IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Urinary Neurotransmitter Profiling Through LC-MS/MS Reveals Key Biomarkers In Autism Spectrum Disorder

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Abstract:

Autism Spectrum Disorder (ASD) raises a challenge in early diagnosis due to its diverse symptoms and unclear etiology. Present study focuses on exploring potential biomarkers associated with the pathogenesis of ASD through the analysis of urinary neurotransmitters using liquid chromatography-tandem mass spectrometry (LCMSMS). A total of 104 urine samples (64 ASD and 40 non-ASD controls) were collected and analyzed. Significant differences were observed in the levels of serotonin, 5-hydroxyindoleacetic acid (5-HIAA), and normetanephrine between ASD and non-ASD groups ($p<0.05$). Principal component analysis (PCA) revealed minimal variation between groups. Correlation analysis indicated weak positive correlations between serotonin and 5-HIAA ($p<0.05$), while negative correlations with metanephrine and normetanephrine ($p<0.05$). Metanephrine and normetanephrine had a moderate negative correlation ($p<0.05$). Heatmap analysis illustrated difference in the concentrations of serotonin, 5-hydroxy indoleacetic acid and normetanephrine and metanephrine among ASD and control group. Tryptophan (impact factor-0.12) and tyrosine (impact factor-0.02) pathways are highlighted in the pathway enrichment analysis ($p<0.05$). Serotonin, 5-hydroxy indoleacetic acid and normetanephrine ($p<0.05$) were identified as the potential biomarkers through Receiver operating characteristic (ROC) analysis, showed high area under the curve (AUC) values and strong sensitivity and specificity while metanephrine showed low performance ($p<0.79$). Present work emphasizes the importance of urinary neurotransmitters as a tool for identifying ASD and provides insight into neurobiological mechanisms underlying the disorder. Further research is essential to validate these findings.

Key words: Autism Spectrum Disorder, Neurotransmitters, Liquid chromatography, Mass spectrometry, Biomarkers.

Introduction:

Autism Spectrum Disorder (ASD), a neurodevelopmental disorder has characteristics like social impairment, restricted and repetitive behavior, sensory along with cognitive issues. Children with ASD display numerous behavioral traits like mood instability, hyperactivity, lack of attention, resistance to change, sensory disintegration, sleep disorders, urinary retention, and gastrointestinal issues (1). Symptoms are usually observed at the earliest of 18 months and above; hence, the diagnosis is possible at or above 18 months, based on direct observation and behavioral assessment. There has been a high rate of prevalence of ASD in the last two decades,

it has been raised from 1 in 100 to 1 in 36 children, according to estimates from the Centre for Disease Control and Prevalence (CDC) in the USA. The rise in prevalence may be due to awareness among the population or changes in the diagnostic criteria that promptly identify autistic features. The diagnostic criteria for ASD is based on the recent classification DSM IV (Diagnostic and Statistical Manual of Mental Disorders, 4th edition) (2) which is based on a child's behavioral symptoms, like impairments in communication, reciprocal social interaction, as well as restricted and repetitive behaviors (3, 4).

The etiology of ASD is complex due to its heterogeneous manifestation, which includes heredity, prenatal and early postnatal environmental exposure, neuroanatomical changes, neurotransmitter abnormalities, and so on. Neurotransmitters (NTs) are chemical signals in the brain, which play a role in regulating various processes such as proliferation, growth, migration, differentiation, and survival of neural precursor cells. At the time of brain maturation neural progenitor cell behavior is influenced by NTs, therefore these are involved in the neural development also (5). Mono-aminergic neurotransmitters like serotonin and dopamine are essential in depressive disorder and anxiety disorders that affect neural circuits in different brain regions and influence mood and mental movement in the CNS (6).

Serotonin is produced in the brain stem and spread throughout the CNS by the serotonergic neurons. It affects different parts of the brain, like the hypothalamus, cortex, hippocampus, amygdala, and striatum (7). Serotonin, with its inhibitory action, regulates emotion and behavior, including the inhibition of aggression. (8) Neural development and synaptic function are also being regulated by serotonin as a neurotransmitter (9, 10, 11). It is produced from the amino acid tryptophan, synthesis which involves two steps. Initially, tryptophan is converted to 5-hydroxytryptophan (5-HTP), followed by 5-HTP to 5-Hydroxytryptamine (5-HT) generally referred as serotonin. These two steps are mediated by tryptophan hydroxylase (TPH), and aromatic acid decarboxylase (AADC), respectively. Further, 5-HT is degraded to its metabolite 5-hydroxy indoleacetic acid (5-HIAA) by monoamine oxidase A (MAOA), which is involved in the conversion of catecholamine to their metabolites. The serotonin system in ASD is altered in terms of serotonin synthesis, transporter binding, and receptor activity (12). Neuroimaging studies showed alterations in focal and global serotonin synthesis in autistic children, which is illustrated by an increase in brain serotonin synthesis capacity in ASD when compared with a normal group, which shows high serotonin in early childhood and then declines to the adult value. A dysregulation in serotonin transporter binding capacity is observed in ASD. Various brain regions showed deprived serotonin transporter binding sites, which are correlated with social cognition and repetitive behavior (13). 5HT2A receptor binding capacity in ASD was found to be reduced when compared to the control group, which was associated with alterations in social interaction and cognition (14).

Normetanephrine and metanephrine are O-methylated metabolites of norepinephrine and epinephrine. The association of metanephrine and normetanephrine with autistic behavior is not clearly explained. However, few studies on MAO-A and MAO-B genes explained that these two monoamines are involved in developmental deficits, including profound mental retardation and autistic-like behavior (15). MAOA deficiency is associated with intellectual disabilities, autonomic abnormalities, and behavioral dysfunction, and MAOB deficiency is associated with intellectual and behavioral impairments. A combined MAO-AB deficiency is associated with intellectual difficulty, recurrent stereotypes, rapid loss of muscle tone, and epileptic seizures (16). MAOA is involved in the degradation of endogenous bioamines metanephrine and normetanephrine (28).

Knowing the neurotransmitter profile among the different populations of ASD may help in personalised treatment strategies that target specific neurotransmitters. Our study aims to find possible biochemical mechanisms and related factors, explaining how neurotransmitter levels can correlate with behavioral changes and detect ASD.

Materials and Methods:

Sample collection:

The study involves 104 urine samples which include 64 autistic and 40 controls. The specimens were collected from Utage Child Neuro Clinic and Developmental Center. Approximately 5 ml of urine sample was collected from each subject and control, age ranging from 2 to 10 years. After collection, samples were stored at -20°C until further processing was carried out. The diagnostic criteria followed for selecting ASD subjects was the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM IV). Children who do not fall under DSM IV criteria were considered as controls in the present study. Parents/ legal guardians of the children who have participated in the study have signed a written consent. Ethical committee has approved the study proposal.

Sample Preparation:

Creatinine estimation was performed in all samples after collection, and samples were stored at -20°C until further processing. Creatinine of each sample was estimated to normalize the concentration of the neurotransmitters to compensate the variation in the urine volume (27). Jaffe's method is used to estimate urine creatinine value.

Solid phase extraction (SPE) was carried out to extract the endogenous NTs before LCMSMS acquisition. C18 (week anion exchange) cartridges were used to extract NTs, Cartridges were initially equilibrated with 1ml methanol followed by 1ml, 5mM ammonium acetate. Approximately 1 ml of samples (1:1 ratio sample: 5mM ammonium acetate) was loaded into each cartridge and then 250 ul of 5% IPA was used to wash out the interference, finally, NTs were eluted in 250ul, 0.1% formic acid then loaded into LCMSMS. 5-hydroxy indoleacetic acid was injected through the dilute and shot method (1:9 sample: methanol).

Chromatography:

Separation of NTs was carried out with Liquid chromatography. Shimpack GIST phenyl column (150 × 4.6 mm, 5µm particle size, Shimadzu) performed better separation of NTs when compared to a few other columns with a short run time of 8 minutes. The program was created to enable an injection volume of 10ul of each sample loaded in the autosampler tray with the capacity of 96 sample vials. A gradient method applied to separate NTs with the combination of two mobile phases (MP) one with 0.05% acetic acid and the second one was 50% methanol. All the solvents including water used for the mobile phase preparation were LCMS grade. Flow rate 0.4ml/min was optimized initial flow 5% MP-A Injection port, autosampler temperature maintained at 4°C. In contrast, the column oven temperature was set at 40°C high-resolution peaks were obtained with all set parameters.

LC time program- 10% MP B was constant for 1 min, then MP B shifted to 90% and flowed constantly till 7 mins then dropped down to 10% at 8 mins holding for 1 min to equilibration of next run.

HPLC parameters

Column	Shimpack GIST Phenyl (150mm x 4.6mm,5 µm)
Mobile phase A	0.1% acetic acid in water
Mobile phase B	0.05% methanol in water
Flow rate:	0.4 mL/min
Column temp.:	40 °C
Injection volume:	10 µL
Time program:	10% B (0-3.0 min), 90% B (3.01 – 7.0min), 10% B (7.1-8.0 min)

Mass Spectrometry detection:

The data acquisition was performed by mass spectrometry (Shimadzu, 8045) coupled with liquid chromatography (I series LC 2050) equipped with electrospray (ESI) ionization, operating in positive and negative modes. The ionization temperature was fixed at 400°C, and Multiple Reaction Monitoring (MRM) was performed to acquire data. Acquired peaks were analyzed with Lab Solutions (Shimadzu) software.

Mass spectrometry parameters

ESI positive mode:	
Interface voltage:	+0.6 kV
Nebulizer gas flow:	3.0 L/min
Drying gas flow:	10 L/min
Heating gas flow:	5 L/min
DL temp.:	200°C
Interface temp.:	400°C
Heat block temp.:	400°C

MRM transitions and parameters

Compound	Polarity	Precursor ion m/z	Product ion m/z	Q1	CE	Q3
Serotonine	+	177.1	160.0	-22.0	-12.0	-15
5HIAA	-	190.1	145.75	9.0	14	25
Metanephrine	+	180.1	148.2	-13.0	-20	-26.0
Normetanephrine	+	166.1	134.0	-18.0	-12.0	-22.0

Statistical analysis

SPSS software was used for the statistical analyses. Initially, the Shapiro-Wilk normality test was applied to check the normality distribution. All variables were analyzed using descriptive statistics and summarized as mean ± standard deviation. Categorical variables were tested using the Chi-square test. Continuous variables were analyzed using Mann-Whitney U test where the assumptions of normal distribution were not met and expressed in p-value and Z-value. The receiver operator characteristics (ROC) curve was used to assess the accuracy of the urinary neurotransmitter levels in the diagnosis of ASD. ROC curves were plotted to determine the cut-off points with Youden's index. The p-value < 0.05 was assessed as statistically significant. Metaboanalyst version 6.0 software was used to perform principal component analysis (PCA), Spearman's correlation coefficient, and heatmap analysis to evaluate the differences among the groups. Logarithmic transformation and scaling were performed before statistical analysis.

Results:

Table 1 Demographic characteristics of participants

Parameters	**Non-ASD (n = 40) (Mean ±SD)**	**ASD (n=64) (Mean ±SD)**	**p-Value**
Male	23 (57.5%)	51 (79.7%)	0.015
Female	17 (42.5%)	13 (20.3%)	
Age (Years)	4.11±2.96	4.14±2.29	0.950

A total of 104 (64 ASD and 40 non-ASD) children were enrolled in the study. The percentage of males (79.7% and 57.5%) was found to be higher when compared to females (20.3% and 42.5%) in ASD and non-ASD children, respectively. There is a significant gender difference observed among ASD and Non-ASD (p-0.015). The mean age of ASD (4.14 ± 2.29) and Non ASD was (4.11 ± 2.76), no statistical difference was observed (Table 1).

Table 2: Neurotransmitter levels in controls and ASD

Parameters	Controls (n=40) Mean ± SD	ASD (n=64) Mean ± SD	p Value	Z Value
Serotonin	95.86 ± 162.19	275.73 ± 742.70	0.005	-2.801
5-HIAA	1154.98± 1424.91	2778.53± 6551.25	0.001	-3.361
Metanephrine	16035.98±56685.46	501.79±1027.85	0.910	-0.114
Normetanephrine	468.78± 976.26	1002.43 ±1787.06	<0.001	-4.744

p value and z value calculated using Mann-Whitney test
Mean and SD were calculated using an independent t-test

The mean levels of serotonin, 5-HIAA, and normetanephrine (275.73 ± 742.70, 2778.53± 6551.25, and 1002.43 ±1787.06) were higher in ASD when compared to Non-ASD (95.86 ± 162.19, 1154.98± 1424.91, and 468.78± 976.26), while the metanephrine mean level was lower in ASD (501.79±1027.85) than in Non-ASD (16035.98±56685.46). However, the mean levels of serotonin, 5-HIAA, and normetanephrine showed significant differences between ASD and non-ASD (p - 0.005, p -0.001, p < 0.001) (Table 2).

Principal component analysis

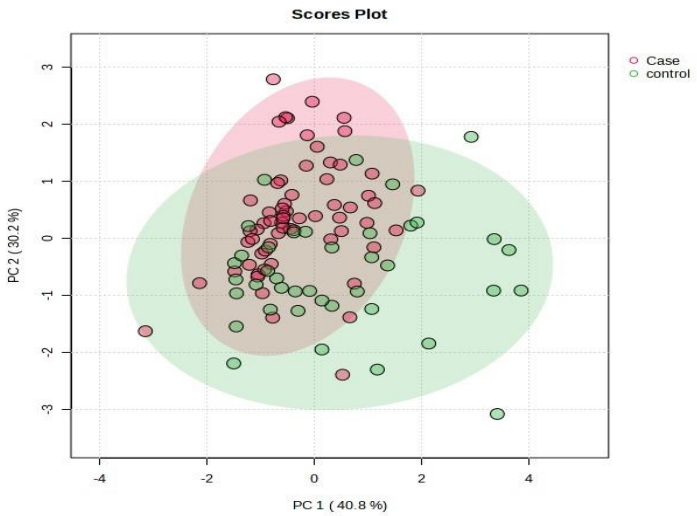

Figure 1 PCA score plot with principal components 1 and 2 shows 71.0% variance among the groups, the red dot indicates the control group and the green one indicates the ASD group.

Principal component analysis (PCA) was performed to identify the differences, similarities, trends, and outliers between ASD and healthy controls. The PCA plot showed an overlap with minimal separation among the groups. This distinguishes the metabolite concentrations of ASD from the healthy group (figure 1).

Correlation analysis:

Spearman's correlation coefficient analysis was performed to find the strength and direction of the relationship between the metabolites (Figure 2).

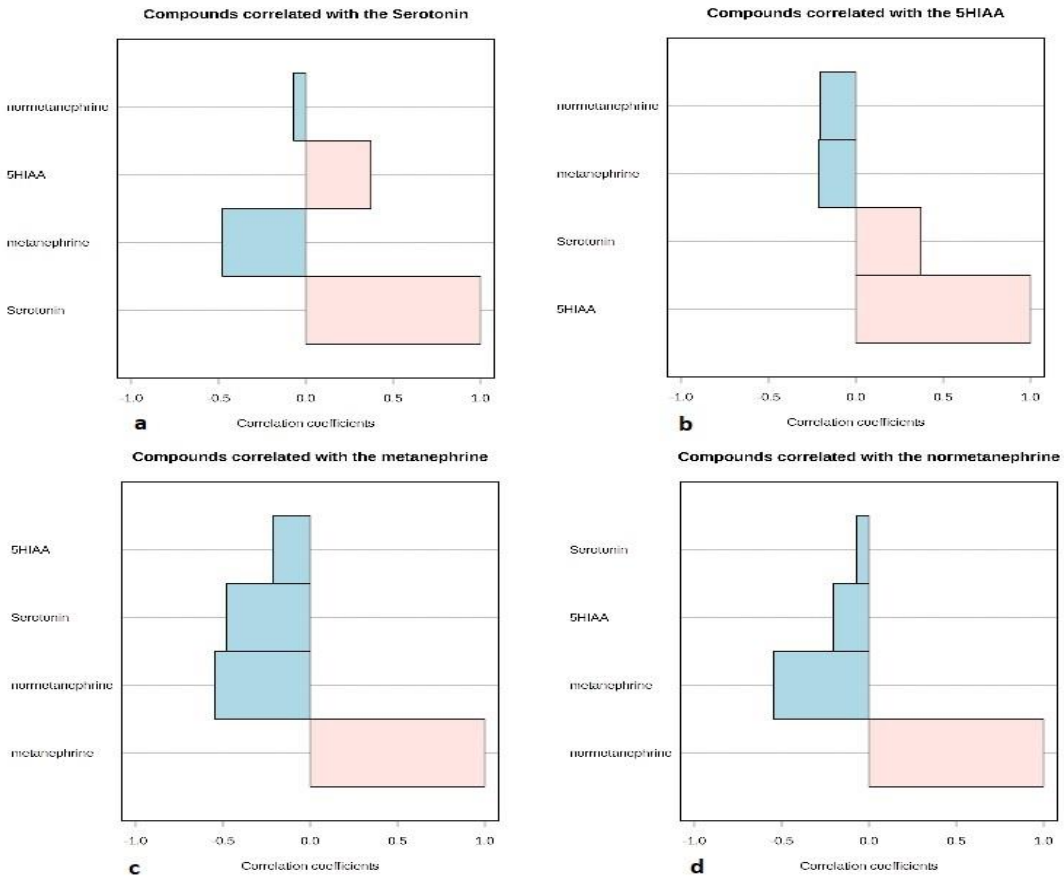

Figure 2 Correlation of individual metabolite with other metabolites in the study (a) compounds correlated with serotonin, (b) compounds correlated with 5-HIAA, (c) compounds correlated with metanephrine, and (d) compounds correlated with normetanephrine

The correlation matrix (Table 3) reveals that serotonin and 5-HIAA have a weak positive correlation. On the other hand, serotonin shows a moderately negative and weakly negative relationship with metanephrine and normetanephrine, respectively. 5-HIAA shows a weak negative correlation with metanephrine and normetanephrine. While metanephrine and normetanephrine are moderately negatively correlated with each other.

Table 3 Correlation between individual metabolites

	Serotonin	**5HIAA**	**Metanephrine**	**Normetanephrine**
Serotonin	1	0.37393	-0.47585	-0.06936*
5HIAA	0.37393	1	-0.21458	-0.20235
Metanephrine	-0.47585	-0.21458	1	-0.54581
Normetanephrine	-0.06936*	-0.20235	-0.54581	1

Note: Spearman's correlation coefficient showing p-value < 0.05
*No significance observed

Heatmap analysis:

Heatmap is a data visualization technique in which individual values in the dataset are represented with a colour, the colour indication ranges from light blue to dark brown, shows the expression of neurotransmitter levels in the ASD and non-ASD populations. The blue colour indicates the downregulation, while the red colour indicates the upregulation of the metabolites. The ASD group is represented in green, and the non-ASD group is represented in red. Expression of neurotransmitter levels was significantly associated with the ASD group (Mann-Whitney test). The NT serotonin and 5HIAA are closely related, which is depicted by the clustering (Figure 3). In the case of serotonin and metanephrine, we observed a difference in the metabolite concentrations of control and patient populations. Whereas in the case of normetanephrine, the control group showed more expression compared to the ASD group.

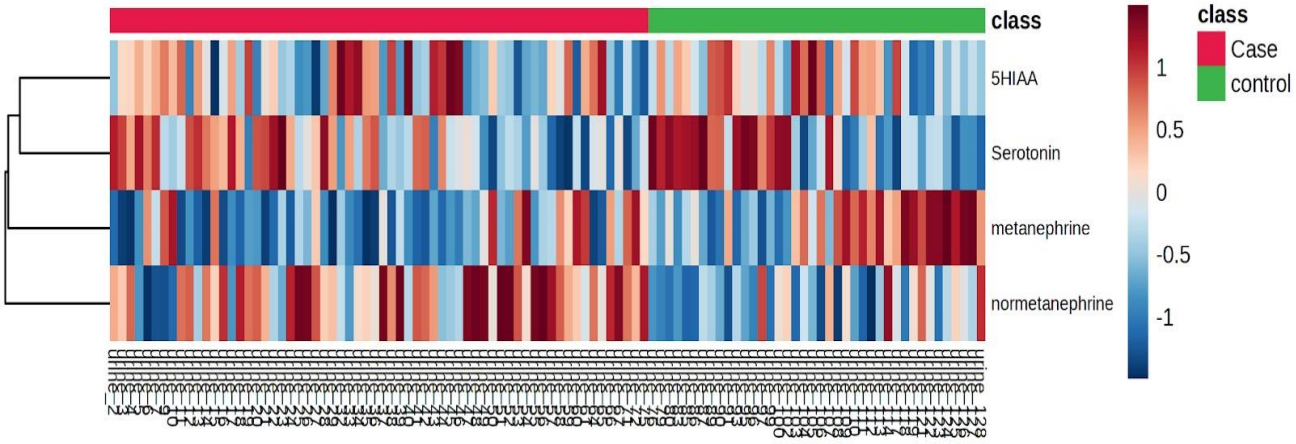

Figure 3 Heatmap depicting the neurotransmitter levels (5-HIAA, serotonin, metanephrine, and normetanephrine) in rows.

Biomarker analysis

The neurotransmitters that showed significant differences among the groups were further evaluated by the ROC curve analysis to determine the potential biomarkers for disease diagnosis. The ROC curve is performed by measuring different parameters, like area under the curve (AUC), sensitivity (true positive), and specificity (true negative) (Figure 4).

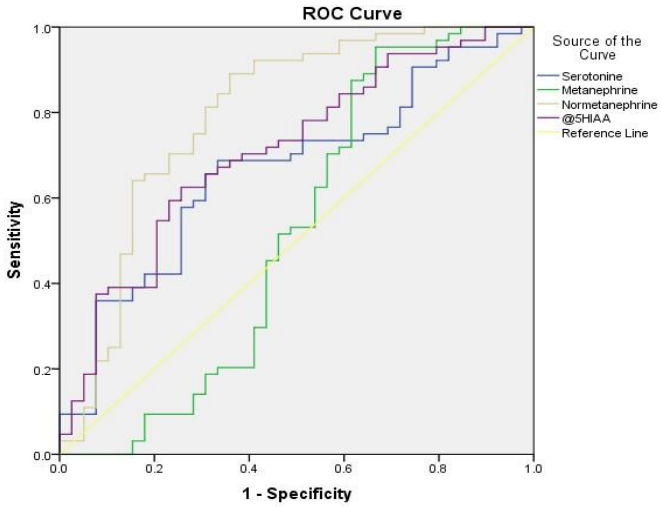

Figure 4 The ROC curve of neurotransmitters, y-axis display sensitivity and x-axis display 1-specificity.

Table 4. Cut-off values of urinary neurotransmitters observed in ASD

Parameters	AUC	Cut-off values	Sensitivity (%)	Specificity (%)	p Value
Serotonin	0.665	59.54	65.6	69.2	<0.05
5-HIAA	0.711	842.86	70.3	61.5	< 0.001
Metanephrine	0.518	227.16	51.6	53.8	0.76
Normetanephrine	0.795	209.48	78.1	69.2	< 0.001

The area under the ROC curve of serotonin and 5-HIAA was 0.665 and 0.711, with 65.6% and 70.3% sensitivity and 69.2% and 61.5% specificity, respectively, with a cut-off of 59.54 umol/gcr and 842.86 umol/gcr. The cut-off
values of metanephrine and normetanephrine were 227.16 umols/g cr and 209.48 umols/g cr respectively; the AUC was 0.518 and 0.795 with 51.6% and 78.1% sensitivity, 53.8% and 69.2% specificity, respectively (Table 4).

The ROC analysis revealed that serotonin, normetanephrine, and 5HIAA have high AUC values and strong sensitivity and specificity, suggesting them as good markers ($p<0.05$) for the identification of ASD from the non-ASD group. On the other hand, metanephrine having low AUC value, sensitivity, and specificity of around 50% may not be reliable marker for the differentiation of ASD from non-ASD accurately, hence determining it as poor performer with p-value 0.79

Pathway enrichment analysis:

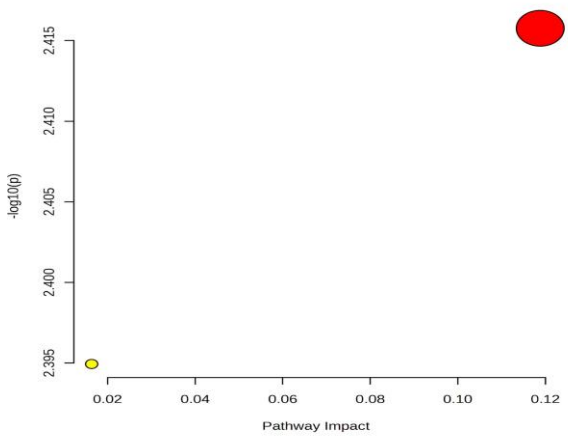

Figure 5 Pathway enrichment analysis, pathway impact and pathway enrichment on the x-axis and y-axis respectively. The larger and darker circle represents the more significant pathway.

Pathway enrichment analysis was carried out to identify the biological pathways involved in the study. Results showed that tyrosine and tryptophan pathways are involved in the metabolism of serotonin, 5-HIAA, metanephrine, and normetanephrine. Tryptophan and tyrosine metabolic pathways were statistically significant with p-value <0.05 and impact factors 0.12 and 0.02 respectively (figure 5).

Discussion:

An early diagnosis of ASD is difficult considering its diverse symptoms and unclear etiology. Urinary LCMSMS analysis provides a comprehensive assessment to detect potential biomarkers of different metabolic pathways (26). Our study was based on LCMSMS analysis of urine samples to evaluate neurotransmitters as potential biomarkers associated with the pathogenesis of ASD. The study group includes ASD children along with age and gender-matched controls. The ASD and non-ASD groups did not show variation in the age group of the study population, and no significance was observed ($p>0.950$). Previous study reported males diagnosed

with ASD were more frequent than females, with about a 4:1 ratio (17). A recent study found that male children show slightly more repetitive and restricted behavior than females (18, 19) while there was no difference in the social interaction or communication among the male and female children (18). Meta-analysis-based studies revealed male vs female ratio of about 3:1 rather than 4:1 as assumed (17). The present study showed substantial differences between the male and female individuals.

Herault et al. study on 23 ASD children and 59 typically developed (TD) children, reported higher 5–HT levels in ASD children than in TD children (20). Aiping Liu et. al., study suggests that 30 ASD cases with age and gender-matched 30 controls revealed high 5-HT levels in urine (21). In contrast studies conducted by Gevi et. al. stated that 5-HT concentration in urine was decreased in ASD children when compared with matched controls. Urine serotonin and serotonin transporter (SERT) levels were high in ASD when compared to controls (22). Our findings were in association with the Herauil et al. and Aiping Liu et al. studies, which suggest urine serotonin levels are higher in the ASD group when compared with the control group.

Deepak et al explained that dysregulation in the serotonergic system was associated with an increase in platelet 5-HT levels and an increase in the excretion of 5HIAA. Few studies found no significant differences in urinary 5-HIAA between children with ASD and control (20, 23). One study reported higher 5-HIAA excretion in the urine of hyperserotonemic individuals with ASD when compared to controls (23). Further studies conducted among Italian autistic children showed a decrease in the concentrations of 5-HT and 5-HIAA (24) our study showed a higher excretion of 5-HIAA along with 5-HT.

To our knowledge, this is the first study that evaluates metanephrine and normetanephrine, along with 5-HT and 5-HIAA as biomarkers in the urine samples of ASD children. Epinephrine and norepinephrine are catabolized to metanephrine (MN) and normetanephrine (NMN), respectively by MAOA. Genetic studies revealed that the defect in MAO is associated with autistic features hence, MN and NMN estimation in urine may serve as useful markers in the diagnosis of ASD. Only one study found high normetanephrine in siblings and very mild increase in their mother (25). In our study we observed, increased concentrations of NMN and low concentrations of MN in ASD when compared to non ASD.

NTs serotonin, normetanephrine and 5-HIAA are the good indicators in discriminating ASD and non-ASD as per the ROC curve investigation, in contrast metanephrine may not be a reliable marker for ASD and non-ASD differentiation.

Conclusion:

In the present study, LCMSMS was used for the urine samples to look at possible biomarkers involved in the etiology of autism spectrum disorder. Urine serotonin, normetanephrine, and 5-HIAA levels differ significantly in ASD individuals compared to matched controls. The present study is the first to assess urine metanephrine and normetanephrine as possible biomarkers in ASD, implying their potential for use in diagnosis. ROC analysis demonstrated that serotonin, normetanephrine, and 5-HIAA have high AUC values, strong sensitivity and specificity, suggesting that they might serve as potential markers in discriminating ASD from non-ASD individuals.

These findings express the role of urine biomarkers, especially 5-HIAA, serotonin, and normetanephrine, may be useful in aiding the early detection of ASD. Future studies in large population may validate these findings and explore the diagnostic utility of these biomarkers with other diagnostic tools to relate the improvements in the accuracy of ASD diagnosis and personalised treatments.

Acknowledgements: We thank Mr. Rajeev Sindhi, MD, Sandor Lifesciences Pvt. Ltd. for providing necessary infrastructure for the study. We thank all the families who had participated in the study.
Funding Information: No funding.

Author contributions SCN participated in the study design, statistical analysis, interpretation of data, and drafting the manuscript. NB carried out technical analysis. NA and PU participated in recruitment of the patients, interpretation of clinical reports, and counseled the affected families. SLG carried out the conception and design of the study, interpretation of data, and final approval of the manuscript.

Conflict of interest: The authors declare that they have no competing interests (financial or non-financial) in the present study.

Ethical approval: The study was approved by the Institutional ethical committee for biomedical research, Institute of Genetics and Faculty of Science, Osmania University (4/EC/NEW/IN ST/2023/4032). This study complied with the ethical principles outlined in the Declaration of Helsinki.

Informed consent: Informed written consents were obtained from patients/guardians along with detailed clinical history during their enrollment for the study

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