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A Student Perspective: N-Acetylaspartate (NAA) Detection by Urine GCMS Analysis in a Rare Genetic Brain Disorder – Canavan Disease

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ABSTRACT

Canavan Disease (CD) is a rare devastating brain disorder with degeneration of the white matter of brain. It is primarily of white matter degeneration disorder, affecting myelin. It characteristically shows spongy degeneration of the brain (vacuoles with fluid) and is a lethal neurodegenerative disorder of infancy with incidence of 1in 100,000. This study follows a retrospective, observational research design based on secondary data analysis of 4 cases accurately diagnosed by the increased N-acetylaspartate using urinary GCMS metabolic profiling. The research focuses on analyzing existing metabolic data to identify patterns and variations associated with Canavan disease, a very rare autosomal recessive genetic disorder in India. This retrospective study emphasises the crucial diagnostic role of non-invasive urinary GCMS analysis in the evaluation of suspected Canavan disease cases. Elevated urinary N-acetylaspartate levels (NAA) were consistently observed in all four patients and correlated with clinical and neuroimaging findings, confirming it as the biochemical hallmark of Canavan disease. In these 4 Canavan cases, there was no correlation found in NAA levels & severity of clinical findings or white matter changes studied.

Keywords: Canavan disease, NAA, GCMS, neurodegeneration, ASPA enzyme

INTRODUCTION

Canavan Disease (CD) is a rare devastating brain disorder with degeneration of the white matter of brain. Hence it is also referred as a "Leucodystrophy", leuco means white. The human brain shows grey matter (mainly full of neurons /brain cells) and the long ramifications of neurons which are incoming (afferent) & outgoing (efferent) fibres covered by the myelin sheath which looks white. The Canavan disease is primarily of white matter degeneration disorder, affecting myelin. It characteristically shows spongy degeneration of the brain (vacuoles with fluid) and is a rare lethal neurodegenerative disorder of infancy (18).

First described by Myrtelle Canavan in 1931, this disease is most prevalent in the Ashkenazi Jewish population (4). When both the parents are of Ashkenazi Jewish descent, the incidence is 1: 6,400 to 1:13,500 births. The Canavan disease occurs worldwide and the incidence of severe Canavan disease in the non-Jewish population is approximately 1:100,000 births. It is very rare brain disorder in Indian population (15).

This neurodegenerative disorder mainly occurs in infants & young children with characteristic features such as large head (macrocephaly), mild to severe neurodevelopmental (motor & mental) delay, loss of muscle tone (hypotonia), seizures (epilepsy), spasticity and sometimes visual impairment (5 & 7). The signs & symptoms slowly become severe & progressive deterioration occurs.

Neuroimaging- Besides clinical examination, the other diagnostic modalities used by the child neurologist are neuroimaging (1 & 14) which are expensive & not afforded by majority of the Indian population from low socio-economic background. These are as below-

1) Brain Magnetic Resonance Spectroscopy (MRS) which shows markedly elevated NAA peaks, and are considered a biochemical hallmark of the disorder.

2) Brain MRI for diffuse white matter degeneration in the cortex & central brain region.

Biochemical & Enzyme Diagnosis - The measurement of enzyme activity of aspartoacylase is measured by certain coupled enzyme assays based on spectromphotometric & fluorometric methods **(11).** Some are high-throughput screening used for population screening but not available easily in our country due to rarity of the disorder. The use of radioactive substance in radiometric assay makes the method non-feasible in routine biochemical laboratory.

The Gas Chromatography-Mass Spectrometry (GCMS) is considered as the definitive "gold standard" for identifying Canavan disease (2 & 16). It works by detecting a specific metabolic footprint in a patient's urine. In a healthy brain, the enzyme aspartoacylase breaks down a substance called N-Acetylaspartic acid (NAA) into acetate and aspartate, while in Canavan disease patient, this enzyme is deficient, causing NAA to build up to toxic levels which can be detected in the urine with high sensitivity, accuracy & reliability (14). However, such advanced mass spectrometry diagnostic laboratories are very few to cope with the demand of 1.4 billion Indian population.

Genetics and Pathological Mechanism:
The inheritance of Canavan disease is an autosomal recessive (OMIM #271900), means if both parents are carrier of the defective gene, the recurrent risk of birth of affected child is 25 % in that family. It is mainly caused by mutations in the ASPA gene located on chromosome 17p13.2, which result in a deficiency of the enzyme Aspartoacylase (12). This enzymatic defect leads to impaired hydrolysis of N-acetylaspartate (NAA) into aspartate and acetate, causing excessive accumulation of NAA in the brain and increased NAA excretion in urine (14). The disease course is often progressive, leading to significant neurological impairment and reduced life expectancy.

In the absence of enzyme Aspartoacylase, the myelin sheath around nerve fibres does not form & triggers a pathological build-up of N-Acetylaspartate (NAA) in the brain and eventually spill over into the blood and urine. This eventually leads to the degradation of oligodendrocyte -the myelin forming glial supporting brain cells (3). A characteristic spongiform degeneration and a failure of myelin development ultimately results into impairment of the efficient transmission of neural signals which are important during neurodevelopmental period.

Once the specific pathologic mutation of ASPA gene is detected in the affected child using molecular genetic methods, both parents are screened for the same gene defect to establish the inheritance from the parents. (12). The pregnancy monitoring and prenatal diagnosis in future pregnancy of that family is then possible to detect whether unborn child (fetus) is affected or not & thereby can prevent the birth of another Canavan disease child.

We describe here a retrospective analysis of 4 cases of rare Canavan Disease diagnosed at GCMS metabolic laboratory in India and present it from the student's perspective (first author who is undergraduate intern student) to emphasise the need of such services for early and accurate diagnosis which is essential for prevention, genetic counseling and prenatal diagnosis, especially in populations with high rates of consanguinity.

MATERIALS AND METHODS

Study Design: This study follows a retrospective, observational research design based on secondary data analysis. The research focuses on analyzing existing metabolic data to identify patterns and variations associated with Canavan disease, a very rare autosomal recessive neurodegenerative disorder in India.

Data Source : The primary dataset consists of anonymized GCMS (Gas Chromatography –Mass Spectrometry) test reports provided by the laboratory at which training for GCMS testing was taken by the student. These reports include metabolic profiles from:

- Individuals diagnosed with Canavan disease
- Healthy control subjects

All data were de-identified prior to access to ensure patient confidentiality and ethical compliance.

Study Population:
The study was conducted as a retrospective observational case series involving four pediatric patients (Patients 1–4 in Table-1) who were referred for metabolic evaluation with clinical suspicion of Canavan disease. The age of the patients ranged from 4 months to 8 years. The cohort included both male and female patients from different ethnic backgrounds. Clinical data were retrieved from medical records and included demographic details, presenting neurological features, developmental history, seizure history, and family history including consanguinity.

All patients had undergone detailed clinical examination by a pediatric neurologist. Neuroimaging findings, including magnetic resonance imaging (MRI) and / CT scan were reviewed wherever available. Urine samples were collected from all patients for GCMS analysis. The study was performed in accordance with institutional ethics standards, and informed consent was obtained from parents or legal guardians prior to metabolic testing.

Methodology:

Urine Sample Collection and Process for GCMS analysis: Random urine samples were collected from each patient in sterile containers. Samples were stored at appropriate temperatures until analysis. Alternatively the urine soaked & dried filter paper were sent to the GCMS laboratory from the remote places (Fig. 1) by post. Prior to GC-MS analysis, urine samples underwent urease pretreatment to remove excess urea, followed by chemical derivatization to convert organic acids and other compounds into volatile derivatives suitable for gas chromatographic separation. Standard protocols used in MILS Laboratory for analysis were followed using method of Matsumoto & Kuhara (1996) who had pioneered the current method (13). The test detects almost 250 metabolites comprising different amino acids, organic acids, sugars, sugar alcohols, nucleic acids, fatty acids etc. which serve as biomarkers for various metabolic conditions.

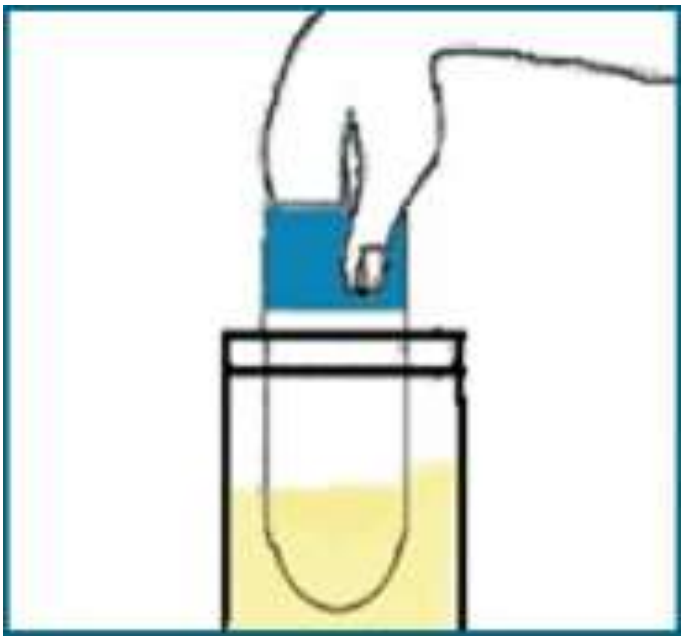

Figure 1: The filter soaked in urine, dried & to be sent to Laboratory

Gas Chromatography–Mass Spectrometry (GCMS) Analysis:

The 2 microL of extract after chemical processing of the urine samples were then injected into GCMS equipment. Separation of metabolites was achieved through gas chromatography, and detection was performed using mass spectrometry under electron impact ionization conditions. Total Ion Chromatograms (TICs) were generated for each sample, enabling comprehensive profiling of urinary metabolic compounds.

Identification of metabolites was performed by comparing retention times and *m/z* mass spectral patterns using established reference libraries. Particular attention was given to the identification and quantification of N-acetylaspartate (NAA), a key biochemical marker of Canavan disease (2,17). Quantitative analysis of NAA was performed, and values were compared against already established laboratory reference ranges. Markedly elevated urinary NAA levels were considered diagnostic of Canavan disease when interpreted in conjunction with clinical and neuroimaging findings.

Data Interpretation

GC-MS findings were correlated with clinical presentation and available neuroimaging results. Elevated urinary N-acetylaspartate levels were interpreted as diagnostic of Canavan disease in the appropriate clinical context. Descriptive analysis was used to summarize clinical, biochemical, and radiological findings across the four patients.

The Table -1 summarizes four cases identified through clinical & GCMS urinary screening:

Table 1 – Details of 4 Canavan Disease cases

Sr No.	Age	Gender	Ethnicity	Available Clinical Information	NAA (Ratio of peak area of NAA & IS)
1	4 Months	Male	Muslim	Hair bleaching, hearing impairment, intellectual disability, Consanguinity present Investigations:CT positive for white matter degeneration.	159.38
2	8 Years	Female	Muslim	Spastic – ataxic developmental delay; seizures at the age of 6 months, Investigations: Brain MRI- s/o glutaric aciduria; sleep EEG- normal	12.68
3	9 Months	Male	Hindu	Born full term out of consanguineous marriage. Cried immediately after birth, H/o Global developmental delay, intermittent dystonic posturing (+), cooing (+). C/o seizures, febrile seizures, hypotonia, scissoring, brisk reflexes, spastic quardriparasis, dystonia with visual and auditory inattention. Investigations: MRI- hypomyelination with increased NAA peak.	6.83
4	5 Months	Male	Muslim	Respiratory distress at birth, macrocephaly, seizures, Consanguinity present. MRI with white matter changes indicative of positive case.	39.82

(*NAA- N-Acetylaspartic Acid; IS- Internal Standard-Creatinine)

RESULTS

Clinical Evaluation

Clinical assessment in all 4 cases revealed neurological manifestations commonly associated with leukodystrophies, including developmental delay, hypotonia or spasticity, seizures, macrocephaly, abnormal posturing, and visual or auditory inattention with varying degrees. The brain white matter involvement in correlation with other clinical findings were suggestive of Canavan Disease and hence referred for the NAA estimation by GCMS analysis.

The age of presentation ranged from 4 months to 8 years. Three patients were male and one was female. Three patients belonged to the Muslim ethnic group and one was from Hindu religion.

The family history with particular attention to consanguinity was recorded due to its relevance in this genetic condition. Out of 4 cases, three (75%) showed consanguinity, confirming the predominant role played by the autosomal recessive inheritance in Canavan disease.

Neuroimaging findings were found to be consistent with white matter degenerative changes. However, in one case, (Table-1:Case No.2), Glutaric aciduria was suspected due to similar brain MRI changes. But it was ruled out by the precise detection of biochemical marker-NAA in urine GCMS analysis in comparison with the control samples.

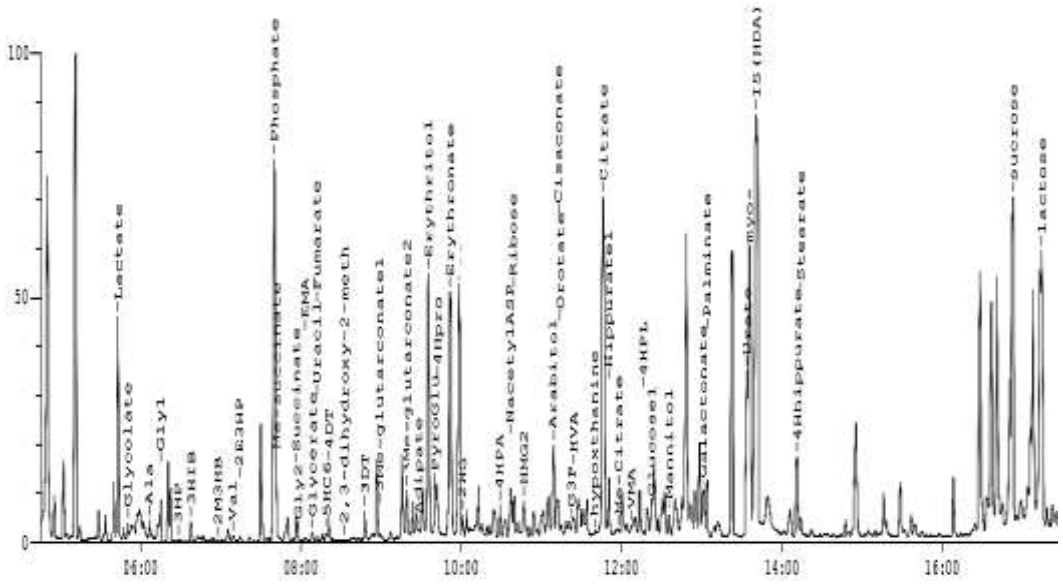

Fig 2- The TIC of urinary GCMS analysis from the healthy control subject showing absence of N-Acetylaspartic Acid (NAA).

The age matched 4 control samples were studied for GCMS urine analysis & their TICs did not reveal NAA marker compound at 10 minutes RT (Fig. 2). However, TIC of all 4 cases could detect NAA and one such example is shown in Fig. 3 with long peak of NAA (red arrow) in TIC. The quantitative measurement of NAA was different in all cases & was not found in correlation with the severity of the disease course (Table 1).

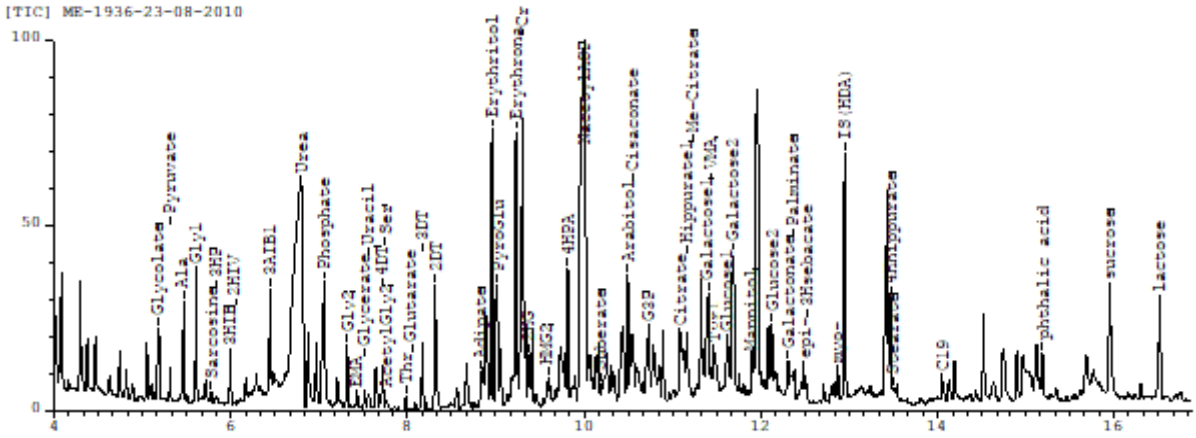

Fig 3- The TIC Urinary GCMS analysis of Canavan Disease patient showing abnormal increased N-Acetylaspartic Acid (red arrow) in TIC.

The parents of these 4 cases were explained about the inherited genetic nature of this devastating condition & further course of the disease, half of them with tele genetic counseling. The future steps for prevention through prenatal tests during pregnancy was also briefed to them.

DISCUSSION

The rare genetic myelin disorders typically emerge in early childhood and present through several clinical signs of neurodevelopmental delay, including low muscle tone (hypotonia), impaired coordination (ataxia) and seizures. Currently, no curative treatment exists for the Canavan Disease, which typically leads to mortality in childhood. There are published reports documenting no obvious correlation between the severity of the white-matter disease and the clinical presentation-one patient with severe white-matter disease was clinically normal (1).

The CT and MR findings in Canavan disease are also reported to be nonspecific and somewhat non-uniform and its pre-autopsy diagnosis relied primarily on biochemical findings (1). A case report from India showed severe spasticity & MRI changes in Hindu girl with non- consanguineous marriage (15) which was also observed in our present study (Case No. 3).

A high NAA peak on a GCMS is considered pathognomonic - meaning it is a "hallmark" sign that effectively confirms the diagnosis of Canavan disease even before molecular genetic testing is completed (17). The Mass Spectrum measures the mass of the fragments and NAA has a unique "mass spectrum" fingerprint (m/z ions) at about 10 minutes Retention Time (RT) that allows the computer to confirm its identity with 100% certainty (Fig. 3). Quantitative urinary NAA values ranged from 159.38 to 6.83 (units as per laboratory reference standards). The highest NAA concentration was observed in Patient 1 (Table 1). A characteristic elevation of NAA at specific retention times on the total ion chromatogram (TIC), distinguished affected individuals from normal controls (2 & 16). Compared to imaging techniques, GC-MS provides a minimally invasive, cost-effective, and reliable diagnostic approach, particularly significant in resource-limited settings. Furthermore, GC-MS allows simultaneous detection of additional metabolic abnormalities, aiding in differential diagnosis of other neurometabolic disorders (6).

In a healthy individual, aspartoacylase is responsible for breaking down N-acetylaspartic acid (NAA) into l-aspartate and acetate. Acetate is crucial for the synthesis of myelin, the protective sheath around nerve fibers. Mutations in the ASPA gene lead to a significant reduction or complete lack of aspartoacylase activity, with high concentration of NAA which creates an osmotic imbalance, drawing water into the myelin layers. This causes the characteristic "spongy" appearance of the brain's white matter (leukodystrophy), leading to the clinical symptoms of macrocephaly and developmental delay (17 & 18).

Molecular diagnosis is the definitive method for confirming Canavan Disease (CD) following initial biochemical screening. It involves identifying specific pathogenic variants in the genetic code that lead to enzyme deficiency. The entire coding region of the ASPA gene is sequenced to identify rare or novel missense, nonsense, or splice-site mutations (8 & 17). The ASPA gene is located on the short (p) arm of chromosome 17 at position 13.3. Identifying healthy parents (carrier screening) who carry one copy of the mutated gene to assess the risk of passing it to offspring is very important from genetic counseling & prevention point of view. The prenatal diagnosis can be achieved by testing fetal cells in chorionic villus sampling (CVS) or amniotic fluid if the parental mutations are known.

More than forty five mutations have so far been identified in the ASPA gene (17 & 20). During this study, it was learnt that pathological missense mutation in ASPA gene with Cysteine to Arginine (C152R) was found in one of the diagnosed cases of Canavan disease. The prenatal molecular DNA diagnosis in the amniotic fluid in the next pregnancy was conducted in the family to ensure the absence of such defect & then pregnancy was continued. Thus, the prevention was possible through a reliable & affordable GCMS diagnosis in the index case.

Interestingly, Patient No. 2 demonstrated MRI findings initially suggestive of Glutaric aciduria, illustrating the overlap in neuroimaging features among metabolic leukodystrophies. In such scenarios, GCMS plays a crucial role in clarifying the diagnosis by providing specific metabolic signatures. The very presence of elevated urinary NAA level in this patient supported Canavan disease despite non-specific or misleading imaging findings, underscoring the GCMS diagnostic value of biochemical confirmation. An early & definitive diagnosis requires a multidisciplinary approach, integrating neuroimaging findings, biochemical (metabolic analysis) and molecular genetic testing (7, 12 & 17). Genetic counseling and genetic testing are thus strongly recommended for parents to prevent the inheritance in subsequent pregnancies.

Gene Therapy is currently in the research phase (using viral vectors to deliver a functional ASPA gene directly to the brain) (9 & 10). Canavan disease is being used as a leucodystrophy model to explore the function of abundant amino acid-NAA in myelin formation/ regeneration and several hypotheses are under study to find out its therapeutic role. It is reported that in addition to NAA, pathophysiological abnormalities seen in CD is possibly due to abnormal metabolic levels of other compounds such as aspartate, acetate, aspartate aminotransferase, glutamate, glutamate dehydrogenase, γ-aminobutyric acid, and ketoglutarate dehydrogenase complex. The research on therapeutical targets and pharmacological manipulations would be based on the understanding of these several metabolic pathways (10, 19).

Limitations: The present study is limited by the small sample size, reflecting the rarity of Canavan disease as one in 100,000. Additionally, molecular genetic confirmation of *ASPA* gene mutations was not available for all patients. Future studies incorporating larger cohorts and comprehensive genetic analysis would strengthen the role of GC-MS in the diagnostic algorithm of Canavan disease.

CONCLUSION

This retrospective study emphasises the crucial diagnostic role of non-invasive urinary GCMS analysis in the evaluation of suspected Canavan disease cases in India. Elevated urinary N-acetylaspartate level (NAA) was consistently observed in all four patients and correlated with clinical and neuroimaging findings, confirming it as the biochemical hallmark of Canavan disease. The urinary GCMS metabolic profiling proved to be a reliable, sensitive, and affordable tool for early detection, particularly in cases with overlapping or non-specific neuroimaging features. Early biochemical confirmation by advanced GCMS technology facilitates timely clinical management, appropriate genetic counseling and improved diagnostic accuracy in children with suspected leucodystrophies. While gene therapy is a

distant hope, prevention through prenatal diagnosis & genetic counseling is the only option to reduce the social & economic burden of the family & nation at large.

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