Research Paper

A comprehensive IEM screening approach for 12 common IEMs in India: Recommendation based on 25 years of diagnostic experience in mass spectrometry

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Abstract The incidence of Inborn Errors of Metabolism (IEMs) is lacking in India as Newborn Screening (NBS) is still an emerging practice and not mandatory. There is yet no population-based genetic epidemiological data. We established IEM Screening for the first time in India in 1998 using mass-spectrometry in high-risk cases when high through-put NBS laboratories & metabolic genetic expertise were inadequate and NBS concept was hardly accepted. Over the last 25 years, we have been providing the non-invasive urinary GCMS metabolic screening and diagnosis for the referral cases with high-suspicion of metabolic disorders. The urine soaked, air-dried filter paper was sent from all over India for GCMS analysis. Out of total 8246 high-risk cases (1998−2024), metabolic abnormality was detected in overall 28% (2289/8246) when compared with the age-matched controls. The 12 IEMs of organic, amino acids, urea cycle & sugar metabolism constituted 13.6% (1124/ 8246), with high detection rate of Methylmalonic acidemia (1 in 26), Galectosemia (1 in 76), Propionic acidemia & Glutaric acidemia type-1 (1 in 94), Maple Syrup Urine Disorder (1 in 85), Hyperglycinemia (1 in 56) & followed by the remaining 6 IEMs with 1 in 100−400, such as Tyrosinemias, Urea Cycle Disorders (UCD), Fructose-1−6-Diphosphatase Deficiency (FDPD), Multiple Carboxylase Deficiency (MCD), Isovaleric Acidemia (IVA) & Beta-Ketothiolase Deficiency (BKT). The significant finding was that the periodic analysis in 2005, 2015, 2020 & 2024 revealed consistent results with highest detection rate of the same 12 IEMs which often cause developmental delay, disabilities or death in the high-risk cohort. The non-invasive urinary analysis using mass spectrometry could reach to the rural untapped regions and covered a large spectrum of various IEMs in a single reliable test. This cohort study offered the evidence of 12 preventable common IEMs with high detection rate like 1 in 26 to 1 in 100- 400 and therefore recommended to include first in State-wise pilot studies in high-risk population to detect the frequency & later in future NBS program with preventive approach in India.

Key words: high-risk screening, IEMs, GCMS, mass spectrometry, newborn screening

Introduction

Metabolic profiling of Inborn Errors of Metabolism (IEMs) using Gas Chromatography Mass Spectrometry (GCMS) and Tandem Mass Spectrometry (TMS) is well accepted laboratory practice in developed countries due to the advent of diagnostic mass spectrometry since its first application by Tanaka in 1966 who discovered isovaleric academia[1)]. Since then, the GCMS urinary metabolic screening has been worldwide used to diagnose number of Inborn Errors of Metabolism (IEMs) because of its high accuracy, sensitivity and power of analyzing multiple compounds simultaneously.

IEMs are congenital metabolic disorders of intermediary metabolism and it is broadly classified into two categories 1) Intoxication type (accumulation of intermediate metabo-

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Received: May 31, 2025. Accepted: August 19, 2025.
Epub November 13, 2025.
DOI: 10.24508/mms.2025.11.005

lites) and 2) Energy deficiency type. Single gene defects sometimes result in deficiencies of an enzyme, membrane transporter or other functional protein. This leads to various consequences like substrate accumulation or deficiency which in turn causes minor to severe neurological and psychiatric manifestations resulting in lifelong disability or death[2]. Hence, the abnormal metabolic markers indicating specific IEMs are always targeted using mass spectrometry which is also helpful in population screening due to high through-put technology like TMS using Dried Blood Spot (DBS).

Newborn Screening (NBS) test popularly known as neonatal screening is conducted on apparently normal healthy newborns. It is developed for prevention of serious developmental, genetic, and metabolic disorders so that important action can be taken before symptoms such as mental and or motor retardation, physical disabilities or death occur. The 3 NBS disorders, mainly Congenital Hypothyroidism (CH), Congenital Adrenal Hyperplasia (CAH) & Glucose-6-Phosphatase Deficiency (G6PD) are generally screened by Enzyme-Linked Immunosorbent Assay (ELISA) in Low-Resource setting. American College of Medical Genetics (ACMG) recommended the expanded panel using TMS and was started in India in late 2010 in High-Resource Setting. Realising the limitations of ELISA or Chemiluminicsence method based tests for NBS, the new Tandem Mass-Spectrometry (TMS) method for neonatal screening for few IEMs (viz. amino, organic & fatty acid disorders) became popular worldwide which used acyl carnitine profiling[3, 4]. During the same time, chemical screening & diagnosis of IEMs by urinary GCMS metabolic analysis was also developed by Professor Matsumoto & his team in Japan and became popular in Asian countries[5].

Majority of the newborn screening disorders are congenital metabolic disorders of organic, amino acids, sugars & fatty acids (short, medium & long-chain fatty acids), comprising of small molecules and can be easily & precisely detected by mass spectrometry. The early detection & reliable diagnosis thus can lead to better prevention in the diagnosed index case. Once the screen positive cases are confirmed by GCMS or enzyme tests, it helps in appropriate genetic counseling to prevent the recurrence and future prenatal diagnosis in the affected family.

A rapid, practical, non-invasive and simultaneous urinary GCMS metabolite analysis method covering several groups of IEMs[5] was found suitable to Indian rural & urban high-risk screening, especially when concept of newborn screening was not even initiated or accepted till year 2000. This was due to various health constraint factors, inadquate high-throughput NBS laboratories and limited metabolic genetic expertise[6]. The high-risk screening differs from NBS in that the metabolic screening is conducted on critically ill sick neonates, infants & children who indicate high suspicion of metabolic abnormalities, indicating IEM on clinical examination & routine biochemical tests. Therefore, in the absence of NBS program and with a technical support of MILS Japan laboratory, we established IEM Screening service for the first time in 1998 in India, using mass-spectrometry for screening and diagnosis of IEMs and continued till date for educating clinicians in early IEM diagnosis & management, spreading awareness & knowledge about newborn screening and high-risk metabolic screening[7].

The present study is the outcome of 25 years of accurate & reliable metabolic screening & diagnosis by urinary GCMS method in genetically diverse Indian high-risk cohort and further data analysis for comparision at the periodic interval of every 5 years.

Material and Methods

The study was conducted on total 8246 high-risk cases across the country (period 1998−2024), using urinary GCMS metabolic analysis. The high-risk cases are those with a high-suspicion of metabolic disorders based on the clinical signs & symptoms, viz. lethargy, failure to thrive, hypotonia, metabolic acidosis, ammonia status, high anion gap, hepato- or splenomegaly or respiratory distress, developmental delay or disability etc. These were referred by neonatologists, intensivists, paediatricians & pediatric neurologists as possible 'IEM' cases. The age, sex, birth & family history, dysmorphism and parental consanguinity were recorded along with the clinical signs & symptoms. The routine liver & kidney function test results were also noted. Brain MRI findings were supportive to metabolic diagnosis and were made available to further confirm GCMS chemical diagnosis in some cases like MSUD or GA type-1 disorder. The TMS analysis on dried blood spot whenever available was done for acyl carnitine profiles, as a supportive complementary testing. The 'Informed Consent' of patients or parents in case of children was received along with the urine or dried blood spot samples which included the infor-

mation about the clinical history & other routine laboratory investigations.

GCMS method

The urinary GCMS metabolic sreening method of Matsumoto & Kuhara[5] was used which describes the method in detail. Urine samples were collected by using one of two different techniques− 1) by blotting urine on the special ADVANTC filter Paper No. 2, air-dried, put in plastic ziplock bag to send to the laboratory from various far away cities & hospitals or 2) by collecting 20−30 mL of urine directly in a sterile container and sending it to the laboratory from nearby places to reach within 12 h. The urine samples soaked on filter paper were allowed to completely dry at room temperature for 2 to 3 h before being shipped to the laboratory. The proper protocol of sample collection avoiding fecal contamination and proper storage was ensured which is essential to avoid fungal growth which may lead to inaccurate misleading conclusions. The samples were considered for processing only if the sample acceptance protocol was met by these samples.

Analysis of urine samples

As described in details in the method[5], the urine sample preparation has various steps such as—first enzyme urease treatment followed by deproteinization, evaporation to dryness and derivitization by trimethylsilylation. In brief, the 100 μL of elute from urine filter paper or direct 100 μL urine was treated with 30 μL urease solution at 37°C for 30 min. It was further deproteinized with ethanol, centrifuged and evaporated to dryness under reduced pressure. The organic compounds in urine were trimethylsilylated (TMS) by adding N, O-bis-trimethylsilyltrifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), and heated at 90°C for 40 minutes. The 2 μL of the derivatized sample was injected into a Shimadzu QP-2010 SE GCMS with Ultra Alloy capillary column (30 m × 0.25 mm). The temperature of GC was kept at 60°C for 1 minute, and then increased up to 350 °C to 360°C at 17°C/min. Each injected sample was automatically injected in 20 : 1 split mode and mass spectrum was scanned with resolution mode from *m/z* 50 to *m/z* 650 every 0.25 s. The data was analyzed with computer-assisted program. Peaks in the Total Ion Chromatogram (TIC) that showed the profile of urinary metabolites were identified from each mass spectrum.

The Shimadzu GCMS system (QP2010SE model) was used throughout the study with the same method protocol and conditions as mentioned above.

Data interpretation

According to the method reported in reference 5, a semi-automatic qualitative analysis was performed on 260 component peaks (some of which had 2−3 peaks), including the internal standard heptadecanoic acid (HAD). The peak detection of quantitative ions (Q-ion) and reference ions (I-ion) was selected based on the pre-set retention time (RT) and mass spectrometry characteristics of each component. Each component was confirmed by three points of RT, Q-ion, and I-ion for qualitative analysis as shown in Table 1 for 12 IEMs. At the same time, the detection level of each component in the tested sample was evaluated based on the ratio of the peak detection area of Q-ion to the intrinsic Creatinine (Cr) peak area of the sample itself. In this interpretation process, data interpretation technicians need to manually confirm the computer-generated qualitative and quantitative peaks. Whether the integral is accurate and correct or if there is any error- it needs to be considered as correction. The ratio of the measured result after confirmation to the cut-off value (mean+2 standard deviation) obtained from healthy age-matched controls was used as the basis for evaluating the degree of abnormality of the measured sample. If the ratio of the measured result to the cut-off value is greater than 2−5 times, it is considered as an increase. The cut-off value used here was obtained from urine analysis results of 30 healthy Indians in different age groups.

Chemical diagnosis

Based on the analysis results obtained by the above data interpretation method, combined with the characteristics of Laboratory Abnormal Items in OMIM (Online Mendelian Inheritance in Man) and the abnormal metabolic components of genetic metabolic diseases reported in the latest related literature, the final chemical diagnosis report was made. This study, as shown in Table 1, analyzed and interpreted 12 pathological conditions (IEMs) using their specific biomarkers and corresponding RT, Q-ion, I-ion for data analysis and pathological interpretation.

The final chemical diagnosis is given along with TIC labelling abnormal markers by arrows as shown in Figs. 1 to 4, along with age-matched control to appreciate the difference. The multiple specific biomarkers for 12 IEMs along with the external standard—Heptadecanoic acid

Table 1. The list of targeted 12 IEMs and their biomarkers used in this study

No	Disorder name	Biomarkers	GCMS Retention Time (R.T)	Quantitative ions (Q-ion) *m/z*	Reference ions (I-ion) *m/z*
1	Methylmalonic Acid (MMA)	MMA	7.16 min	218	247
		Me-citrate	12.04 min	287	479
2	Tyrosinemia	Tyrosine	12.21 min	179	310
		4-Hydroxyphenyllactate	12.26 min	179	308
		Succinylacetone	10.38 min	157	169
		N-acetyl tyrosine	13.55 min	179	179
		4-hydroxyphenylpyruvate	13.20 min	325	381
3	Hyperglycinemia	Glycine	6.25 min	102	204
4	Glutaric Aciduria I	Glutarate	8.55 min	158	261
		3-Hydroxyglutaric acid	10.15 min	185	349
5	Galactosemia	Galactose	12.01 min	435	204
		Galactitol	12.52 min	205	319
		Galactonate	13.18 min	292	333
6	Maple Syrup Urine Disorder (MSUD)	Leucine	7.50 min	158	218
		Isoleucine	8.02 min	158	218
		Valine	7.25 min	144	218
		2-Hydroxyisovalerate	6.53 min	145	219
		3-Hydroxyisovalerate	7.16 min	131	247
		2-Hydroxyisocaproic Acid	7.38 min	159	261
7	Propionic Acidemia (PA)	3-Hydroxypropionic acid	6.39 min	219	177
		Propionylglycine1	8.40 min	159	188
		Propionylglycine2	9.07 min	102	158
		Tiglylglycine	10.17 min	170	154
		Methyl-citrate	12.04 min	287	479
8	Urea Cycle Disorder (UCD)	Uracil	8.22 min	241	256
		Orotic Acid	11.22 min	254	357
9	Fructose 1,6 Disphosphate Deficiency (FDPD)	Glycerol-3-Phosphate	11.33 min	357	299
		Fructose	11.56 min	217	437
10	Multiple Carboxylase Deficiency (MCD)	Me-citrate	12.04 min	287	479
		3-Methylcrotonylglycine	10.02 min	170	139
		3-Hydroxyisovalerate	7.16 min	131	247
11	Isovaleric Acidemia (IVA)	Isovalerylglycine	9.37 min	172	216
		3-Hydroxyisovalerate	7.16 min	131	247
12	Beta Ketothiolase Deficiency (BKT)	Tiglylglycine	10.17 min	170	154
		2-Methyl-3-Hydroxybutyric acid	7.15 min	117	247
		2-Methylacetoacetic acid	8.11 min	171	245
*	Internal standard (I.S)	HAD: Heptadecanoic Acid	13.05 min	117	327
**	Concentration conversion standard	Cr: Creatinine	10.08 min	329	314

(HDA) & internal standard Creatinine (Cr) for the method are shown in Table 1 with their Retention Time (RT) & masses (*m/z*) which are helpful in detection of individual IEMs. Each laboratory, after standardization of its method, determines RT & control values under each peak area of Total Ion Chromatogram (TIC) as seen in Figs. 1 to 4. The

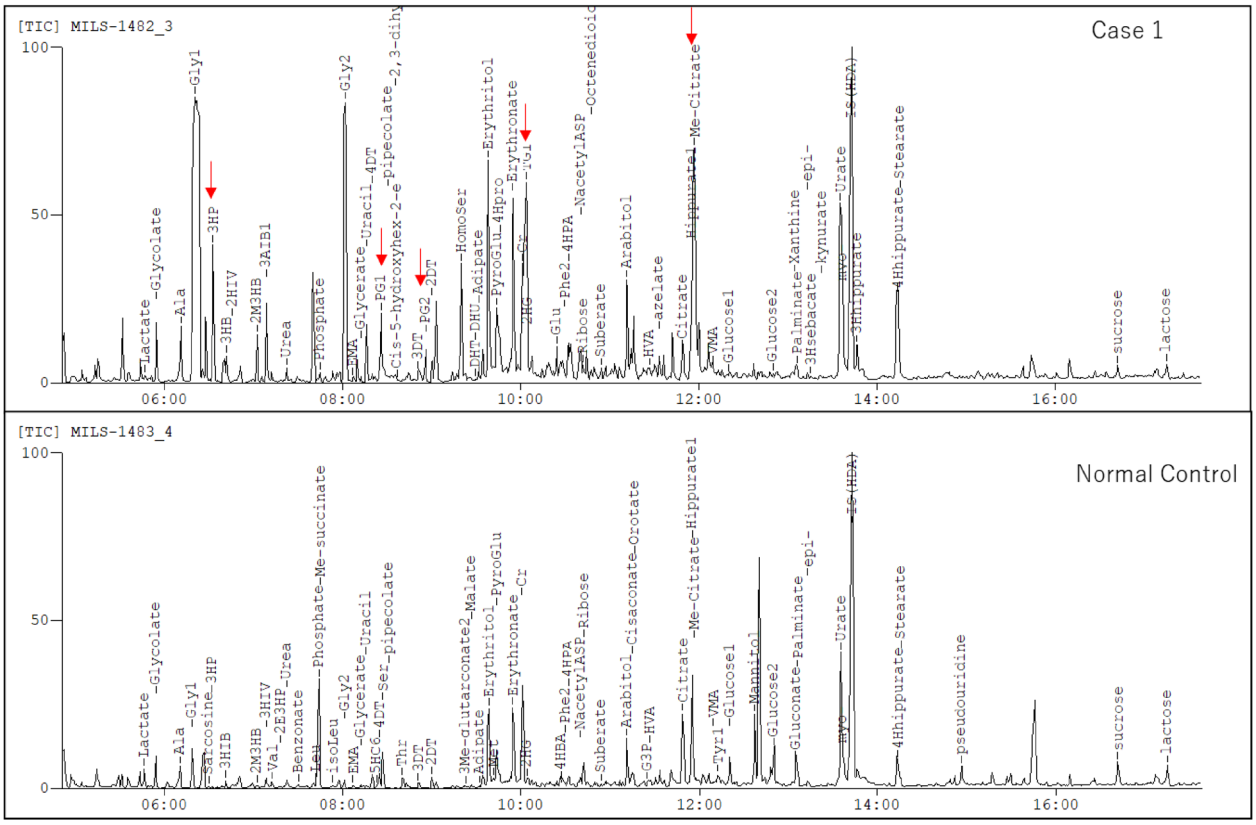

Fig. 1. Case 1 (2 years 5 months, male) GCMS data analysis result.
Upper half showing the TIC chromatogram of urinary metabolites from a patient with Propionic acidemia (PA) & lower half showing the normal control.
Biomakers of Propionic acidemia are marked with arrows-3HP: 3-hydroxyprpionic acid, PG1: propionylglycine peak 1, PG2: propionylglycine peak 2, TG: tiglylglycine, Me-citrate: methylcitric acid.

experienced metabolic GCMS analyst does the interpretation & analysis of data for final reporting.

Abnormal excretion of marker compounds for the specific metabolic disorders along with the age-specific controls are shown as examples in 4 IEM cases, viz. Propionic acidemia (Fig. 1), Methylmalonic acidemia (MMA) (Fig. 2), Maple Syrup Urine Disease (MSUD) (Fig. 3) & Urea Cycle Disorder (UCD) (Fig. 4).

Results

During the last 25 years (from 1998 till December 2024), total 8246 high-risk cases were screened for metabolic conditions using urinary GCMS analysis method (as explained above) and it revealed overall 28% (2289/8246) metabolic abnormality. However, the 12 common IEMs of organic, amino acids & sugar metabolism constituted 13.6% (1124 out of 8246) (Table 2-A). A very high detection rate was observed in 6 out of 12 IEMs, viz. MMA (1 in 26), Galectosemia (1 in 76), PA & GA type-1 (1 in 94), MSUD (1 in 85), Hyperglycinemia (1 in 56). The remaining 6 IEMs, (three of these form the IEM groups e.g. Tyrosinemias and or hepatic dysfunction, Urea Cycle Disorders, & Multiple Carboxylase Deficiency), Isovaeric Acidemia, FDPD, & BKT were found with overall positive detection rate as 1 in 100−400 (Table 2-A) in high-risk cohort. The abnormal metabolic biomarkers to identify each disorder are given in Table 1 for 12 common IEMs detected.

In the final analysis in year 2024, the first 9 IEMs comprising of organic acids, amino acids, UCDs & sugars (Table 2-A) showed alarmingly high positive detection rate in the range of 1 : 26 to 200 & next 3 IEMs (from no. 10 to 12) revealed the positive detection rate as 1 : 200−400.

The abbreviations used for the 12 IEMs are given in Table 2-B which shows the total number of each IEM detected till 2024 along with the percentage out of total 8246 cases. The MMA was found to be the most common (3.74%) followed by the remaining 11 IEMs in the range of 0.25% to 1.8%. Interestingly, the periodic data analysis in 2005, 2015, 2020 & 2024 revealed consistent results with highest detection rate of the same 12 IEMs as shown in

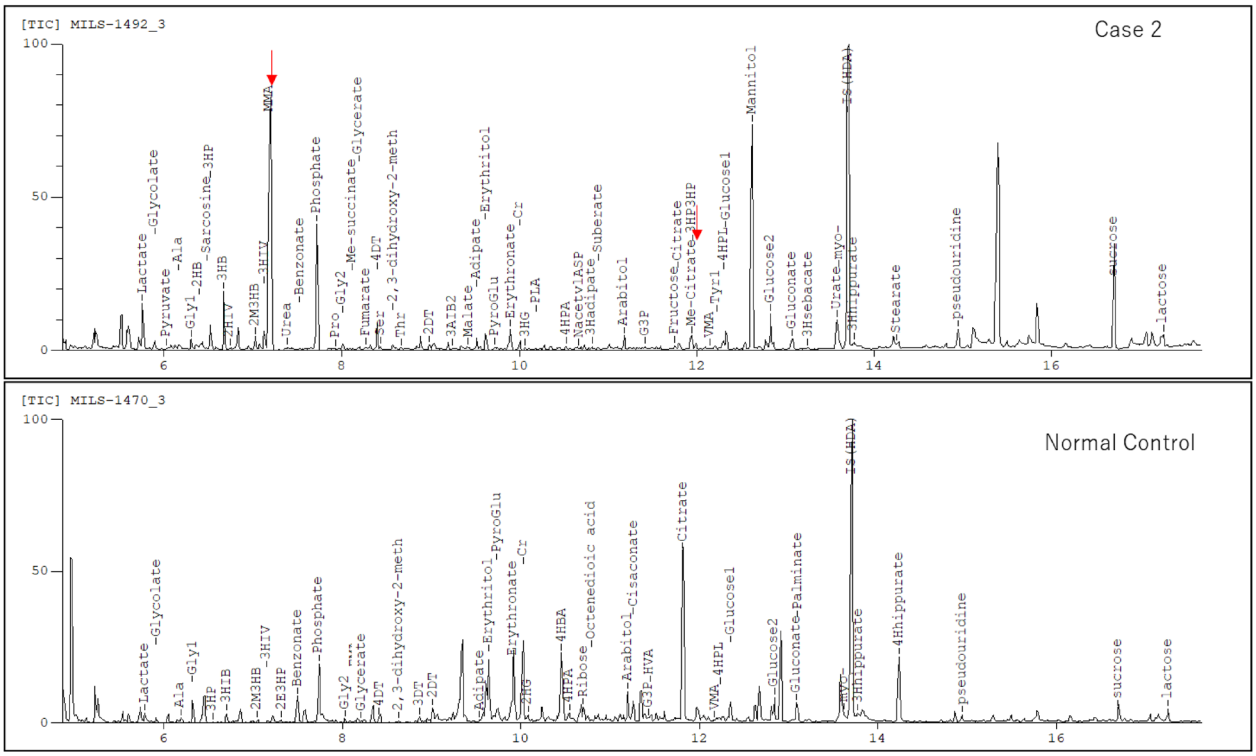

Fig. 2. Case 2 (5 years, male) GCMS data analysis result.
Upper half showing the TIC chromatogram of urinary metabolites from a patient with Methylmalonic acidemia (MMA) & lower half showing the normal control TIC chromatogram.
Biomakers of Methylmalonic acidemia were marked with arrows. MMA: methylmalonic acid, Me-citrate: methylcitric acid.

Table 2-A. The percentage of 12 IEMs was found to be 8.6−8.7% in the beginning years till 2015. There was increase in 2020 (10.4%) and in 2024 (13.6%) in these 12 common IEMs as seen in Table 2-A.

Since the urinary metabolic screening by GCMS method is a powerful tool with high sensitivity & specificity based on *m/z* identification of each molecule, it was decided to explore the data for various groups of IEMs. Out of total 8246, the 2289 cases were found with metabolic abnormality (28%) and these were grouped into 13 categories to understand according to their metabolism (Table 3). The disorders of branched chain amino acids (26.64%) and Tricarboxylic Cycle (TCA) and mitochondrial disorders (32.06%) constituted the highest detection groups, followed by the disorders of carbohydrate metabolism (13.10%) as compared to the remaining groups which ranged from 0.04% to 5.11% (Table 3).

The list of metabolic abnormalities in a single urinary test GCMS covers a large spectrum (about 140+) of abnormalities as shown in Table 4. It is beyond the scope of this article to enumerate several metabolic conditions which could also be detected based on the compounds with their molecular weight and structural formula by the expert IEM specialist with experience in medical masspectrometry. However, out of 2289 metabolic abnormality cases, the number of cases diagnosed in each group are categorized in Table 3.

Overall, in 28% (2289 of total 8246) metabolic abnormality cases, the low birth weight (33%), convulsions (32%), premature birth (26%), acidosis & refusal to feed (13%) and respiratory distress (13%) were found, with consanguinity (15−20%), history of mental retardation (8%) and death of earlier sibs (4%).

The 12 preventable common IEMs consistently found till the year 2024 also satisfy the Wilson & Jungner criteria endorsed by WHO for selection of NBS disorders[8].

Discussion

In the developed countries like USA, Australia and Japan and some European countries, all newborns are screened for a substantial number of metabolic diseases by TMS method or also referred as LC-MS/MS[9,10]. Though individually rare, the overall collective incidence of IEMs is worldwide known to be 1 in 1500−2500. NBS programs are

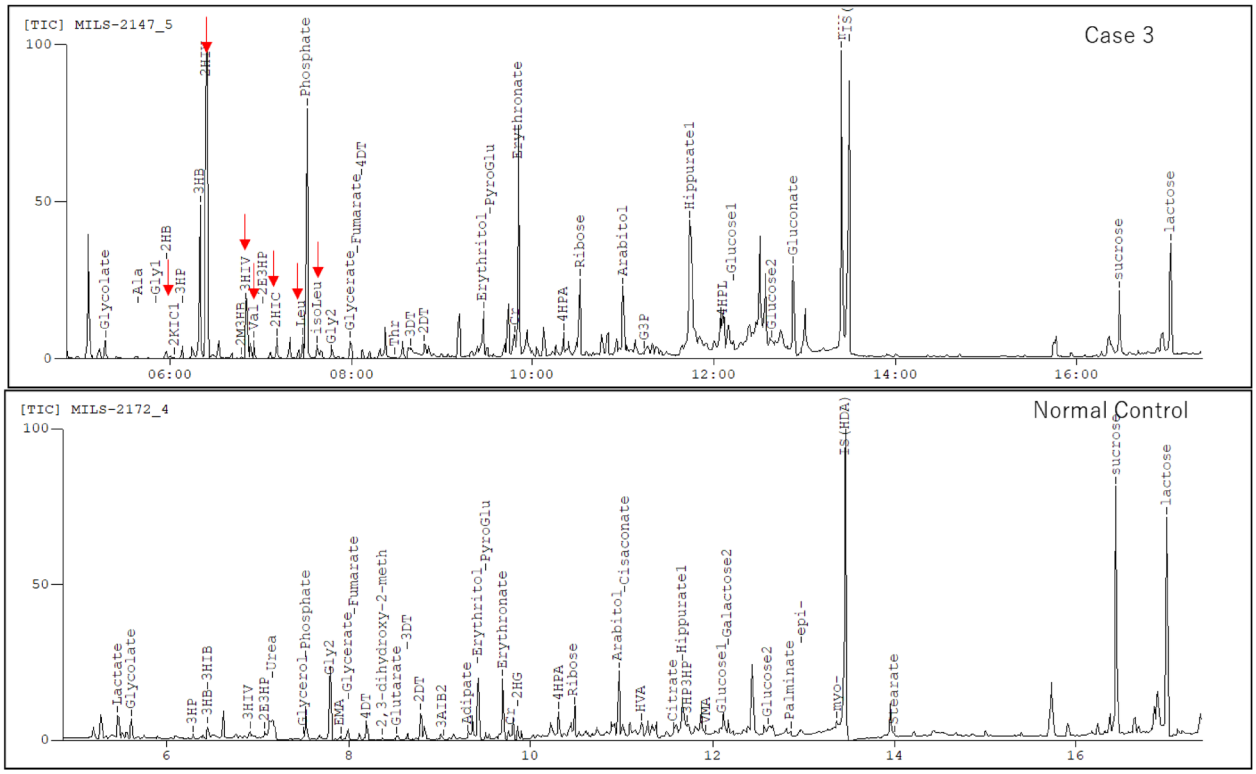

Fig. 3. Case 3 (1 year, female) GCMS data analysis result.
Upper half showing the TIC chromatogram of urinary metabolites from a patient with Maple Syrup Urine Disease (MSUD) & lower half showing the normal control TIC chromatogram.
Biomakers of MSUD were marked with arrows. 2KIC: 2-ketoisocaproic acid, 2HIV:2-Hydroxyisovalerate, 3HIV: 3-Hydroxyisovalerate, 2HIC: 2-Hydroxyisocaproic acid.

being implemented as a mandatory health policy in USA & some European countries soon after the introduction of high through-put Tandem Mass (TMS) analysis using Dried Blood Spot (DBS) method[2,3]. More & more information about incidence of several metabolic conditions was soon available which guided the selection of NBS candidate disorders.

India is much more lagging behind in both mass spectrometry technology & its use in screening every newborn as a universal screening due to several other health priorities and constraints. According to WHO, congenital malformations and genetic disorders are the third most common cause of mortality in newborns in urban India, while data on rural areas is unavailable. Over 1.4 billion population & high birth rate pose a huge genetic burden on nation, considering the racial, ethnic & genetically diverse Indian population. Indian Council of Medical research (ICMR) conducted a study and recommended only 2 ELISA-based disorders (CH and CAH) in a pilot project of 0.1 million newborns in 2011 which reflected inadequacy in preventing childhood disabilities & death due to IEMs considering 28 million annual births[11]. Nevertheless, in this decade, India has witnessed, neonatal screening programs atleast for 2 disorders. NBS is also slowly gaining popularity in many Asian countries[12], including India, but has remained limited to only few metabolic disorders, like CH, CAH, G6PD, Galectosemia, Biotidinase deficiency, PKU, & Cystic fibrosis. The results of the present study has emphasized additional 12 most common IEMs showing high positive detection rate throughout 25 years of analysis period and therefore recommended in high-risk cohort first to determine the frequency in pilot projects and later consider as NBS candidate disorders. These 12 IEMs can be screened by urinary GCMS analysis and except FDPD & Galectosemia, the remaining 10 IEMs are already a part of screening by TMS analysis (Table 2-A).

As India does not have population-based genetic epidemiology studies, the exact burden & incidence of NBS disorders is yet not known. We simply follow the Western data. In the present high-risk cohort, the incidence of Phenylketonuria (PKU) remains unknown as evident by its absence in the present result data. The PKU is the first &

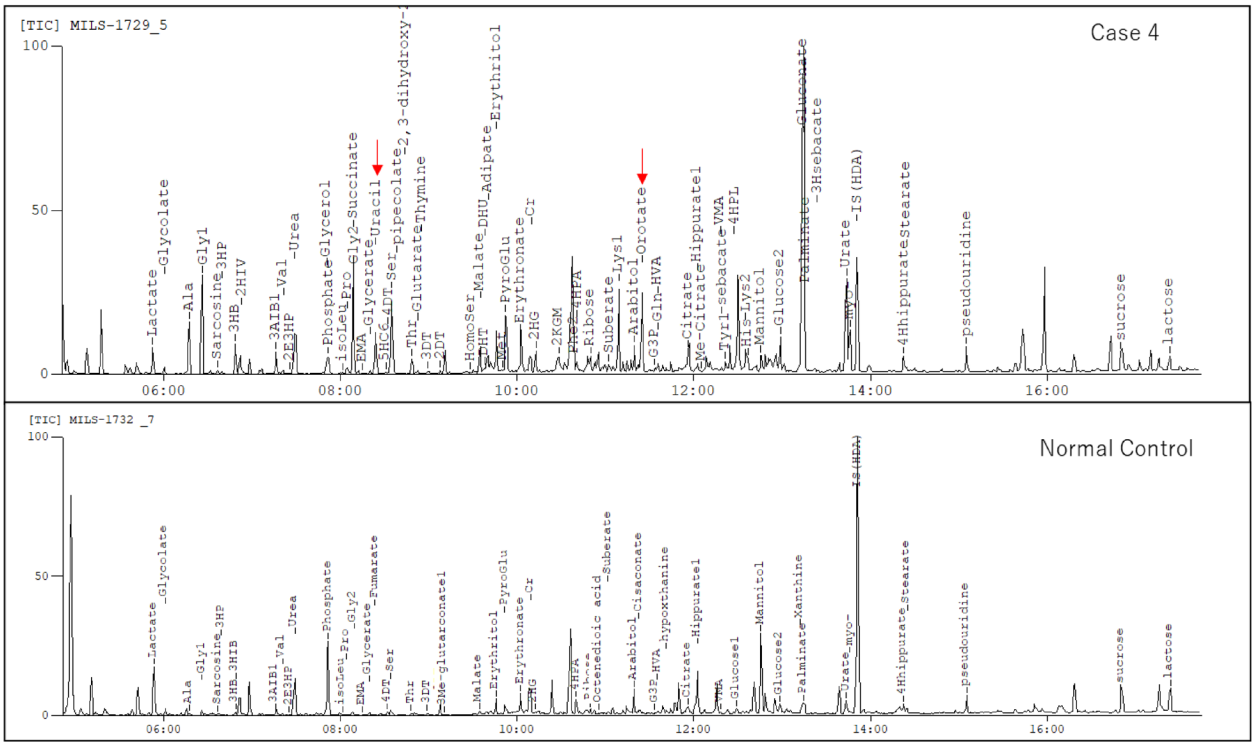

Fig. 4. Case 4 (3 years 6 months, female) GCMS data analysis result.
Upper half showing the TIC chromatogram of urinary metabolites from a patient suspected as Urea Cycle Disorder (UCD), lower half showing the normal control TIC chromatogram.
Biomakers of urea cycle disorder were markerd with arrows. Uracil and Orotate.

most common IEM selected for newborn screening and widely used globally as one of the common NBS disorders. However, in the present study, PKU is not among the recommended common 12 IEMs (Table 2-A) in Indian scenario. It reflects the difference between the choice of NBS disorder from country to country due to genetically different populations.

The dire need of neonatal/newborn screening in India was clear in the last decade[13], and has been recently highlighted with NBS disorder studies from the few States of India emphasising the issues, challenges and integration of universal NBS recommendation in Indian health policies[14]. The three disorders viz. CH, CAH & G6PD are still the preferred conditions which are done by the conventional immunoenzyme assays (ELISA).

The first application of Tandem Mass Spectrometry (TMS) test using DBS was reported in 2020 from Goa State of India which was the public-private partnership project for only Newborn Testing in about 50% of the births (~48,000) during 2008−2013 of 5 years period[15]. However, it was not a NBS program with comprehensive care & management of screened positive cases and no follow-up. The urine-based GCMS analysis emphasising the non-invasive nature, reliability and accuracy of metabolic test was reported as a suitable method for IEM screening & diagnosis in Indian context[16]. The high molecular weight fatty acids are not detected by GCMS analysis which focuses on detection of low molecular weight & volatile compounds like organic and amino acids, nucleic acids & also sugars, sugar alcohols (Table 4).

In 2011, National Neonatology Forum (NNF), India recommended expanded NBS panel of 46 conditions using TMS test in affordable patients. Facilities for diagnosis and treatment is not easily available at the screening site or at the referral NBS laboratory. The disorders screened by Tandem Mass spectrometry (fatty acids and organic & amino acid disorders) are not often the part of the screening panels due to resource constraints, viz. significant capital costs, few experts, lack of treatment facilities. The common 3 NBS panel comprising of CH, CAH and G6PD is generally advised. The high cost of diets is another hurdle for the therapy management of the screen positive cases. In short, NBS is not yet under one umbrella of health services as a program using advanced mass spectrometry and taking care of screening, diagnosis, therapy management & care at one Centre in India.

Table 2-A. Detection of 12 IEMs in screening urinary GC/MS analysis in high-risk cases (2005−2024)
Detection of 12 IEMs is 13.6% (1124 /8246 in Year 2024)

Sr. No	Name of IEM	2005 N=2040; Abn=171 8.6%		2015 N=3341 Abn=286 8.7%		2020 N=6510 Abn=676 10.4%		2024 N=8246 Abn=1124 13.6%	
		Detection Rate	Positive Cases	Detection Rate	Positive Cases	Detection Rate	Positive Cases	Detection Rate	Positive Cases
1.	Methylmalonic Acidemia (MMA)	1 : 55	37	1 : 64	52	1 : 30	214	1 : 26	308
2.	Tyrosinemia/Hepatic Dysfunction	1 : 78	26	1 : 88	38	1 : 72	55	1 : 93	88
3.	Hyperglycinemia	1 : 146	14	1 : 119	28	1 : 171	38	1 : 56	148
4.	Glutaric Aciduria 1 (GA Type-1)	1 : 102	20	1 : 90	38	1 : 90	73	1 : 94	87
5.	Galactosemia	1 : 136	15	1 : 176	19	1 : 130	50	1 : 76	108
6.	Maple Syrup Urine Disease (MSUD)	1 : 156	13	1 : 239	14	1 : 99	66	1 : 85	97
7.	Propionic Acidemia (PA)	1 : 170	12	1 : 176	19	1 : 105			
	62	1 : 94	87						
8.	Urea Cycle Disorders (UCD)	1 : 170	12	1 : 134	25	1 : 186	35	1 : 206	40
9.	Fructose-1,6-Diphosphatase Def. (FDPD)	1 : 136	15	1 : 134	25	1 : 217	30	1 : 155	53
10.	Multiple Carboxylase Def. (MCD)	1 : 510	4	1 : 257	13	1 : 260	25	1 : 206	40
11.	Isovaleric Acidemia (IVA)	1 : 680	3	1 : 835	4	1 : 591	11	1 : 392	21
12.	Beta-Ketothiolase deficiency (BKT)	Nil	Nil	1 : 304	11	1 : 383	17	1 : 175	47

(Keys: N=Total number of referred cases; Abn=number of abnormal metabolism detected)

Table 2-B. High-risk screening by urinary GC/MS Analysis (2024)
*** Overall incidence of 12 IEMs is 13% (1124/8246)**
Total referral cases=8246
Normal=5957 (72%); abnormal=1124 (13.6%)

Sr. No	Disorders name	Disorder abbreviation	Total positive cases till 2024	% IEM in total cases
1	Methylmalonic Acidemia	MMA	308	3.74%
2	Tyrosinemia/Hepatic Dys	TYR	88	1.1%
3	Hyperglycinemia	HyperGL	148	1.8%
4	Glutaric Aciduria Type-1	GA Type I	87	1.1%
5	Galactosemia	GALT	108	1.31%
6	Maple Syrup Urine Disease	MSUD	97	1.2%
7	Propionic Acidemia	PA	87	1.1%
8	Urea Cycle Disorder	UCD	46	0.56%
9	Fructose-1,6-Diphosphatase Def.	FDPD	53	0.64%
10	Multiple Carboxylase Def.	MCD	40	0.55%
11	Isovaleric Acidemia	IVA	21	0.25%
12	Beta-Ketothiolase deficiency	BKT	47	0.57%

Table 3. Detection of IEMs in various groups
Total high-risk referral cases N=8246
Normal=5957 (72%); total abnormal=2289 (28%)

	IEM Groups by Urinary GCMS Analysis	Abnormal N=2289	%
1	Branched-Chain Amino Acid Metabolism	610	26.64%
2	Aromatic Amino Acid Metabolism	114	4.98%
3	TCA Cycle Disorder and Mitochondrial Disorder	734	32.06%
4	Metabolism of Proline, Glycine, Histidine, β-Alanine and Others	117	5.11%
5	Metabolism of Lysine, Tryptophan and Ornithine	88	3.84%
6	Sulfur-Containing Amino Acids, Folate, Cobal l	1	0.04%
7	Urea Cycle Disorder and Citrin Deficiency	52	2.27%
8	Fatty Acid Metabolism and Vitamins	91	3.97%
9	Carbohydrate Metabolism	300	13.10%
10	Metabolism of Purine and Pyrimidine	9	0.39%
11	Transport and Other Disorders	44	1.92%
12	Miscellaneous Disorders	119	5.19%
13	Neuroblastoma (not IEM)	10	0.43%

(Note: Please refer Table 4 for various groups of above IEMs)

There are numerous analytical techniques available like Mass Spectrometry (MS), Liquid Chromatography-Mass Spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR) which are used to identify various metabolite levels in urine samples. However, like the most developed countries these technologies are not readily available to meet the great demand of socio-economically deprived population. In the present cohort, the GCMS method used simple non-invasive urine collection which needs no DBS collection kit or the skilled person. It is one of the most widely used techniques for urinary metabolomics studies because of its higher sensitivity, resolution, reproducibility, reliability, and ease of operation[5,16], though it is not high-throughput testing. The preference of TMS is well documented as a high-throughput method for a large population in many countries and should be considered by the Goverement of India as a public health free service. Presently, the expenses for the NBS testing is out of patient's or parents' pocket and it is not covered under health insurance or free. We preferred GCMS urinary metabolic analysis which coveres a diversed groups of IEMs (Tables 3 and 4) in high-risk patients. The interpretation of GCMS was also enhanced by using MS data-handling assisted programs with computer database analysis as conducted in the present study and the reporting was possible in next 24 hours of receiving the sample at the GCMS laboratory. It was evident that the 12 IEMs, by whichever method you use can be recommended as candidate disorders for future NBS program in India. The intention of the present study is not to compare the cost-effectiveness of methodology such as TMS versus GCMS, but to emphasize the frequency of positive detection rate that we consistenetly found over a long period, possibly giving a true picture in Indian genetically diverse population.

There are limited published studies on newborn screening in India using Mass Spectrometry approach as mentioned above, despite the very high prevalence of IEMs[7]. Considering a comprehensive coverage of a large number of metabolic abnormalities simultaneously in one test (Table 4), the present urinary GCMS screening is recommended as a rapid test for routine metabolic screening for IEMs in Indian setting. Additionally, the metabolic clinicians are available in handful of tertiary centres and this diagnostic GCMS approach is found suitable in starting the early treatment of patients. The air-dried urine collection on a filter paper is also the most feasible national laboratory accreditated sample collection method. The efforts to train and educate the younger doctors is on the anvil of Indian Society for Inborn Errors of Metabolism (ISIEM), India and 'Rare Disorder Policy' has also been formed by the Indian Government to help the patients with rare disorders.

The common 12 IEMs, (Table 2-A) cover a larger spectrum of IEM groups which are well known for newborn crisis, disabilities & death in neonates, infants & children. To reduce the under 5 years age morbidity & mortality in India, it is important to undertake, integrate & implement

Table 4. Metabolic conditions screened & diagnosed by urinary GCMS analysis

Aromatic Amino acid Metabolism
1 Phenylketonuria
2 Hyperphenylalaninemia
3 Tyrosinemia type I
4 Tyrosinemia type II
5 Tyrosinemia type III
6 Aromatic amino acid decarboxylase deficiency
7 Hawkinsinuria
8 Alcaptonuria
9 Defects in the synthesis of BH4
10 Defects in the recycling of BH4
11 Neonatal transient hyperphenylalaninemia
12 Secondary mild phenylketonuria due to total parenteral nutrition,
13 Secondary mild phenylketonuria due to methotrexate
14 Neonatal transient tyrosinemia
15 Secondary tyrosinemia due to other drugs
16 Secondary tyrosinemia due to other inborn errors of metabolism

Branched-Chain Amino Acid Metabolism
17 Hypervalinemia
18 Hyper leucine isoleusinemia
19 Maple syrup urine disease
20 Dihydrolipoyl dehydrogenase deficiency
21 Isovaleric acidemia
22 α-Methylbutyryl-CoA dehydrogenase deficiency
23 Multiple acyl-CoA dehydrogenase deficiency
24 Isobutyryl-CoA dehydrogenase deficiency
25 β-Methylcrotonylglycinuria
26 α-Methyl-β-hydroxybutyryl-CoA dehydrogenase deficiency
27 Multiple carboxylase deficiency due to HCSD
28 Multiple carboxylase deficiency due to biotinidase deficiency
29 β-Methylglutaconic aciduria
30 β-Hydroxy-β-methylglutaric aciduria
31 β-Ketothiolase deficiency
32 β-Hydroxyisobutyryl-CoA deacylase deficiency
33 β-Hydroxyisobutyrate dehydrogenase deficiency
34 Methylmalonic semialdehyde dehydrogenase deficiency
35 Propionic acidemia
36 Methylmalonic acidemia (mutase)
37 Methylmalonic acidemia (isomerase)
38 B12-Responsive methylmalonic acidemia

Primary Hyperammonemias and Citrin Deficiency
39 Carbamoylphosphate synthetase deficiency
40 *N*-Acetylglutamate synthetase deficiency
41 Ornithine transcarbamylase deficiency
42 Citrullinemia
43 Argininosuccinic aciduria
44 Hyperargininemia
45 Lysinuric protein intolerance
46 Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome
47 Citrin deficiency
48 Transient neonatal hyperammonemia
49 Hyperammonemia due to other origin

Transport and Other Disorders
50 Cystinuria
51 Hartnup disease
52 Dibasic amino aciduria
53 Iminoglycinuria
54 Secondary iminoglycinuria
55 Transient neonatal iminoglycinuria
56 Fanconi syndrome
57 Hereditary renal hypouricemia
58 Hypophosphatasia

Metabolism of Lysine, Tryptophan and Ornithine
59 Glutaric aciduria type I
60 Saccaropinuria
61 Pipecolic acidemia
62 α-Aminoadipic aciduria
63 Tryptophanuria
64 Xanthurenic aciduria (kynureninase deficiency)
65 Xanthurenic aciduria due to B6 deficiency
66 α-Aminoadipic α-ketoadipic aciduria
67 Hydroxylysinuria
68 Hyperornithinemia

Sulfur-Containing Amino Acids, Folate, Cbl
69 Homocystinuria type I (cystathionine β-synthase (CBS) deficiency)
70 Homocystinuria type II, 5-methyltetrahydrofolate-homocysteine methyltransferase deficiency
71 Homocystinuria type III (5,10-methylenetetrahydrofolate reductase deficiency)
72 γ-Cystathionase deficiency (cystathionuria)
73 Hypermethioninemia
74 Hereditary folate malabsorption

Metabolism of Proline, Glycine, Histidine, β-Alanine and Others
75 Hyperprolinemia type I
76 Hyperprolinemia type II
77 Hydroxyprolinemia
78 Hyperglycinemia
79 Sarcosinemia
80 Hyper β-alaninemia
81 Malonyl-CoA decarboxylase deficiency
82 Hyperhistisinemia
83 Urocanic aciduria
84 Primary hyperoxaluria type I, alanine: glyoxylate aminotransferase (AGT) deficiency
85 Primary hyperoxaluria type II, D-glycerate dehydrogenase/ glyoxylate reduetase deficiency
86 D-glycerate kinase deficiency
87 Succinic semialdehyde dehydrogenase deficiency, 4-hydroxybutyric aciduria
88 Glycerol kinase deficiency
89 Canavan disease
90 5-Oxoprolinuria due to glutathione synthetase deficeincy
91 5-Oxoprolinuria due to 5-oxoprolinase deficiency
92 Prolidase deficiency

Carbohydrate Metabolism
93 Galactosemia type I
94 Galactosemia type II
95 Galactosemia type III
96 Galactosemia type IV
97 Fructose intolerance
98 Renal glucosuria
99 Diabetes mellitus
100 Glucose-6-phosphatase deficiency
101 Fructose-1,6-diphosphatase deficiency

TCA Cycle Disorders and Mitochondrial Disorders
102 Pyruvate carboxylase deficiency
103 Lactic aciduria (lactic acidemia)
104 α-Ketoglutaric aciduria
105 Fumaric aciduria
106 Pyruvate dehydrogenase deficiency
107 Dihydrolipoyl transacetylase deficiency
108 Pyruvate dehydrogenase phosphatase deficiency
109 Thiamine-responsive pyruvate dehydrogenase deficiency
110 Defect in electron transport system
111 Dihydrolipoyl dehydrogenase deficiency
112 Short-chain enoyl-CoA hydratase (SCEH, OMIM*602292) deficiency
113 3-Hydroxyisobutyryl-CoA hydrolase (HIBCH, OMIM 250620) deficiency
114 Mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase (HMCS2) deficiency
115 Cytochrome C oxidase deficiency (COX deficiency)
116 Combined malonic and methylmalonic aciduria
117 Barth syndrome (3-Methylglutaconic Aciduria TypeII)
118 MEGDEL Syndrome (3-Methylglutaconic Aciduria TypeV)
119 Other Secondary 3-methylglutaconic aciduria
120 Ethylmalonic encephalopathy (EE, OMIM # 602473)

Neuroblastoma (not IEM) and Zellwerger Syndrome
121 Neuroblastoma
122 Zellwerger syndrome

Metabolism of Purine and Pyrimidine
123 Molybdenum cofactor deficiency
124 Xanthine oxydase deficiency, xanthinuria
125 Dihydropyrimidine dehydrogenase deficiency
126 Dihydropyrimidine hydrolase deficiency
127 β-Ureidopropionase deficeincy
128 Lesch Nyhan syndrome
129 Hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency
130 Adenine phosphoribosyltransferase (APRT) deficiency
131 2,8-Dihydroxyadenine lithiasis (APRT deficiency)
132 Orotic aciduria

Fatty Acid Metabolism and Vitamins
133 Trifunctional protein deficiency
134 3-Hydroxyacyl-CoA dehydrogenase deficiency
135 Medium chain acyl-CoA dehydrogenase deficiency
136 Medium chain β-ketothiolase deficiency
137 Short chain acyl-CoA dehydrogenase deficiency
138 Medium/short chain 3-hydroxyacyl-CoA dehydrogenase deficiency
139 Molybdenum deficiency
140 Biotin deficiency
141 Folate deficiency
142 B12 deficiency

Others
143 Mevalonic aciduria (MEVA)
144 Urocanic aciduria
145 Pyridoxine Dependent Epilepsy(ALDH7A1 deficiency)

Note:
* Some diseases need test during episode and urine comparison in remission.
* Some diseases need to give results in combination with other test analysis.

the NBS program under one umbrella using TMS as an initial screening and confirmation by the GCMS method. The noteworthy point is that the TMS method using dried blood spots was introduced for metabolic screening in India almost 10 years after our GCMS services began, by initially private organisation and later in government hospitals. Therefore, whenever available, GCMS result was correlated in later period (2015−2024) in this study with the

findings of acyl carnitine TMS results, in addition to EEG, brain MRI, and other laboratory test findings. Currently, TMS screening test and urinary GCMS diagnostic analysis complement each other in supporting the diagnosis.

Limitations and proposed role of GCMS

The diagnostic panel by GCMS metabolic screening (Table 4) is found important in high-risk cohort and can be proposed as a diagnostic panel for confirmation of the ‘screen positive cases’ in a NBS program while implementing at the national level. This is especially true because there are limitations to adopt the GCMS method comprehensively as a primary screening method in India. Considering the 28 million annual birth rate in India, nationwide implementation of the GCMS method as a sole primary screening tool is not feasible because the time required to do one analysis is about 18 minutes per test against 2−3 min per test by TMS screening method. It is also the fact that detectable substances are limited in asymptomatic newborns. Additionally, the long-chain fatty acid oxidation disorders such as VLCAD deficiency and CPT2 deficiency and even medium chain fatty acid disorders like MCAD are equally important to include in NBS program. In MCAD deficiency, the substances detected by the GCMS method (hexanoylglycine, suberylglycine) are not detected in all asymptomatic cases which is the prerequisite of newborn screening test. It is apparent in metabolic practice that some diseases need to give results in combination with other test analysis and also some diseases need test during episode and urine comparison in remission. We therefore strongly recommend that the GCMS panel elaborated in Table 4 as the diagnostic test in primarily targeted high-risk groups. Through this study, we propose the “Tiered Approach” of TMS high-throughput screening for any NBS program, followed by the confirmatory diagnostic approach by urinary GCMS method. The current IEM metabolic screening by GCMS method is emphasized as a rapid ‘High-Risk Diagnostic Tool’ for symptomatic children in a setting where universal NBS is not yet widespread.

In our experience, urinary GCMS metabolic screening in India is the preferred test along with supportive TMS test in clinical practice for confirmation of diagnosis when DNA mutation tests are not easily available & or affordable. No doubt, the TMS using DBS is worldwide accepted and used as an universal newborn screening test. However, in hypoketotic hypoglycemia suspected patients, the TMS is the preferred choice to rule out or diagnose fatty acid disorder. Presently, in India, the referral treating doctor finds the combined use of urinary GCMS & DBS-TMS screening as the most suitable panel covering a large spectrum of IEMs in high-risk cases as it is evident by larger number of patients from 2015−2025 period (Table 2-A) due to the increased awareness among medical profession about congenital metabolic conditions. At times, urinary GCMS metabolic screening is the preferred test of choice in strongly suspected organic & amino acid disorders in non-affordable patients as both screening & confirmatory chemical diagnosis is possible in one test. It is well endorsed that screen positive cases detected by the TMS method must be confirmed for diagnosis by the GCMS analysis, as per the international NBS protocol. Therefore, in the recent years, a non-invasive urinary screening for 140+metabolic conditions by our MILS method (Table 4) is also the most acceptable IEM metabolic screening test in tertiary centres in high-risk patients and also by the far a way district level cities or rural regions.

Majority of the referral cases in the present study were sent by the clinical metabolic experts, neonatologists and paediatricians in private or public hospital practice to diagnose suspected IEMs. Additionally, the tele-interaction with the referral doctor was always found helpful in avoiding misdiagnosis & proper guidance during genetic counseling for the further metabolic and or molecular genetic work-up in consanguineous parents or storage of urine & blood samples in case of death of the patient[17]. In many cases, the appropriate guidance & interaction with the doctor was found helpful in genetic counseling and prevention of recurrence in the diagnosed families for not so rare IEMs in India due to genetic diversity and population. The autosomal recessive inheritance of the most IEMs could be explained to the parents during tele-counseling for prevention and also prenatal diagnosis in future from the remote areas.

The American College of Medical Genetics (ACMG) has given evidence-based clinical guidelines for exome & genome sequencing for pediatric patients with congenital anomalies or intellectual disability which indicates transition towards better care for patients using advanced technologies like next generation sequencing (NGS)[18]. We, in India look forward to have this transition with advanced technology for the care of metabolic patients.

Conclusion

The present urinary GCMS screening can be opted in a rural & tribal high-risk population where trained medical / metabolic experts & logistics for heel-prick DBS samples are difficult & almost lacking in India. The samples can be easily sent to the tertiary centres for analysis. Thus, the metabolic screening analysis using mass spectrometry can reach to the rural, tribal & untapped regions of India, covering a large spectrum of multiple IEMs in a single reliable test.

In brief, over 25 years of experience in a large high-risk cohort of 8246 offered the evidence of 12 preventable common IEMs of organic, amino acids, urea cycle & sugar disorders with high positive detection rate (1 in 26 to 400). This high-risk cohort demonstrates that these 12 IEMs are found with high frequency among symptomatic individuals in India. Therefore, these metabolic disorders should be considered as high-priority candidate diseases for future NBS pilot studies to determine their true incidence and the feasibility of screening in the general newborn population.

Secondly, besides the currently advocated 3 NBS disorders (CH, CAH and G6PD), the other organic, amino, fatty acids & sugar disorders are equally important & need to be considered as significant candidate NBS disorders because these are also well recognized for causing high mortality & morbidity. We also conclude that there is a dire need of many NBS Centres with comprehensive laboratory testing, therapy management experts & metabolic diets under one umbrella. Considering the huge 1.4 billion population & every 6th child born in the world is Indian, we recommend the "Referral NBS Centre" in each State of India to cope with screening, diagnosis and care of IEM patients. In India, the Regional Pilot Studies can be conducted using GCMS screening as a valuable tool for investigating disease frequency in specific states or regions before a nationwide implementation of NBS program. The study underscores the significance of the present urinary GCMS analysis method as a 'Confirmatory Diagnostic Tool' and the importance of establishing GCMS infrastructure as the international standard for the confirmatory diagnosis of positive cases screened by the DBS-TMS method.

Acknowledgements

The authors greatly acknowledge the help of Ms. Sumaiya Ansari, PG research fellow in data analysis. We are also thankful to the referring doctors, patients & parents for their valuable cooperation & support.

Funding

No funding available.

Conflict of Interest

None.

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