and Technology [2018/196].

Conflicts of Interest

The authors disclose none.

References

[1] Martin, N. (2009). "Art therapy and autism: Overview and recommendations." Art Therapy 26(4): 187-190.
[2] Mitchel, G.E. and K.D. Locke. (2015). Lay beliefs about autism spectrum disorder among the general public and childcare providers. Autism 19(5): 553-561.
[3] Lovinger, D. M. (2008). "Communication Networks in the Brain: Neurons, Receptors, Neurotransmitters, and Alcohol." Alcohol Research & Health 31(3): 196-214.
[4] Fürst, P. (2009). "Basics in clinical nutrition: Proteins and amino acids." e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism 4(2), e62-e65.
[5] Zhou, Y., & Danbolt, N. C. (2014). Glutamate as a neurotransmitter in the healthy brain. Journal of neural transmission, 121(8), 799-817.
[6] Best, J., Nijhout, H.F., reed, M. (2010). "Serotonin synthesis, release and reuptake in terminals: a mathematical model." Theoretical Biology and Medical Modelling 7(1): article no. 34.
[7] Gelenberg, A. J. and C. J. Gibson (1984). "Tyrosine for the Treatment of Depression." Nutrition and Health 3(3): 163-173.
[8] Bani Rashaid, A., Jackson, G. P., Harrington, P. de B. (2014). "Quantitation of amino acids in human hair by trimethylsilyl derivatization gas chromatography." Mass Spectrometry. Enliven: Bio Anal Techniques 1(1): 1-12.
[9] Tirouvanziam, R., et al. (2012). "Distinct plasma profile of polar neutral amino acids, leucine, and glutamate in children with Autism Spectrum Disorders." J Autism Dev Disord 42(5): 827-836.
[10] Arnold, G. L., Hyman, S.L., Mooney, R.A. Kirby, R.S. (2003). "Plasma amino acids profiles in children with autism: potential risk of nutritional deficiencies." Journal of autism and developmental disorders 33(4): 449-454.
[11] Zheng, Z., et al. (2016). "Blood Glutamate Levels in Autism Spectrum Disorder: A Systematic Review and Meta-Analysis." PloS one 11(7): e0158688-e0158688.
[12] Kempson, I. M. and E. Lombi. (2011). Hair analysis as a biomonitor for toxicology, disease and health status. Chem Soc Rev 40(7): 3915-3940.
[13] Gubi, A., et al. (2014). DSM-5 and school psychology: Changes to DSM-5 schizophrenia diagnosis.
[14] Chlebowski, C., et al. (2010). "Using the childhood autism rating scale to diagnose autism spectrum disorders." Journal of autism and developmental disorders 40(7): 787-799.
[15] Alqhazo, M., & Rashaid, A. B. (2018). The concentrations of bioelements in the hair samples of Jordanian children who stutter. International journal of pediatric otorhinolaryngology, 112, 158-162.
[16] Rashaid, A. H., Harrington, P. D. B., & Jackson, G. P. (2015). Profiling amino acids of Jordanian scalp hair as a tool for diabetes mellitus diagnosis: a pilot study. Analytical chemistry, 87(14), 7078-7084.
[17] Tu, W.-J., et al. (2012). "Application of LC-MS/MS analysis of plasma amino acids profiles in children with autism." Journal of clinical biochemistry and nutrition 51(3): 248-249.
[18] Fontanarosa, C., et al. (2017). "Quantitative determination of free D-Asp, L-Asp and N-methyl-D-aspartate in mouse brain tissues by chiral separation and Multiple Reaction Monitoring tandem mass spectrometry." PloS one 12(6), e0179748.
[19] Hajian-Tilaki, K. (2013). "Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation." Caspian

journal of internal medicine 4(2): 627-635.
[20] Zaki, M. M., et al. (2017). "Assessment of plasma amino acid profile in autism using cation-exchange chromatography with postcolumn derivatization by ninhydrin." Turk J Med Sci 47(1): 260-267.
[21] Aldred, S., et al. (2003). "Plasma amino acid levels in children with autism and their families." Journal of autism and developmental disorders 33(1): 93-97.
[22] Black, M. M. (2008). "Effects of vitamin B12 and folate deficiency on brain development in children." Food and nutrition bulletin 29(2 Suppl): S126-S131.
[23] Briani, C., et al. (2013). "Cobalamin deficiency: clinical picture and radiological findings." Nutrients 5(11): 4521-4539.
[24] Rojas, D. C. (2014). "The role of glutamate and its receptors in autism and the use of glutamate receptor antagonists in treatment." Journal of neural transmission (Vienna, Austria: 1996) 121(8): 891-905.
[25] Zieminska, E., et al. (2018). "Glutamate, Glutamine and GABA Levels in Rat Brain Measured Using MRS, HPLC and NMR Methods in Study of Two Models of Autism." Frontiers in Molecular Neuroscience 11: 418.
[26] Schousboe, A., Sonnewald, U., & Waagepetersen, H. S. (2003). Differential roles of alanine in GABAergic and glutamatergic neurons. Neurochemistry international, 43(4-5), 311-315.
[27] Ruzzo, E. K., et al. (2013). "Deficiency of asparagine synthetase causes congenital microcephaly and a progressive form of encephalopathy." Neuron 80(2): 429-441.
[28] Naushad, S. M., Jain, J. M. N., Prasad, C. K., Naik, U., & Akella, R. R. D. (2013). Autistic children exhibit distinct plasma amino acid profile.