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Preface

MMS Special Issue “Newborn Screening and Mass Spectrometry”

Guest Editor: Seiji Yamaguchi, MD[1,2]

Recently, mass spectrometry has seen expanding applications in the field of clinical medicine. Among these, GC/MS and MS/MS (tandem mass spectrometry) have been widely used to the diagnosis of inherited metabolic disorders (IMDs), and the newborn screening using MS/MS (TMS screening) is now spreading globally.

Currently the TMS screening employs flow injection analysis, which directly introduces samples extracted from newborn blood filter paper absorbed with minute amount of blood, into MS/MS. It enables simultaneous analysis of amino acids and acylcarnitines, allowing for simple and rapid screening for amino acidemias, organic acidemias, and fatty acid oxidation defects.

This special issue featured six papers, including one in “Short communication”, and five in “Research papers”, respectively. In the “Short communication”, a case report of hereditary spherocytosis found in the TMS screening was presented. In the “Research papers”, 1) development of a highly sensitive analytical method for succinylacetone which is a diagnostic marker for hereditary hypertyrosinemia type 1; 2) experience and significance of prenatal diagnosis combining mass spectrometry and genetic testing for the severest types of IMD cases detected in the NBS; 3) an attempt of screening for urea cycle disorders through the development of orotic acid detection method, 4) a summary of disease distribution and frequency in cases detected via GC/MS in clinically high-risk patients in India; and 5) development of a rapid, highly sensitive MPS screening method combining RapidFire and MS/MS, were presented.

We believe the papers featured in this special issue contribute to expanding the application scope of MS/MS in NBS and improving diagnostic accuracy.

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Short Communication

Hereditary spherocytosis is associated with elevated C3 levels in dried blood spots used for newborn screening

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Abstract High propionylcarnitine (C3) levels and a high C3/acetylcarnitine (C2) ratio are used in newborn screening to indicate propionic acidemia and methylmalonic acidemia. Hyperbilirubinemia and maternal vitamin B12 deficiency are other factors that can cause high C3 levels during newborn screening, but hereditary spherocytosis (HS) has recently been identified as an important differential disorder. We experienced two cases of HS that were diagnosed through detailed examinations of high C3 levels. In Case 1, C3 level and C3/C2 ratio in the dried blood spots on day 4 after birth were 6.28 nmol/mL (cutoff value 3.60 nmol/mL) and 0.25 (cutoff value 0.25), respectively. In Case 2, C3 level and C3/C2 ratio in the dried blood spots on day 5 after birth were 9.32 nmol/mL and 0.42, respectively. In both cases, C3 levels in serum were not elevated, and a diagnosis of HS was confirmed at an early stage by a peripheral blood hemogram, thus avoiding unnecessary testing and treatment for suspected organic acidemia. When performing a detailed examination in the case of high C3 levels and a high C3/C2 ratio, it is important to confirm possible HS using a peripheral blood hemogram and to test for inherited metabolic diseases.

Key words: hereditary spherocytosis, propionylcarnitine, dried blood spots, liquid-chromatography tandem mass spectrometry, newborn screening

Introduction

In Japan, high propionylcarnitine (C3) levels and high C3/acetylcarnitine (C2) ratios in dried blood spots (DBSs) are used in newborn screening (NBS) to detect propionic acidemia (PA) and methylmalonic acidemia (MMA)[1], which require prompt detailed examination. Other known factors can contribute to high C3 levels besides inherited metabolic diseases (IMDs), such as hyperbilirubinemia[2] and maternal vitamin B12 deficiency[3]. In 2021, van Dooijeweert et al. reported that acylcarnitine analysis of DBSs derived from patients with hereditary spherocytosis (HS) showed elevated C3 levels[4]. We have experienced two cases of HS diagnosed on the basis of high C3 levels, and this report describes the clinical course and laboratory data of each case.

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Case Presentation

Case 1: The patient was delivered vaginally at 38 weeks and 4 days of gestation, and his birth weight was 2660 g (−1.1 standard deviation). His parents were non-consanguineous, and he had no family history of IMD or HS. He was the

second child of his parents. He was treated with phototherapy for jaundice on days 2, 6, and 10 after birth. An NBS sample was collected on day 4 after birth, and acylcarnitine analysis of the DBSs showed a C3 level of 6.28 nmol/mL (cutoff value 3.60 nmol/mL) and a C3/C2 ratio of 0.25 (cutoff value 0.25) (Table 1). He was transferred to our hospital on day 13 after birth for a detailed examination. Blood tests showed no metabolic acidosis or hyperammonemia, his vitamin B12 level was 405 pg/mL (reference range 180 to 914), his total homocysteine level was 5.2 nmol/mL (reference range 5.3 to 15.2), and plasma amino acid analysis showed no abnormalities. Serum acylcarnitine analysis showed a C3 level of 0.75 nmol/mL (reference ＜2.5 nmol/mL) and a C3/C2 ratio of 0.07 (reference ＜0.2) (Table 1), and urinary organic acid analysis showed mild increases in methylcitrate and 3-hydroxypropionic acid. Urinary organic acid analysis at 14-month-old showed no increases in methylcitrate and 3-hydroxypropionic acid. The clinical course is shown in Fig. 1A. Because of anemia, erythrocyte transfusions were performed on day 13 and day 52 after birth. A peripheral blood hemogram showed numerous spherical erythrocytes. Genetic testing showed no abnormalities in the genes responsible for PA or MMA (including *PCCA*, *PCCB*, *MMUT*, *ABCD4*, *HCFC1*, *LMBRD1*, *MMAA*, *MMAB*, *MMACHC*, and *MMADHC*). Genetic testing revealed a heterozygotic c.1602+1 G＞A variant in the *ANK1* gene, which was unreported. Familial analysis showed no variants in the parents; therefore, this was considered a *de novo* variant. We determined the variant to be pathogenic on the basis of the American College of Medical Genetics and Genomics guidelines[5] (PVS1+PS2+PM2+PP3+PP4). Finally, the patient was diagnosed with HS. The patient is now 18 months old and showed no metabolic crisis at the time of infection, and he has no evidence of progressive anemia and normal growth and development. No recurrence of a high C3 level in DBSs was observed after the neonatal period.

Table 1. Acylcarnitine analysis results of Case 1

Acylcarnitine	DBSs at day 4	Cut-off	Serum at day 13	Reference
C0	53.23	(≤9.00)	45.81	(20−60)
C2	28.49		11.45	(10−40)
C3	<u>6.28</u>	(≥3.60)	0.75	(≤2.5)
C4	0.46		0.25	(≤1.0)
C5	0.33	(≥1.00)	0.33	(≤0.5)
C5:1	0.02		0.01	(≤0.02)
C5-DC	0.18	(≥0.29)	0.06	(≤0.25)
C5-OH	0.21	(≥1.00)	0.06	(≤0.15)
C6	0.07		0.07	(≤0.2)
C8	0.09	(≥0.30)	0.06	(≤0.3)
C10	0.10	(≥0.40)	0.09	(≤0.3)
C12	0.16	(≥0.40)	0.09	(≤0.2)
C14	0.34		0.03	(≤0.15)
C14:1	0.14	(≥0.40)	0.06	(≤0.2)
C16	1.97	(≥6.00)	0.04	(≤0.2)
C16-OH	0.009	(≥0.050)	＜0.01	(≤0.03)
C18	0.66		0.04	(≤0.3)
C18:1	1.39		0.09	(≤0.4)
C18:1-OH	0.016	(≥0.050)	＜0.01	(≤0.05)
C3/C2	<u>0.25</u>	(≥0.25)	0.07	(≤0.2)

Unit: nmol/mL, DBSs: dried blood spots, C0: free carnitine, C2: acetylcarnitine, C3: propionylcarnitine, C4: butyrylcarnitine, C4-OH: 3-hydroxybutyrylcarnitine, C5: isovalerylcarnitine, C5:1: tiglylcarnitine, C5-DC: glutarylcarnitine, C5-OH: 3-hydroxyisovalerylcarnitine, C6: hexanoylcarnitine, C8: octanoylcarnitine, C8:1: octenoylcarnitine, C10: decanoylcarnitine, C10:1: decenoylcarnitine, C12: dodecanoylcarnitine, C12:1: dodecenoylcarnitine, C14: tetradecanoylcarnitine, C14:1: tetradecenoylcarnitine, C16: palmitoylcarnitine, C16-OH: hydroxy-hexadecanoylcarnitine, C18: stearoylcarnitine, C18:1: oleylcarnitine, C18:2: linoleylcarnitine, C18-OH: hydroxy-octadecanoylcarnitine, C18:1-OH: hydroxy-octadecenoylcarnitine, Abnormal date are underlined.

Case 2: The patient was delivered vaginally at 39 weeks and 4 days of gestation, with a birth weight of 3330 g (+1.1 standard deviations). Her parents were non-consanguineous, and she had no family history of IMD, but her mother was diagnosed with HS and had been splenectomized because of this disease. She was treated with phototherapy for jaundice from day 1 to day 4 and on day 6 after birth. A peripheral blood hemogram showed numerous spherical erythrocytes; therefore, HS was suspected on the basis of family history. An NBS sample was collected on day 5 after birth, and acylcarnitine analysis of the DBSs showed a C3 level of 9.32 nmol/mL (cutoff value 3.60 nmol/mL) and a C3/C2 ratio of 0.42 (cutoff value 0.25) (Table 2). The patient was transferred to our hospital on day 12 after birth for a detailed examination. Blood tests showed no metabolic acidosis or hyperammonemia, her vitamin B12 level was 272 pg/mL, her total homocysteine level was 5.5 nmol/mL, and plasma amino acid analysis results were normal. Serum acylcarnitine analysis showed a C3 level of 0.55 nmol/mL (reference ＜2.5 nmol/mL) and a C3/C2 ratio of 0.08 (reference ＜0.2) (Table 2). Urine organic acid analysis showed no abnormal findings. The clinical course is

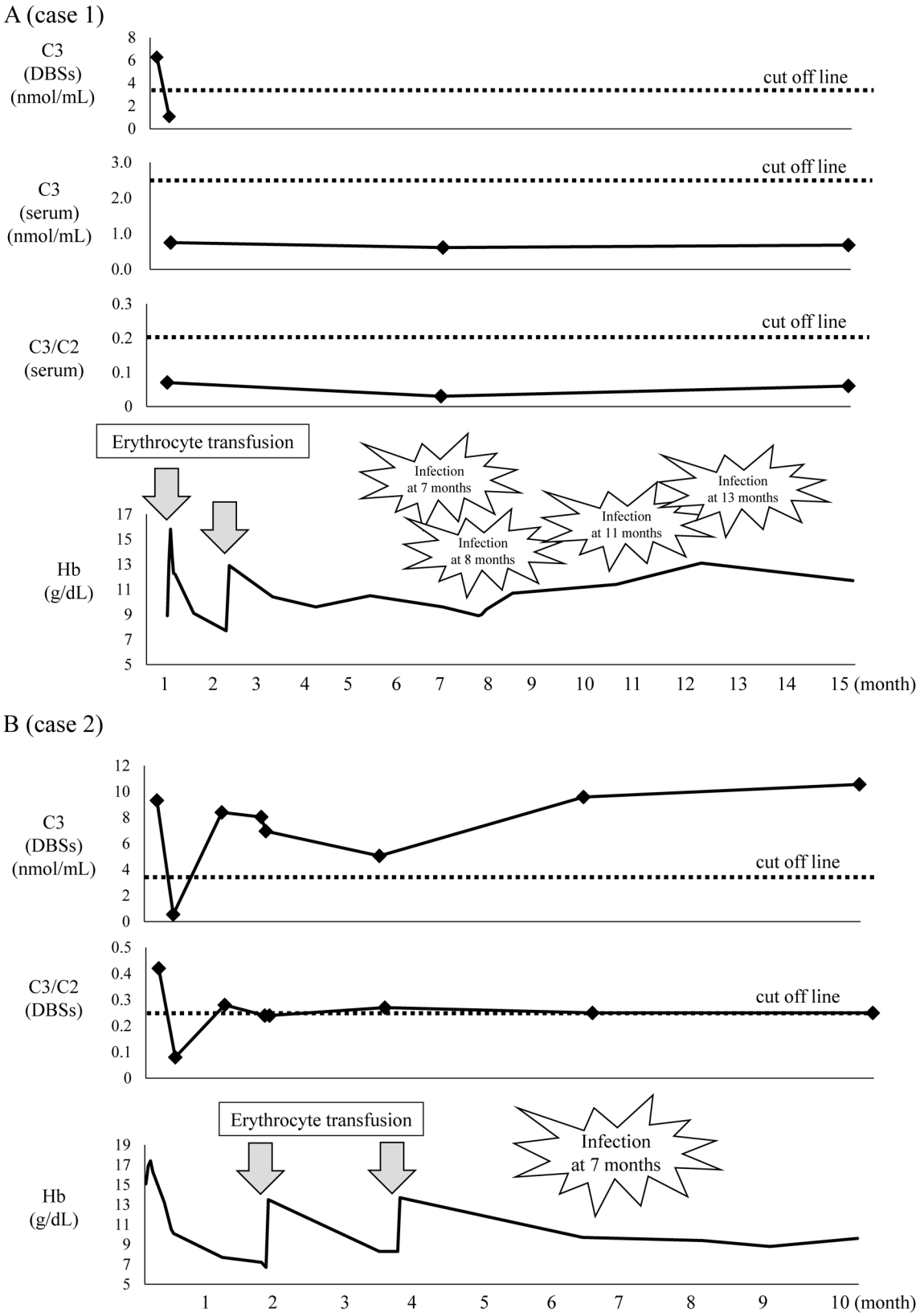

Fig. 1. Changes in C3 level, C3/C2 ratio, and hemoglobin levels over the clinical course. A, The clinical course of Case 1. B, The clinical course of Case 2. C3: propionylcarnitine, C2: acetylcarnitine, Hb: hemoglobin, DBSs: dried blood spots.

Table 2. Acylcarnitine analysis results of Case 2

Acylcarnitine	DBSs at day 5	Cut-off	Serum at day 12	Reference	DBSs at 10 mo	Reference	Serum at 10 mo	Reference
C0	34.34	(≤9.00)	70.78	(20−60)	75.21	(20−60)	43.77	(20−60)
C2	23.90		6.91	(10−40)	45.00	(4.5−45)	6.40	(10−40)
C3	9.32	(≥3.60)	0.55	(≤2.5)	11.90	(≤3.5)	0.70	(≤2.5)
C4	0.47		0.16	(≤1.0)	0.28	(≤0.8)	0.14	(≤1.0)
C5	0.35	(≥1.00)	0.18	(≤0.5)	0.21	(≤0.8)	0.09	(≤0.5)
C5:1	0.01		0.01	(≤0.02)	0.01	(≤0.05)	<0.01	(≤0.02)
C5-DC	0.19	(≥0.29)	0.01	(≤0.25)	0.10	(≤0.25)	0.02	(≤0.25)
C5-OH	0.35	(≥1.00)	0.03	(≤0.15)	0.48	(≤1.0)	0.03	(≤0.15)
C6	0.08		0.04	(≤0.2)	0.07	(≤0.15)	0.04	(≤0.2)
C8	0.07	(≥0.30)	0.04	(≤0.3)	0.05	(≤0.3)	0.05	(≤0.3)
C10	0.06	(≥0.40)	0.06	(≤0.3)	0.06	(≤0.4)	0.07	(≤0.3)
C12	0.13	(≥0.40)	0.06	(≤0.2)	0.08	(≤0.4)	0.06	(≤0.2)
C14	0.27		0.03	(≤0.15)	0.31	(≤0.4)	0.04	(≤0.15)
C14:1	0.10	(≥0.40)	0.05	(≤0.2)	0.05	(≤0.3)	0.04	(≤0.2)
C16	1.84	(≥6.00)	0.08	(≤0.2)	2.35	(0.5−3.0)	0.20	(≤0.2)
C16-OH	0.017	(≥0.050)	<0.01	(≤0.03)	0.01	(≤0.08)	<0.01	(≤0.03)
C18	0.32		0.02	(≤0.3)	0.93	(0.2−2.0)	0.07	(≤0.3)
C18:1	1.11		0.06	(≤0.4)	2.08	(0.37−2.8)	0.22	(≤0.4)
C18:1-OH	0.015	(≥0.050)	<0.01	(≤0.05)	0.02	(≤0.05)	<0.01	(≤0.05)
C3/C2	0.42	(≥0.25)	0.08	(≤0.2)	0.26	(≤0.2)	0.11	(≤0.2)

Unit: nmol/mL, mo: months, Abnormal date are underlined.

shown in Fig. 1B. Because of anemia, erythrocyte transfusions were performed on day 52 and day 109 after birth. Genetic testing revealed no abnormalities in the genes responsible for PA or MMA (same as in Case 1). Genetic testing revealed a heterozygotic c.856C>T (p.Arg286Ter) variant in the *ANK1* gene. This variant was reported as pathogenic[6], and therefore the patient was diagnosed with HS. Familial analysis showed no variant in the father, but her mother had the same variant. The patient had persistently high C3 levels and C3/C2 ratios until 10 months of age only when tested using the DBSs. A discrepancy between the serum and the DBSs acylcarnitine analysis was observed at 10 months (Table 2). The patient is now 12 months old, and her growth and development are within the normal range.

Materials and Methods

Sample preparation for serum acylcarnitine analysis

Serum acylcarnitine analysis was conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Sample preparation was performed using NeoSMAAT® AC (SEKISUI Medical Co., Tokyo, Japan), a standardized acylcarnitine extraction reagent designed to ensure consistent quantification for clinical testing. The kit used for acylcarnitine analysis included standard compounds for C0, C2, C3, C4, C5, C5-OH, C5-DC, C8, C10, C12, C14, C14:1, C16, and C18. Additionally, the kit contained stable isotope-labeled internal standards for each of these analytes to ensure accurate quantification. Quantification of hydroxylated (−OH) and unsaturated acylcarnitine species was performed using internal standards consisting of structurally similar acylcarnitines, following the method used for DBSs analysis.

A 5 μL aliquot of human serum was mixed with 5 μL of 1% formic acid in ethanol and 5 μL of internal standard solution. Then, 200 μL of ethanol was added, and the mixture was vortexed for 10 s before centrifugation at 10,000 rpm at 4°C for 5 min. The resulting supernatant was carefully transferred to a separate container and evaporated under a 40°C nitrogen stream for 60 min. The residue was reconstituted in 50 μL of 0.5% formic acid in acetonitrile, followed by vortexing for 30 s. Subsequently, 50 μL of acetonitrile was added, and the sample was vortexed again for 30 s. The final mixture was centrifuged at 2000 rpm at 4°C

for 3 min, and the entire supernatant was transferred to a high-performance liquid chromatography plate for LC-MS/MS analysis. The injection volume was 10 μL.

For calibration standard (CAL) samples, a minimum five-point calibration curve required for quantification was constructed by incorporating a portion of the quality control samples into the CAL series. The quality control and CAL samples were prepared using 5% fat-free human serum albumin in phosphate-buffered saline as the matrix, and commercial acylcarnitine mixtures were used when available. The processing steps, including ethanol precipitation, centrifugation, and nitrogen evaporation, were identical to those used for clinical samples.

LC-MS/MS analysis

Samples were measured using a Nexera MP System equipped with an SIL-30ACMP Multi-Plate auto-sampler and an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Co. Kyoto, Japan) with a positive electrospray ionization source. Chromatographic separation was achieved using a Triart Diol-HILIC column (2.0×100 mm, 1.9 μm, YMC, Japan) maintained at 40°C. The mobile phase consisted of 0.05% formic acid in water (Phase A) and 0.05% formic acid in acetonitrile (Phase B), with a gradient elution mode at a flow rate of 0.2 to 0.3 mL/minute over a 10-min runtime. The mass spectrometer was operated in positive electrospray ionization mode with multiple reaction monitoring for each acylcarnitine quantification.

Results

Case 1: The results of acylcarnitine analysis of serum at day 13 and DBSs at day4 using LC-MS/MS were shown in Table 1. The results of serum showed no C3 elevation and high C3/C2 ratio.

Case 2: The results of acylcarnitine analysis of serum at day 12, 10-month-old and DBSs at day 5, 10-month-old using LC-MS/MS were shown in Table 2. The specimens at 10-month-old were collected at the same time. The results of serum showed no C3 elevation and high C3/C2 ratio.

Discussion

High C3 levels and C3/C2 ratios detected by acylcarnitine analysis of DBSs are used to screen for MMA and PA in Japan[1]. There is no relationship between the degree of C3 level or C3/C2 ratio elevation and the severity of the disease[7]. Therefore, all cases in which the C3 and/or C3/C2 values exceed the cutoff require prompt diagnosis and careful follow-up.

There are two known causes of falsely high C3 and C3/C2 levels in DBSs analysis, hyperbilirubinemia[2] and vitamin B12 deficiency in newborns resulting from maternal vitamin B12 deficiency[3]. Elevated C3 has been reported in cases of neonatal hyperbilirubinemia, but the detailed mechanism is unclear. The vitamin B12 levels in neonates correlate with maternal vitamin B12 levels. Mitochondrial methylmalonyl-CoA mutase (MUT) requires vitamin B12 as a coenzyme, and when vitamin B12 is deficient, the enzyme activity of MUT decreases. Decreased MUT enzyme activity induces increases in methylmalonic acid and propionyl CoA, which is further converted to C3, and finally the C3 level increases[3].

In 2021, van Dooijeweert et al. analyzed DBSs from HS patients and reported that several polyamines and acylcarnitines including C3 and 2,3-diphosphoglycerid acid were significantly elevated in DBSs from HS patients[4]. Among them, C3 was suggested to be strongly correlated with red blood cell hydration and deformability. When comparing the acylcarnitine analyses of Case 1 and Case 2, the C3 value was higher in Case 2 than in Case 1. In Case 1, hemolytic findings were relatively mild, and C3 elevation was observed in the DBSs only on day 4. In contrast, in Case 2, hemolytic findings were relatively severe, and C3 elevation in the DBSs persisted beyond the neonatal period. A significant increase in C3 was also reported in the DBSs analysis of pyruvate kinase deficiency (#OMIM 266200)[8], an inherited hemolytic disease that causes hemolytic anemia similar to HS, suggesting that C3 may be elevated in patients with hemolytic anemia in general. The detailed mechanism by which C3 is elevated in HS is unknown. It is also unclear whether the C3 peak detected in DBSs analysis of HS patients using LC-MS/MS is only C3 or complex of C3 and another substance. In 2019, Haijes et al. reported that direct-infusion high-resolution mass spectrometry (DI-HRMS) can correctly measure both DBSs and plasma C3 levels in patients with PA[9]. van Dooijeweert et al. also measured C3 in DBSs of HS patients using DI-HRMS[4]. We measured high C3 levels in DBSs of patients with HS using LC-MS/MS; however, C3 levels in serum were very low, even in specimens collected at the same time. The limitation of this report is that we have not been able to identify the substance responsible for the high C3 levels in DBSs. Some reports have shown that carnitine is involved in the

stabilization of erythrocyte membranes[10], but the detailed mechanism is still unclear and further studies are needed.

HS is a hemolytic anemia disease caused by extravascular hemolysis because of the destruction and phagocytosis of erythrocytes in the spleen caused by defects in the genes encoding erythrocyte membrane proteins (alpha spectrin, beta spectrin, ankyrin, band 3, and protein 4.2)[11]. The frequency of this disease in Japan is estimated to be 1 in 50,000 to 100,000 persons. Ankyrin is an erythrocyte membrane protein that is encoded by the *ANK1* gene, which is inherited in an autosomal dominant manner. Laboratory findings for this condition include hyperbilirubinemia with a predominance of indirect bilirubin, high lactate dehydrogenase, increased reticulocytes, increased urinary urobilinogen, and polychromatic erythroblasts, which reflect hemolysis, as well as small spherical erythrocytes in peripheral blood hemograms. However, it should be noted that approximately one-third of HS patients showed no spherical erythrocytes in peripheral blood hemograms in the neonatal period[11].

We recognized the necessity of considering HS as another differential diagnosis for C3 and C3/C2 elevations through these two cases. When conducting a detailed examination of high C3 and C3/C2 levels, it is important to test for IMDs and to repeatedly check peripheral blood hemograms. Early diagnosis of HS is important to avoid unnecessary testing and treatment for suspected organic acidemia.

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Conflict of Interest

Hideo Sasai has received an endowed chair funded by Gifu Research Center for Public Health. The other authors declare no conflict of interest.

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Research Paper

Importance of blood succinylacetone measurement using liquid chromatography-tandem mass spectrometry in high-risk screening for hereditary tyrosinemia type I

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Abstract Newborn screening for hereditary tyrosinemia type I (HT1) is performed worldwide by measuring succinylacetone (SA) in dried blood spots using flow-injection tandem mass spectrometry with cut-off values for SA ranging from 1.29 to 10 μM. In Japan, HT1 is not a target disorder for newborn screening, and high-risk screening is performed only after disease onset. Blood SA concentrations in patients with HT1 who develop liver cirrhosis have been reported to be lower than those in the neonatal period, and there is an urgent need for a reliable diagnostic method. Therefore, in this study, we developed a highly sensitive method for SA analysis using liquid chromatography-mass spectrometry. This method allows quantification of SA concentrations at 0.005 μM and enables measurement of SA concentrations not only in patients with HT1 who have liver cirrhosis but also in asymptomatic patients or patients undergoing drug therapy.

Key words: succinylacetone, newborn screening, hypertyrosinemia type I, liquid chromatography-tandem mass spectrometry, diagnosis

1. Introduction

Hereditary tyrosinemia type I (HT1; McKusick 27670) is an autosomal recessive disorder caused by a deficiency in fumarylacetoacetate hydrolase in the tyrosine degradation pathway[1)]. This disease can cause severe liver disease and renal tubular dysfunction via the accumulation of fumarylacetoacetate (FAA) and maleylacetoacetate (MAA), which are readily converted to succinylacetone (SA). The diagnosis of HT1 relies on the detection of increased levels of SA in the blood and/or urine. Treatment with 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione has now become the standard treatment for the management of HT1 and provides significant improvement of symptoms in patients by reducing the levels of FAA, MAA, and SA in biological fluids.

Newborn screening for HT1 in Europe using SA as a screening marker was first reported in 2007[2)]. In the initial study, screening was performed using a non-kit method; SA in dried blood spots (DSB) was derivatized into the oxime form. Then, flow injection-tandem mass spectrometry (FI-MS/MS) analysis was performed with a cutoff value of 10 μM. A review paper published in 2017[3)] reported that the methods used by screening laboratories included both non-kit and kit methods and that the cutoff values of these methods were not consistent, ranging from 1.29 to 10 μM. These discrepancies raised concerns about the accuracy of screening.

In Japan, HT1 has not been a target disorder for newborn screening due to the low frequency of patients with the disease and difficulties with the derivatization method. Instead, urinary organic acid analysis was performed to detect increased SA excretion for infants with symptoms of liver failure together

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with high tyrosine levels. However, we encountered a patient with HT1 who had mildly elevated serum SA levels without pathological increases in urinary SA due to advanced liver cirrhosis[4]. In this case, the serum SA levels measured by liquid chromatography (LC)-MS/MS were lower than the cutoff values for newborn screening using FI-MS/MS in the review paper mentioned above. Notably, serum SA levels in patients with asymptomatic HT1 and treated patients with HT1 measured by gas chromatography (GC)-MS were much lower than those of untreated patients with HT1[5].

In the current study, we improved our LC-MS/MS method to measure serum SA levels in patients with HT1, as described above, and examined whether this approach could be useful in clinical applications.

2. Materials and Methods

2.1. Materials

2.1.1. Biological samples

DBS samples from two patients with HT1 prepared during the newborn period for mass screening using MS/MS were stored in a refrigerator in screening laboratories and then transported to our laboratory at the University of Fukui and measured using LC-MS/MS after obtaining permission from the parents of each patient. DBS and serum and urine samples from some patients with HT1 were collected at the time of disease onset. Three patients with HT1 had liver cirrhosis at diagnosis, and diagnoses of HT1 were confirmed by gene analysis. Informed consent was obtained from all subjects involved in the study.

2.1.2. Chemicals

A NeoSMAAT kit for MS/MS newborn screening, which contained labeled acyl-carnitines and amino acids, was purchased from Sekisui Medical Co. (Tokyo, Japan). Succinylacetone was purchased from Tokyo Chemical Industry (Tokyo, Japan), and succinylacetone-$^{13}C_5$ was purchased from the VU Medical Center Metabolic Laboratory (Amsterdam, The Netherlands).

2.2. Methods

For routine LC-MS/MS analysis of serum SA, 6 μL serum was placed in a small test tube with a punched piece of unused filter paper for newborn screening, and 0.22 mL of acetonitrile solution containing succinylacetone-$^{13}C_5$ (0.2 nmol/mL), 0.1% hydrazine H_2O, and 0.1% formic acid was added. The tube was shaken slowly for 45 min. The supernatant, which contained oxime derivatives of SA (SA-oxime) and SA-$^{13}C_5$, was obtained by centrifugation, dried, and then redissolved in 60 μL of 2% formic acid/methanol. For the analysis using 120 μL serum, the combined supernatant obtained from ten test tubes with 12 μL serum and 0.44 mL reaction mixture was dried and then redissolved in 2% formic acid/methanol.

To determine the linearity, we analyzed non-patient serum spiked with SA to a final concentration of 0.005−10 μM.

For the analysis of SA in DBS, a 3-mm-diameter punch of DBS was used instead of a serum sample.

An aliquot (10 μL) of the redissolved solution was introduced into the LC mobile phase flow (flow rate: 0.4 mL/min) using a 150 mm×3.0 mm Scherzo SM-C18 column (Imtakt, Portland, OR, USA). Gradient elution of the analytes was achieved using a program with mobile phase A as aqueous 0.5% formic acid and mobile phase B as 0.5 M ammonium formate and 0.5 M NH_4OH (9:1)/methanol (1:9).

A Triple Quad 4500 LC-MS/MS system (Sciex, Tokyo, Japan) was used for LC-MS/MS. The MS/MS analyses were performed in multiple reaction monitoring (MRM) mode using transitions at *m/z* 155/137 for SA-oxime and *m/z* 160/142 for $^{13}C_5$-SA-oxime. Suitable measurement conditions for the designated transitions for SA-oxime and $^{13}C_5$-SA-oxime were identified with the automatic tune function in Analyst software. For quantification, the recorded peak areas of the designated MRM ion set were used.

3. Results

No measurable SA peak was observed on the chromatogram of non-patient serum without spiked SA (n=15). In the analysis of non-patient DBS (n=6), the SA concentration ranged from less than 0.05 to 0.14 μM (Table 1). When non-patient serum containing spiked SA (SA concentration: 0.05−10.0 μM) was prepared and analyzed using our routine method, good linearity (R^2=0.9995) was obtained and intra- and interday assay coefficients of variation (CVs) for SA at a concentration of 0.05 μM were 3.5% and 5.1%, respectively.

In the analysis using 120 μL serum (SA concentration: 0.005−1.0 μM), good linearity (R^2=0.9995) was obtained, and intra- and interday assay CVs for SA at a concentration of 0.005 μM were 4.1% and 5.4%, respectively (Fig. 1). Several unknown peaks in MRM chromatograms for the transition at *m/z* 155/137 (representing SA-oxime) were observed, together with an apparent SA-oxime peak (Fig. 2).

In HT1 patient A, the SA concentrations in DBS were

Table 1. Succinylacetone levels in serum samples, DBSs, and urine samples from patients with HT1

HT1 patient	Age at diagnosis	Measurement at diagnosis			Newborn screening
		SA in urine (mmol/molCr)	SA in DBS (μM)	SA in serum (μM)	SA in stored DBS (μM)
A	5 months	103	2.6	—	28.7
B	2 years 6 months	<4	1.6	1.1	—
C	2 months	<4	—	0.78	5.5
Controls		<4	<0.05–0.14	<0.05	<0.05–0.14

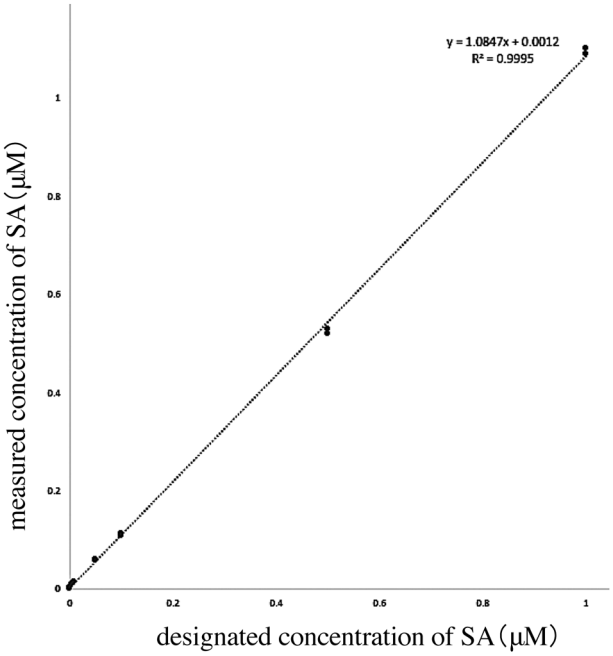

Fig. 1. Regression line of measured SA versus designated serum concentrations of SA in our analysis using 120 μL serum samples.

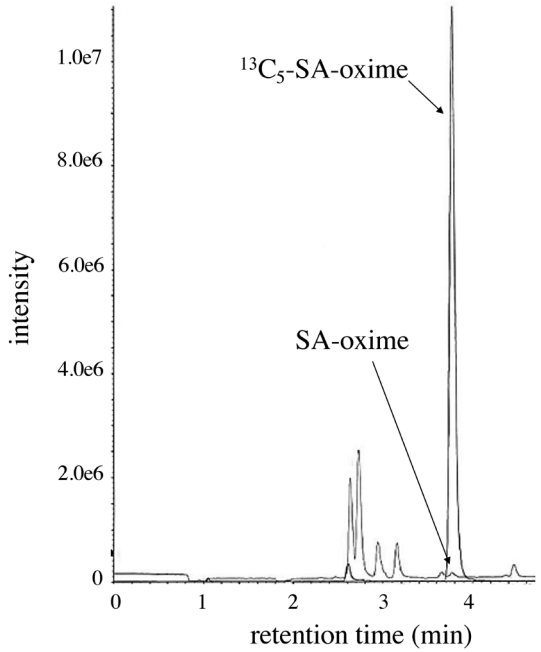

Fig. 2. MRM chromatograms for SA-oxime (*m/z* 155/137) and $^{13}C_5$-SA-oxime (*m/z* 160/142), showing an SA-oxime peak of 0.01 μM together with several unknown peaks.

28.7 μM in the neonatal period and 2.6 μM at the time of diagnosis (5 months of age). In patient B, the SA concentrations at 2.5 years of age were 1.6 μM in DSB, 1.1 μM in serum, and less than 4 mmol/mol Cr in urine. In patient C, the serum SA concentrations at 2 months of age were 0.78 μM and less than 4 mmol/mol Cr in urine, whereas the SA concentration in newborn DBS was 5.5 μM (Table 1).

4. Discussion

According to a review paper on HT1 screening in Europe and the United States[3], the cutoff value for SA in neonatal DBS by FI-MS/MS ranges from 1.29 to 10 μM. The uncertainty regarding test accuracy is assumed to be related to differences in sample preparation methods. Many of the laboratories surveyed in the review paper used non-kit methods, although SA was derivatized in different ways in both laboratories using kit and non-kit methods. A recent report from a screening facility using the Neobase2 kit[6] showed that the SA cutoff value was 1.5 μM and that the non-patient control value was 0.60±0.07 μM. However, according to a report from the 2020 CDC Newborn Screening Quality Assurance Program (NSQAP)[7], the cutoff value for the SA concentration in seven screening facilities using the Neobase2 kit ranged from 0.9 to 2.8 μM. They also reported large differences in SA concentrations determined using the FI-MS/MS method at various facilities. In addition, the detection limit for SA using FI-MS/MS may be affected by the presence of nonspecific compounds, which could appear as unknown peaks, as shown in our MRM chromatogram (*m/z* 155/137) for SA-oxime.

In an analysis of SA in blood samples using LC-MS/MS

and SA derivatives of butyl ester and oxime obtained when using dansylhydrazine[8], the SA concentrations in neonatal DBS from patients with HT1 were reported to range from 3.3 to 65 μM, and the lower limit of measurement was 0.2 μM. In present study, the lower limit of measurement was 0.05 μM, although our derivatization method using only hydrazine was simpler than that of the above report.

In an analysis of plasma SA concentrations using GC-MS (lower limit of measurement: 0.024 μM)[5], the plasma SA concentrations were reported to be 0.11−0.14 μM in asymptomatic HT1 cases, 0.021−0.056 μM in treated patients with HT1, and 0.003−0.021 μM in controls, although 0.5 mL of sample was required for measurement. In addition, complex derivatization using fluoride compounds was performed for GC-MS analysis, resulting in four isomer peaks for SA; this seems to be disadvantageous in terms of quantitation. In our LC-MS/MS method, the sample volume required to obtain the same sensitivity as in the above GC-MS was just 0.12 mL, and the simple derivatization procedure was advantageous in terms of accuracy.

Using LC-MS/MS, we found that blood SA concentrations in patients with HT1 with liver cirrhosis were markedly lower than those collected from patients during the neonatal period. Regarding the difference between serum and DBS in our SA analysis, SA concentrations in DBS were higher than that in the serum sample from patient B, as shown in Table 1. These findings suggest that SA may accumulate more markedly in blood cells than in the liquid component of biological fluids from patients with HT1.

It is difficult to obtain consistent quantitative values using the FI-MS/MS method owing to the ion suppression phenomenon[9]. In addition, as shown in Fig. 2, a few unknown peaks appeared in the MRM chromatogram close to the peak for SA-oxime. These peaks may significantly influence SA quantification using FI-MS/MS. Thus, measurement of blood SA concentrations by LC-MS/MS may be essential for the chemical diagnosis of patients with HT1 with a variety of conditions.

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Institutional Review Board Statement

This study was approved by the Institutional Ethics Committee at the University of Fukui (#20130055, #20180029).

Conflict of Interest

The authors declare no conflicts of interest.

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Research Paper

Prenatal diagnosis of severe metabolic disorders in diseases targeted by newborn screening in Japan

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Abstract The introduction of tandem mass spectrometry (TMS) analysis into newborn screening (NBS) has improved the prognosis of patients with organic acidemias and fatty acid oxidation disorders through early detection and timely intervention. However, some severe phenotypes remain unresponsive to treatment and often result in death during early infancy. Genetic counseling and a prenatal diagnosis may be the only options for the families who have newborns with such forms of the disease and strongly wish to have another baby. Prenatal diagnostic evaluations were conducted in 134 cases, using gas chromatography/mass spectrometry (GC-MS), TMS analysis, and/or genetic testing, at the request of families with an affected proband. Of the 134 fetuses, 46 were diagnosed as being affected, and all 46 pregnancies were therefore terminated. All cases diagnosed as unaffected were confirmed to have no postnatal abnormalities. Given the direct impact of the prenatal diagnosis on the continuation of pregnancy, it is essential that the analytical methods used are reliable. A mass spectrometry (GC-MS and TMS) analysis of amniotic fluid was found to be highly sensitive for prenatal diagnosis. This article suggests that mass spectrometry, combined with genetic testing, significantly improves the accuracy of prenatal diagnosis.

Key words: organic acidemia, fatty acid oxidation disorder, prenatal diagnosis, gas chromatography mass spectrometry, tandem mass spectrometry, newborn screening

Introduction

Nationwide newborn screening using tandem mass spectrometry (TMS)[1–3] was introduced in Japan in 2014. This screening program expanded the target diseases beyond amino acid metabolism disorders to include organic acidemias (OAs) and fatty acid oxidation disorders (FAODs). Consequently, many patients identified early through this screening can be protected from the onset of disease and disability through the timely initiation of treatment.

However, some of the OAs and FAODs present as a lethal neonatal-onset phenotype, which remains resistant to all available treatment. These cases often result in death within days to weeks of birth or lead to severe neurological sequelae in survivors. Genetic counseling is crucial for families with a history of such disorders who are considering subsequent pregnancies, and a prenatal diagnosis (PND) may be the only viable option.

The prenatal diagnosis of OAs and FAODs can be performed through the analysis of metabolites in amniotic fluid, enzyme activity assays evaluating amniotic cells, or genetic analyses[4–8]. However, genetic analysis requires prior knowledge of the proband's genotype.

At the Faculty of Medicine, Shimane University, we provided genetic counseling and a prenatal diagnosis for a family with a history of a proband exhibiting severe pheno-

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types. In 2005, we reported on 28 cases of PND for OAs[5]. Since then, including cases of FAODs, we have conducted a total of 134 PNDs through 2016. In this report, our diagnostic experience and the clinical outcomes with PND of these cases are described.

In particular, in a family affected by mitochondrial trifunctional protein (TFP) deficiency, a type of long-chain FAOD, PND based on acylcarnitine analysis was highly effective in distinguishing affected from unaffected fetuses. Therefore, the details of this representative case are presented with supporting data.

Materials and Methods

This study was conducted in accordance with the ethical principles outlined in the Guidelines for Genetic Testing and Diagnosis in Medical Practice[9] and was approved by the Ethics Committee of Shimane University Faculty of Medicine (formerly Shimane Medical University) under the title "Prenatal Diagnosis of Severe Inborn Errors of Metabolism" (Research Management Number: 20150227-6).

1. Subjects

This study included 134 cases of PND performed at the Faculty of Medicine, Shimane University, between 1998 and 2016. The cases met the following criteria:

1) The proband had a severe OA or FAOD that resulted in death during the neonatal or early infantile period or led to severe sequelae.
2) The family expressed a strong desire for PND in genetic counseling, and the diagnosis was deemed appropriate based on the Guidelines for Genetic Tests and Diagnosis in Medical Practice[9].
3) In cases involving genetic analysis, pathogenic variants in the proband and parents were identified.

2. Sample collection

Amniotic fluid samples were collected via amniocentesis between the 12th and 16th weeks of pregnancy at designated medical facilities. The samples were transported at <4°C and subsequently centrifuged to separate the supernatant and sediment fractions.

3. Amniotic fluid analysis

The supernatant fraction was used for an organic acid analysis by gas chromatography-mass spectrometry (GC-MS) and an acylcarnitine analysis by tandem mass spectrometry (TMS). For FAODs, in addition to mass spectrometry, genomic DNA was extracted from the pellet fraction of centrifuged amniotic fluid for genetic analysis.

(i) Mass spectrometry

Sample preparation, reagent use, and analytical procedures for GC-MS and TMS followed established methodologies[5,10,11]. The GC-MS system consisted of a Shimadzu GCMS-QP 5050 Model (Shimadzu, Kyoto, Japan) until 2007, followed by a GCMS-QP 2020plus Model (Shimadzu), and N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (t-BDMS) derivatization was used for the organic acid analysis. Quantification was carried out in the selective ion monitoring (SIM) mode. The detection of methylmalonic acid using this method is illustrated in Fig. 1.

The TMS system used for acylcarnitine analysis was an API 3000 triple-quadrupole tandem mass spectrometer in combination with an SIL-HTc autosampler (Shimadzu).

(ii) Genetic analysis

Genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). Target genes identified in the proband were sequenced according to previously established methods[12].

(iii) Individual identification in genetic analysis

In cases in which a maternal-origin mutation was identified by genetic analysis, a microsatellite analysis was performed to confirm individual identification by comparing DNA from amniotic cells and maternal blood.

Results

1. Proportion of affected fetuses

Of the 134 fetuses considered at risk for OA and FAODs, 118 were categorized as at risk for OAs and 16 for FAODs, based on the diagnostic information of the probands (Table 1). The key diagnostic markers (organic acids and acylcarnitines) used for each disorder are summarized in Table 2.

Of the OA cases that asked for PND, methylmalonic acidemia (MMA) was the most common disease (85 cases), followed by propionic acidemia (PPA; 17 cases), glutaric acidemia type I (GA1; 11 cases), and multiple carboxylase deficiency (MCD; 5 cases). Forty-six of these cases were ultimately diagnosed as affected. Thirty-five cases were diagnosed as MMA, 8 as PPA, 2 as GA1, and 1 as MCD (Table 1).

Regarding FAODs, 8 cases with mitochondrial trifunc-

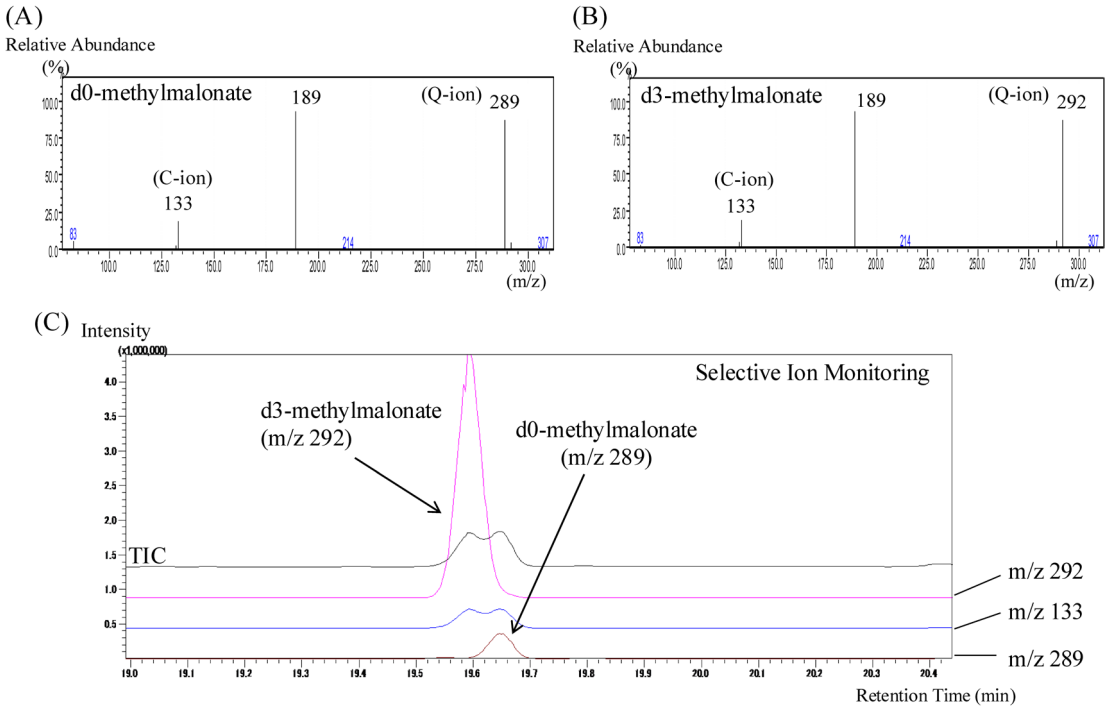


Fig. 1 Extracted ion chromatogram and mass spectrum of t-BDMS derivatives of native (d0) and stable-isotope-labeled (d3) methylmalonate in a case at risk for methylmalonic acidemia.

(A) Mass spectrum of d0-methylmalonate. (B) Mass spectrum of d3-methylmalonate. (C) Extracted ion chromatogram of d0-methylmalonate (*m/z* 289) and d3-methylmalonate (*m/z* 292).

Due to differences in m/z values, retention times in mass spectrometric detection may vary, and quantification is therefore performed based on the ratio of peak areas.

Abbreviations: Q- and C-ion; selected ion for quantification and confirmation, respectively.

Table 1. Number of prenatal diagnoses

Disease	Cases	Affected	Analytical method
1) Organic acidemias	118	46	
Methylmalonic acidemia	85	35	GC-MS TMS
Propionic acidemia	17	8	
Glutaric acidemia type I	11	2	
Multiple carboxylase deficiency	5	1	
2) Fatty acid oxidation disorders	16	3	
Mitochondrial trifunctional protein (TFP) deficiency	8	1	Gene TMS GC-MS
Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency	3	0	
Glutaric acidemia type II	3	1	
Carnitine acylcarnitine translocase deficiency	2	1	
Total	134	49	

tional protein (TFP) deficiency, 3 cases each of very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency and glutaric acidemia type II (GA2), and 2 cases of carnitine acylcarnitine translocase (CACT) deficiency were evaluated. Of these cases, one each of TFP deficiency, GA2, and CACT deficiency were judged to be affected (Table 1).

All fetuses diagnosed as unaffected were confirmed to have no metabolic abnormalities by a postnatal biochemical analysis. The unaffected individuals have been followed for 8 to 20 years postnatally; however, as of the end of 2024, none has developed any clinical symptoms suggestive of late-onset disease.

Table 2. Target diagnostic compounds for organic acidemias and fatty acid oxidation disorders

Disease	Target compounds	
	Organic acids	Acylcarnitines
1) Organic acidemias		
Methylmalonic acidemia	Methylmalonate and Methylcitrate	C_3, C_3/C_2
Propionic acidemia	Methylcitrate (without Methylmalonate)	C_3, C_3/C_2
Glutaric acidemia type I	Glutarate	C_5DC
Multiple carboxylase deficiency	Methylcitrate and Methylcrotonylglycine	C_3, C_3/C_2, C_5-OH
2) Fatty acid oxidation disorders		
TFP deficiency	—	C_{14}-OH, C_{16}-OH, C_{18}-OH, $C_{18:1}$-OH
VLCAD deficiency	—	$C_{14:1}$
Glutaric acidemia type II	—	C_6, C_8, C_{10}, C_{12}
Carnitine acylcarnitine translocase deficiency	—	$C_{14:1}$, C_{16}, $(C_{16}+C_{18})/C_0$

Abbreviations: C_0: free carnitine, C_2: Acetylcarnitine, C_3: Propionylglycine, C_5DC: Glutarylcarnitine, C_5-OH: 3-Hydroxyisovalerylcarnitine, C_6: Hexanoylcarnitine, C_8: Octanoylcarnitine, C_{10}: Decanoylcarnitine, C_{12}: Dodecanoylcarnitine, $C_{14:1}$: Tetradecenoylcarnitine, C_{14}-OH: 3-Hydroxymyristoylcarnitine, C_{16}: Palmitoylcarnitine, C_{16}-OH: 3-Hydroxypaimitoylcarnitine, C_{18}: Stearoylcarnitine, $C_{18:1}$: Oleoylcarnitine, C_{18}-OH: 3-Hydroxystearoylcarnitine, $C_{18:1}$-OH: 3-Hydroxyoleoylcarnitine.

In all cases in which a fetus was diagnosed as affected, the pregnancy was terminated. In cases in which a mass spectrometry analysis was performed using cord blood or other postnatal samples, the prenatal and postnatal diagnoses were consistent in all instances.

Furthermore, in cases in which genetic analysis was performed, the results were fully consistent with the classification of fetuses as affected or unaffected based on mass spectrometry.

2. Comparison between methylmalonic acidemia (MMA) and propionic acidemia (PPA)

In both MMA and PPA, organic acid analyses demonstrated increased concentrations of methylcitrate, whereas acylcarnitine analyses showed elevated concentrations of propionylcarnitine (C3). However, MMA exhibited a marked increase in methylmalonate, which facilitated the differentiation between the two diseases (Table 3). The methylcitrate and C3 concentrations were generally higher in the PPA group than in the MMA group. As illustrated in Fig. 2, this reflects the metabolic pathway position of propionyl-CoA carboxylase upstream of methylmalonyl-CoA mutase.

Table 3. Concentrations of methylcitrate, methylmalonate, and propionylglycine (C3) in amniotic fluid from the index cases

Disease	Organic acid (GC-MS)		Acylcarnitine (TMS)
	Methylcitrate	Methylmalonate	C3
Propionic acidemia	13.31	0.37	11.62
	32.94	2.44	13.85
	10.10	0.41	5.74
	26.66	0.24	16.75
	18.72	2.28	19.72
Methylmalonic acidemia	9.52	43.09	7.86
	7.01	36.91	8.10
	3.18	28.20	7.39
	9.18	42.37	10.74
	10.28	45.89	8.26
Control	0.18−1.56	0.30−3.70	0.43−1.72

(Unit: μmol/L)

The values of five cases are shown for each disease. Underlined values indicate elevated concentrations.

3. Prenatal diagnosis of TFP deficiency

A case of TFP deficiency, one of the FAODs, was analyzed in a family with a history of this disease. The proband was born via a normal delivery but died on day 6 of life due to cardiomyopathy that did not respond to treatment. A postmortem acylcarnitine analysis using dried blood spots strongly suggested a TFP deficiency, and genetic analysis confirmed the presence of compound heterozygous mutations in *HADHA* (c.1392+1G>A and c.1689+2T>G).

Subsequently, two PND tests were performed in subse-

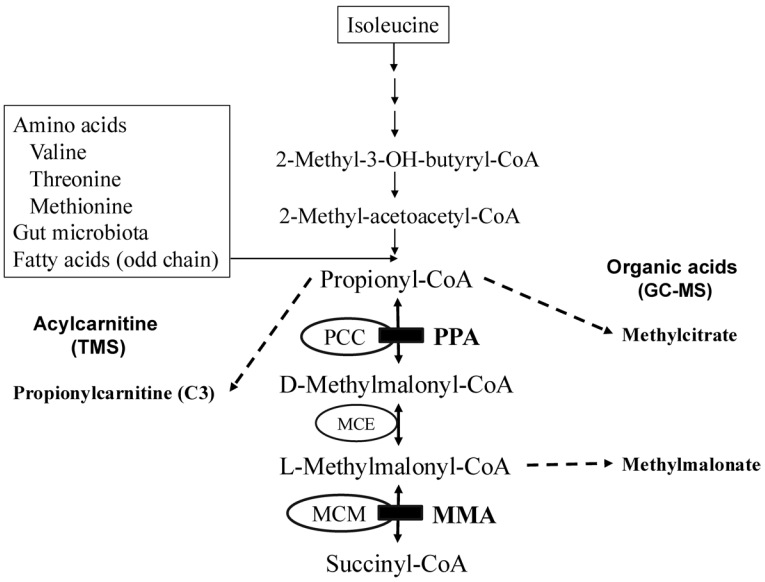
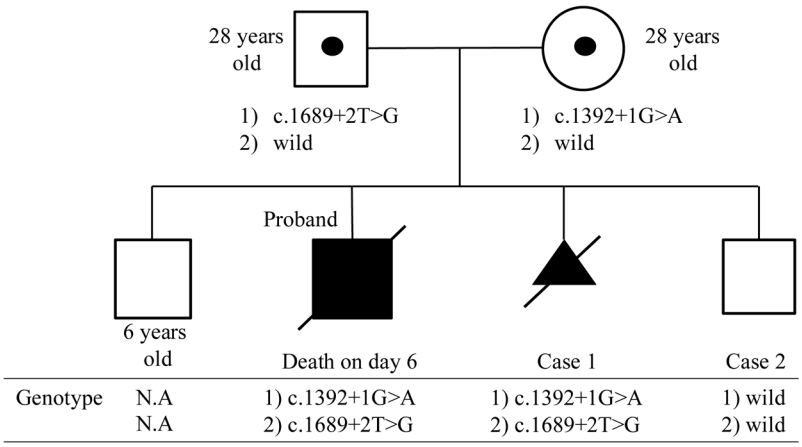


Fig. 2. Metabolic pathway of propionic acidemia (PPA) and methylmalonic acidemia (MMA).
Abbreviations: PCC: Propionyl-CoA carboxylase, MCE: Methylmalonyl-CoA epimerase, MCM: Methylmalonyl-CoA mutase.



Genotype	N.A	1) c.1392+1G>A	1) c.1392+1G>A	1) wild
	N.A	2) c.1689+2T>G	2) c.1689+2T>G	2) wild

Fig. 3. Family tree and genotypes with TFP deficiency.
Abbreviation: TFP: mitochondrial trifunctional protein, N.A: not analyzed.

quent pregnancies, which identified 1 affected fetus (Case 1) and 1 unaffected fetus (Case 2) (Fig. 3).

*Case 1 (Affected)

The same pathogenic variants as those in the proband (c.1392+1G>A and c.1689+2T>G) were identified. An acylcarnitine analysis of the amniotic fluid supernatant showed markedly elevated concentrations of 3-hydroxypalmitoylcarnitine (C16-OH) and 3-hydroxystearoylcarnitine (C18-OH), which are characteristic markers of TFP deficiency (Table 4). The final diagnosis of the affected status was confirmed by individual identification using a microsatellite analysis.

*Case 2 (Unaffected)

No abnormalities were detected in either the genetic analysis of the amniotic cells or the acylcarnitine analysis of the amniotic fluid supernatant. Therefore, the fetus was diagnosed as unaffected. In addition, a postnatal acylcarnitine analysis of the blood showed no abnormalities (no data available).

Discussion

Severe forms of OAs and FAODs often lead to rapid deterioration in the neonatal period, resulting in death or severe sequelae despite medical interventions. These devastating outcomes cause profound grief and distress in affected families. According to the "Guidelines for Genetic Tests and Diagnosis in Medical Practice" issued by the Japanese Association of Medical Science[9], PND of these

Table 4. Concentrations of acylcarnitines in amniotic fluid for prenatal diagnosis of TFP deficiency

Acylcarnitine	Amniotic fluid (Unit: nmol/L)			Dried blood spots (Unit: nmol/mL)	
	Case 1	Case 2	Control (n=9)	Proband	Cut-off
C14	27	0	(4.6±3.4)	1.16	(<0.4)
C14:1	21	0	(5.8±6.5)	0.43	(<0.3)
C14:1-OH	50	0	(5.0±4.8)	0.31	(<0.1)
C16	52	0	(6.9±8.0)	6.26	(<3.0)
C16-OH	120	0	(0.9±1.5)	3.1	(<0.1)
C18	14	10	(2.9±4.6)	0.78	(<2.0)
C18-OH	31	0	(3.6±7.8)	0.76	(<0.08)
C18:1-OH	44	0	(5.1±5.0)	1.90	(<0.08)

Underlined values indicate elevated concentrations.
Compared with the fetus unaffected by TFP deficiency, the concentration of C16-OH in the affected fetus was approximately 133 times higher, and C18-OH showed about an 8.6-fold increase.
Abbreviations: C14: Tetradecanoylcarnitine, C14:1: Tetradecenoylcarnitine, C14:1-OH: 3-Hydroxytetradecenoylcanitine, C16: Palmitoylcarnitine, C16-OH: 3-Hydroxypalmitoylcarnitine, C18: Stearoylcarnitine, C18-OH: 3-Hydroxystearoylcarnitine, C18:1-OH: 3-Hydroxyloleoylcarnitine.

inborn errors of metabolism is considered ethically permissible with careful consideration. When a fetus is diagnosed as affected, it is highly likely to follow the same clinical course as the proband, making the continuation of pregnancy potentially unbearable to the family. In this study, all families opted for termination in cases where the fetus was diagnosed as affected. Conversely, of the cases diagnosed as unaffected, all neonates were confirmed to be postnatally healthy. These findings underscore the critical importance of making an accurate PND, which necessitates comprehensive and multifaceted testing.

In the present study, multiple diagnostic methods were used whenever possible to enhance diagnostic accuracy, including organic acid and acylcarnitine analyses, genetic testing, and enzyme activity assays.

In a previous study, we demonstrated the utility of amniotic fluid mass spectrometry for the PND of OAs[5]. Other studies have similarly reported that the combination of multiple diagnostic tests (e.g., concurrent measurement of enzyme activity and metabolites[7], the simultaneous analysis of chorionic villi and amniotic fluid[13], and the use of multiple mass spectrometry techniques[8]) improves diagnostic reliability.

In addition, the previous study confirmed that acylcarnitines in amniotic fluid become unstable at room temperature[5]. Therefore, in addition to organic acid and acylcarnitine analyses, genetic testing was performed concurrently to enhance diagnostic accuracy and prevent false-positive results. Special attention was paid to the storage and transportation conditions of the specimens, and genetic analyses were conducted when applicable.

The present study also demonstrated that the combination of an acylcarnitine analysis and genetic testing of amniotic fluid is an effective approach for diagnosing FAODs. The prenatal diagnosis of FAODs has been reported since 1996. It has been conducted using methods such as characteristic acylcarnitine profiling and enzyme activity assays using amniotic cells[11,14]. Similarly, the PND of VLCAD deficiency has been achieved through an acylcarnitine analysis of amniotic fluid supernatants and genetic testing[15], as demonstrated in the present study. Although amniocentesis is an invasive procedure, it remains an essential and widely used prenatal diagnosis technique.

In conclusion, a mass spectrometry-based metabolite analysis using amniotic fluid supernatant with genetic analysis is a valuable tool for the PND of OAs and FAODs. An acylcarnitine analysis provides rapid and reliable results if specimens can be stored and transported in a frozen state to prevent degradation.

When pathogenic variants in the proband are identified, the integration of genetic analysis with other diagnostic methods enhances diagnostic reliability. Furthermore, individual identification through a chimerism analysis is essential for differentiating maternal cell contamination and further improving the accuracy of PND.

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Dr. Hasegawa summarized the overall analysis, interpreted and verified the organic acid analysis, and contributed to the composition of the manuscript. Dr. Kobayashi was responsible for the acylcarnitine analysis. Dr. Bo made significant contributions to the analysis of patients with TFP deficiencies. Prof. Taketani provided review and editing. Furthermore, Dr. Hasegawa is grateful to Emeritus Prof. Yamaguchi for providing critical insights into analytical methods and research design, and for his supervision of this study.

Conflict of Interest

The authors declare no conflicts of interest in association with the present study.

Abbreviations

NBS	newborn screening
TMS	tandem mass spectrometry
GC-MS	gas chromatography/mass spectrometry
t-BDMS	N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide
OAs	organic acidemias
FAODs	fatty acid oxidation disorders
PND	prenatal diagnosis
TFP	mitochondrial trifunctional protein
MMA	methylmalonic acidemia
PPA	propionic acidemia
GA	glutaric acidemia
MCD	multiple carboxylase deficiency
VLCAD	very long-chain acyl-CoA dehydrogenase
CACT	carnitine acylcarnitine translocase

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Research Paper

Development and pilot evaluation of an add-on orotic acid assay in dried blood spots for newborn screening of ornithine transcarbamylase deficiency

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Abstract Background: Ornithine transcarbamylase deficiency (OTCD) is the most common urea cycle disorder and can cause lifethreatening hyperammonemia. Biochemical diagnosis typically relies on elevated orotic acid (orotic acid (ORA)) and decreased citrulline (citrulline (Cit)) levels. However, in the newborn screening (NBS) setting, ORA measurement from dried blood spots (DBS) has been technically challenging and is not routinely implemented.

Methods: We developed a tandem mass spectrometry (MS/MS) method for quantifying ORA in DBS using negative electrospray ionization, performed concurrently with routine positive-mode amino acid and acylcarnitine analysis. Reference ranges and cutoffs were determined from retrospective analysis of 4,605 healthy newborn DBS specimens. A prospective pilot screening of 6,562 newborns (September 2018−March 2019) was conducted. DBS from OTCD patients in various clinical states were also analyzed.

Results: The assay showed excellent linearity (0.5−100 nmol/mL; R^2=0.9999) with acceptable precision (CV＜15% above 0.5 nmol/mL). In healthy newborns, the 99.5th percentile was 2.99 nmol/mL for ORA and 0.34 for the ORA/Cit ratio. No positives were detected in the pilot screening. Acutephase OTCD samples had markedly elevated ORA and ORA/Cit, while stablephase values often overlapped with normal ranges. Among four lateonset OTCD patients' newborn DBS, only one exceeded the cutoffs; this patient later developed severe hyperammonemia.

Conclusions: This ORAbased assay can be incorporated into existing NBS workflows with minimal modification. Our study suggests that ORAbased NBS might detect OTCD newborns who are already hyperammonemic as well as a subset of asymptomatic lateonset newborns.

Key words: orotic acid; urea cycle disorder; newborn screening; tandem mass spectrometry; ornithine transcarbamylase deficiency

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Introduction

Ornithine transcarbamylase deficiency (OTCD, MIM #300461) is the most common urea cycle disorder (UCD), and is inherited in an X-linked manner[1)]. In Japan, the overall incidence of UCDs is estimated to be 1 in 50,000 births, with OTCD accounting for approximately 60% of cases. Therefore, the incidence of OTCD is 1 in 80,000 births[2-4)].

The prognosis of OTCD depends on frequency and duration of hyperammonemic episodes, as well as the peak value of blood ammonia. Rapid and appropriate interventions to prevent and treat hyperammonemic episodes are essential to improve outcomes[4, 5]. Thus, implementing newborn screening (NBS) for OTCD during the presymptomatic neonatal period could significantly benefit patients. However, OTCD is not currently included in NBS panels in most countries due to the lack of a simple and reliable screening method, and only a few studies have reported on the feasibility of using orotic acid (ORA) measurement for NBS of OTCD[6–11].

The presence of ORA in urine is a useful diagnostic finding for OTCD. The enzymatic defect in ornithine transcarbamylase (OTC), which catalyzes the conversion of carbamyl phosphate (CP) and ornithine to citrulline, results in the accumulation of CP, which is subsequently converted to ORA[1]. Urinary ORA levels are typically high in symptomatic male OTCD patients, but can be variable in heterozygous female carriers.

In this study, we developed and validated a specific and sensitive method for quantifying ORA in dried blood spots (DBS) using tandem mass spectrometry (MS/MS) with negative electrospray ionization (ESI). By incorporating this assay into routine NBS, alongside conventional amino acid and acylcarnitine profiling conducted in positive ion mode, we aimed to enable effective screening for OTCD. We evaluated the assay performance through retrospective analysis of patient and control DBS specimens and subsequently conducted a small prospective pilot study in a general newborn population.

This study was conducted with the approval of the Institutional Review Board (IRB) at Shimane University Faculty of Medicine (approval number 2734, 2889, and 2908).

2. Materials and Methods

2.1. Reagents

ORA and stable-isotope-labeled ORA, [1,3-$^{15}N_2$] ORA, was purchased from Sigma-Aldrich (Darmstadt, Germany), and Cambridge Isotope Laboratories (Tewksbury, MA, USA), respectively. Acetonitrile, methanol, and deionized water of the LCMS grade were from Wako (Osaka Japan). NeoBase Non-derivatized MSMS Kit (PerkinElmer. Waltham, MA, USA) was used for analysis of routine amino acids except for ORA. Filter paper for NBS and for quality control were obtained from ADVANTEC (Tokyo, Japan).

2.2. Preparation of calibration samples

To prepare calibration standards for ORA in DBS, known amounts of ORA were spiked onto blank newborn DBS punches. Specifically, DBS samples with final ORA concentrations of 0 (blank), 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 nmol/mL were created by applying ORA solution onto blank filter paper blood spots. After air-drying, the spiked DBS samples were sealed and stored at −30°C until analysis. An internal standard (IS) stock solution of [1,3-$^{15}N_2$] ORA was prepared at 1 mg/mL (6.3 mM) in distilled water and stored at 4°C.

2.3. DBS extraction and MS/MS analysis

The assay was performed according to the NeoBase kit protocol for DBS, with modifications to include ORA measurement. In brief, a 3.2 mm disk punch from each DBS was placed into a well of a 96-well plate. A 100 μL volume of working extraction solution was added to each well. The working solution consisted of the NeoBase kit's internal standard mixture (for amino acids and acylcarnitines) supplemented with the [1,3-$^{15}N_2$] ORA internal standard at 0.1 mg/mL (approximately 0.63 mM). The plate was agitated on a shaker at 700 rpm for 45 min at 45°C. After extraction, the supernatant from each well was transferred to a fresh 96-well plate for MS/MS analysis.

MS/MS analysis was conducted by flow injection analysis on a Nexera MP System utilizing the SIL-30ACMP Multi-Plate autosampler and LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The system was programmed to perform sequential acquisitions in positive and negative ESI modes. The mobile phase provided in the NeoBase kit (a methanol-based solution) was used at a flow rate according to the kit's specifications. The injection volume was 1 μL per sample, and the total run time for each analysis was approximately 1 min.

The quantitative analysis in ORA was performed with multiple reaction monitoring (MRM) mode of the following transitions *m/z* 155.10＞111.05 for OA and *m/z* 157.10 ＞113.05 for OA IS in negative ESI mode. Other analytes were quantified in positive ESI mode. The dwell time for each MRM channel was 0.01 s. The source temperature was set to 250°C. Nitrogen was used as the nebulizing and drying gas, at a flow rate of 3 L/min.

2.4. Retrospective analysis of normal newborn DBS

4,605 of leftover DBS samples after NBS, which were presumably normal, were used as normal control in this study. DBS samples were collected between 4 days and 6 days after birth in accordance with the NBS program in Japan. These samples had been stored at −30°C after routine NBS analysis for at longest 2 years. Histograms of ORA and ORA/Cit ratio were illustrated in Fig. 1. We determined summary statistics (mean, standard deviation, and 99.5th percentile) to establish tentative cutoff values for prospective screening.

2.5. Prospective newborn screening pilot study

Using the cutoffs derived from the retrospective study, we conducted a prospective pilot screening. The study was carried out from September 2017 to March 2019. A total of 6,562 consecutive newborn DBS samples (collected for routine NBS) were analyzed with the expanded panel including ORA measurement. ORA and ORA/Cit values were measured for all samples (Fig. 2).

2.6. Analysis of OTCD patient DBS samples

To assess the method's ability to detect OTCD, DBS samples from 34 patients with OTCD were analyzed. They included 19 samples collected in acute metabolic decompensation (14 samples in neonatal onset form, 5 of late-onset form), 11 in stable condition (7 samples in neonatal onset form, 4 of late-onset form), and 4 NBS samples of

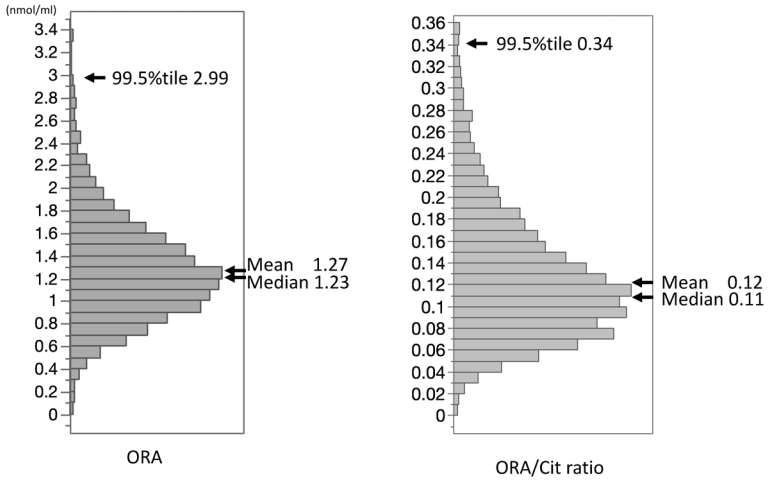

Fig. 1. Histogram of ORA and ORA/Cit ratio in 4,605 specimens of retrospective analysis.
Summary statistics (mean, standard deviation, and 99.5th percentile) were determined to establish tentative cutoff values for prospective screening.

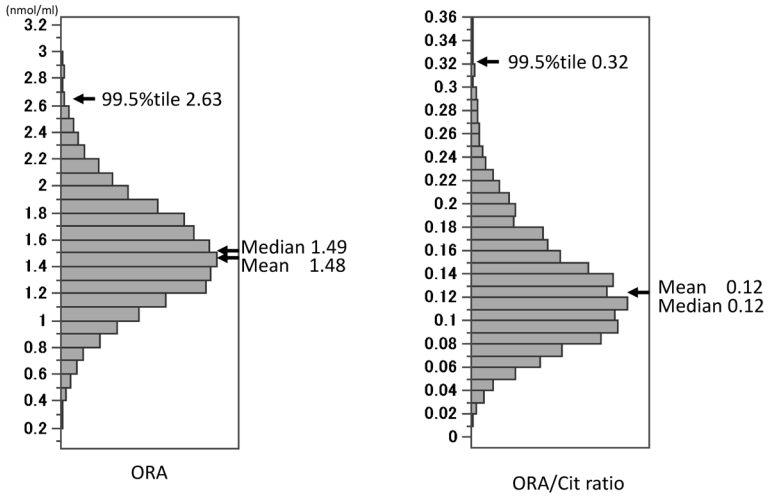

Fig. 2. Histogram of ORA and ORA/Cit ratio in 6,562 prospective pilot study.
The mean±SD of ORA was 1.49±0.40 nmol/mL. The mean±SD of ORA/Cit ratio was 0.124±0.052. The 99.5th percentile values of ORA and ORA/Cit ratio were 2.63 nmol/mL and 0.32, respectively.

patients with late onset form. All OTCD diagnoses were confirmed by either genetic testing of OTC gene or by specific biochemical examination. DBS samples of the late onset cases in NBS period were retrospectively identified and retrieved from leftover DBS storage after diagnosis later in life.

Two of the four NBS samples were obtained from sibling patients with late-onset form (Case 1 and 2 in Fig. 3). Since their older brother had been diagnosed with late-onset OTCD, they received dietary therapy and/or glucose infusion although they were asymptomatic when their NBS samples were collected. The other patient (Case 3 in Fig. 3) was a girl and was born in a member of late-onset OTCD family. She had not developed any acute metabolic decompensation such as hyperammonemia without any treatment until 3 years old. The other boy (Case 4 in Fig. 3) in one of the four cases developed hyperammonemia (highest NH_3 564 μM) when he had upper respiratory infection at the age of 1 year and 1 month, with consequent neurological sequelae.

3. Results

3.1. Validation of ORA quantification in DBS (Table 1)

Table 1 summarizes the validation results of the ORA quantification assay in DBS. The ORA assay showed a linear response over the tested range of 0.5−100.0 nmol/mL (spiked DBS concentrations). The regression equation was y=1.1172x+1.3558, with R^2=0.9999. At the low concentrations of 0 and 0.1 nmol/mL spiked), the coefficients of variation (CV) were 31.4% and 20.8%, respectively. For concentrations at or above 0.5 nmol/mL, CVs were all below 15%, which is generally acceptable on FDA guidance for industry, Bioanalytical Methods Validation.

3.2. Distribution of ORA and ORA/Cit in retrospective newborn samples

Fig. 1 shows the distributions of ORA concentrations and ORA/citrulline (Cit) ratios in 4,605 routine newborn DBS samples. Normal distribution was seen in ORA, with a

Table 1. Validation results of DBS sample spiked ORA using new method

Spiked concentration of ORA in DBS (nmol/mL)	mean (n=7)	S.D.*	CV** (%)
0	1.35	0.42	31.37
0.1	1.37	0.29	20.78
0.5	1.83	0.20	11.17
1	2.18	0.27	12.24
5	6.62	0.80	12.08
10	12.77	1.44	11.26
50	58.14	1.87	3.22
100	112.61	6.70	5.95

*; Standard deviation, **; coefficient of variation

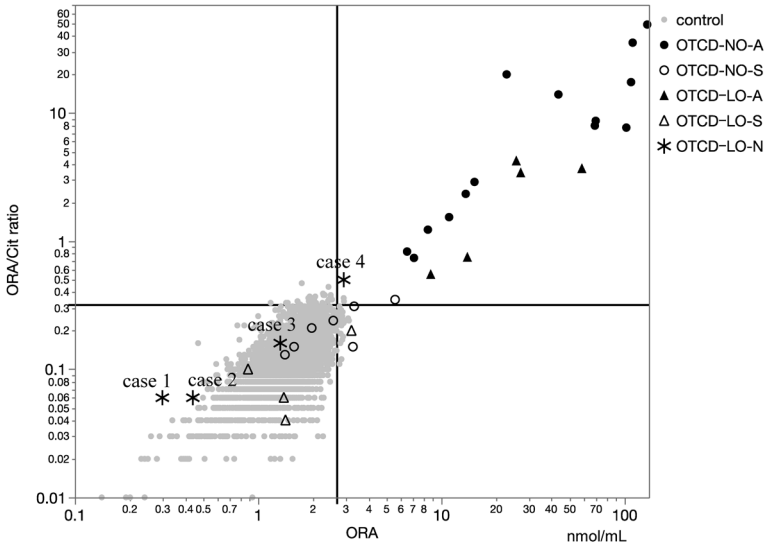

Fig. 3. Scatter plot of ORA versus ORA/Cit ratio in presumably normal newborns and patients with OTCD.
Both axes are plotted on a logarithmic scale. Each gray dot represents a newborn from the prospective cohort (n=6,562). Symbols denote OTCD patient samples: ●, neonatal-onset acute-phase; ○, neonatal-onset stable-phase; ▲, late-onset acute-phase; △, late-onset stable-phase. The four late-onset OTCD patient newborn DBS samples are indicated by asterisks (*). Case 4 lies above the normal cluster, exceeding both ORA and ORA/Cit cutoffs (dashed lines), indicating it would be positive by the screening. Cases 1−3 fall within the normal range and would not be identified by ORA screening alone.

mean of 1.27 nmol/mL and standard deviation (SD) of 0.48 nmol/mL. The 99.5th percentile was 2.99 nmol/mL. The ORA/Cit ratio also approximated a normal distribution, with a mean±SD of 0.12±0.06 and a 99.5th percentile of 0.34. Based on these findings, cutoff values for the prospective pilot study were set at 2.99 nmol/mL for ORA and 0.34 for the ORA/Cit ratio.

3.3. Prospective pilot newborn screening results

The distribution of OA and OA/Cit ratio in the prospective pilot study on 6,562 newborns are shown in Fig. 2, which closely mirrored that of the retrospective analysis. The mean±SD of ORA was 1.49±0.40 nmol/mL. For the ORA/Cit ratio, mean±SD was 0.124±0.052. The values in 99.5%tile of ORA and ORA/Cit ratio were 2.63 nmol/mL and 0.32, slightly lower than retrospective cutoffs.

3.4. ORA values in OTCD patient samples

Table 2 summarizes the ORA and ORA/Cit results for DBS from OTCD patients under various conditions, and Fig. 3 provides a visual comparison of select cases to the normal range. ORA values in the neonatal-onset OTCD cases ranged from 6.5 to 132.9 nmol/mL, and the median and mean values were 33.1 and 51.5, respectively. The ORA/Cit ratio was between 0.74 and 49.1 and the median and mean values were 7.7 and 11.6, respectively. Abnormal values exceeding the cutoff are underlined. In cases of acute phase of late-onset patients, the ORA ranged from 8.75 to 58.45, and the median and mean values were 25.64 and 26.76 nmol/mL, respectively. The OA/Cit ratio was from 0.55 to 4.23, and the median and mean values were 3.42 and 2.53, respectively. In the analysis in the stable metabolic condition in patients with late-onset type, ORA and ORA/Cit ratio were between 0.88 and 3.23 The median and mean values of ORA were 1.96, 2.42, respectively, and those of the ORA/Cit ratio were 0.15 and 0.18, respectively. In analysis of DBS samples collected in the neonatal period of 4 patients with late-onset type, OA was 030, 0.44, 1.32, and 2.96 nmol/mL, while the OA/Cit ratio of the 4 cases was 0.06, 0.06, 0.16, 0.50, respectively. These values reveal that only Case 4 exceeded the screening cutoffs (ORA 2.96 vs cutoff−3.0 nmol/mL, and ORA/Cit 0.50 vs cutoff−0.34). Cases 1, 2, and 3 all had ORA and ORA/Cit well within the normal newborn range. Fig. 3 plots ORA vs ORA/Cit for these cases against the distribution of the 6,562 pilot study newborns. Cases 1−3 fall squarely within the cluster of normal newborns, whereas Case 4 is an obvious outlier (high ORA and ORA/Cit), indicating a positive screen.

Discussion

In this study, we developed a novel screening method for

Table 2. Data are shown for acute-phase and stable-phase samples from neonatal-onset and late-onset OTCD cases, as well as neonatal DBS collected from late-onset patients

	ORA	ORA/Cit ratio
Normal control DBS in NBS (N=4,605)	1.27±0.48	0.124±0.061
[cut off value]	[>2.99]	[>0.34]
Prospective DBS in pilot study (N=6,562)	1.49±0.40	0.124±0.061
[cut off value]	[>2.63]	[>0.32]
Acute condition		
Neonatal onset form case (N=14)	6.5 to 132.9	0.74 to 49.1
Late onset form case (N=5)	8.75, 13.86, 25.64, 27.1, 58.45	0.55, 0.75, 3.42, 3.69, 4.23
Stable condition		
After neonatal samples from neonatal onset form case (N=7)	1.40, 1.57, 1.96, 2.57 3.29, 3.34, 5.59	0.13, 0.15, 0.15, 0.21, 0.24, 0.31, 0.35
After neonatal samples from late onset form (N=4)	0.88, 1.38, 1.41, 3.23	0.1, 0.04, 0.06, 0.2
NBS sample from late onset form (N=4)	0.30 (case 1), 0.44 (case 2), 1.32 (case 3), 2.96 (case 4)	0.06 (case 1), 0.06 (case 2), 0.16 (case 3), 0.50 (case 4)

For patient sample evaluation, cutoff values derived from the prospective cohort (ORA: 2.63 nmol/mL; ORA/Cit: 0.32). The normal control values are presented as mean±SD (standard deviation). Abnormal values exceeding the respective cutoffs are underlined.

OTCD using MS/MS without any derivatization steps. The quantification of amino acids and acylcarnitine in the NBS has been performed in the positive ESI mode, but this method allows the quantification of ORA by analyzing it separately in negative ESI mode[8]. Previously, detecting acidic compounds like ORA in the same run as basic amino acids was challenging because older MS/MS instruments could not rapidly switch polarities[9]. However, modern MS/MS systems have improved substantially and can alternate between positive and negative ion modes within a single injection[9]. This capability allows ORA assay to be incorporated into routine NBS assay at many NBS laboratories with minimal changes to the existing workflow.

Our method showed excellent linearity between 0.5 and 100.0 nmol/mL with a detection limit of less than 1.0 nmol/mL. The only modification to the routine NBS protocol was the addition of a labeled ORA internal standard to the extraction solution. No extra extraction or derivatization steps were needed, and the sample preparation remained simple. The cost implications are negligible—the labeled ORA standard adds a very small expense, and there are no additional consumables or instruments required. In addition, our method allows us to perform analysis without increasing the analysis time. This ORA assay is straightforward to implement in existing NBS laboratories: it does not appreciably increase labor, time, or cost, making it a practical addition to current screening markers.

Through the retrospective analysis of over 4,605 normal newborn samples, we established preliminary reference ranges for blood ORA and the ORA/Cit ratio in healthy neonates. Both distributions were approximately normal (bell-shaped), which allowed us to use a high percentile (99.5th) as a cutoff for defining abnormal results. We did observe that the stored DBS (up to 2 years old at −30°C) had slightly higher ORA and ORA/Cit values on average than the fresh samples from the prospective pilot. The exact reason for this difference is unclear. All measurements were done with the same instrument and protocol, so instrument drift is an unlikely factor. One possible explanation is that prolonged storage might cause a mild increase in measured ORA[12,13]. Strnadová et al. reported that citrulline concentrations decreased by approximately 18.5% per year in DBS stored under freezer conditions, and other amino acids likewise declined over several years[14]. Similarly, Dijkstra et al. reported that 19 out of 22 amino acids, including citrulline, significantly degraded in neonatal DBS stored at +4°C and ambient temperature over up to five years[11]. Taken together, gradual loss of citrulline over time may artificially elevate the ORA/Cit ratio, even if ORA remains stable or changes minimally. Although ORA-specific degradation data are limited, similar physicochemical mechanisms could explain the small increase in ORA units observed in older samples.

The results of retrospective analyses of DBS from OTCD patients revealed that the present method can detect biochemical abnormalities such as increased ORA and ORA/Cit ratio in the acute phase in both neonatal-onset and late-onset forms. On the other hand, the analysis of stable condition samples indicates that there are instances where ORA is not increased even in the neonatal-onset form, which is consistent with the fact that urinary ORA excretion is absent in well-controlled OTCD patients. Analysis of neonatal DBS in patients with late-onset OTCD showed that some patients could be identified by screening before their onset. The patient (Case 4) who was positive in the neonatal DBS analysis had subsequently developed severe hyperammonemia. Such patients represent a potential group that might benefit from NBS by this method. No biochemical abnormality was found in the other three patients. The results may have been influenced by their having received certain interventions at the time of blood collection because they were family cases, or by the fact that they were female. It is important to be aware that screening for OTCD using this method cannot identify all patients with mild forms. Lee et al. reported that pre-symptomatic blood citrulline concentrations measured by MS/MS-NBS were significantly lower in late-onset OTCD[15]. Further large prospective studies are needed to determine the severity spectrum of patients that can be detected in the neonatal period by screening with ORA and citrulline. In the present study, we were unable to evaluate screening performance using citrulline alone. This was because validation of citrulline measurements at low concentrations had not yet been performed in present study, making re-analysis of samples with low citrulline values impractical. Additional data including citrulline concentrations for both retrospective and prospective pilot studies are provided in the Supplementary Data (Table S1). With the advancement of high sensitivity mass spectrometers, low concentrations of citrulline measurement have become feasible. In future work, we plan to investigate a combined screening approach incorporating both low citrulline concentrations and elevated ORA levels.

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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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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

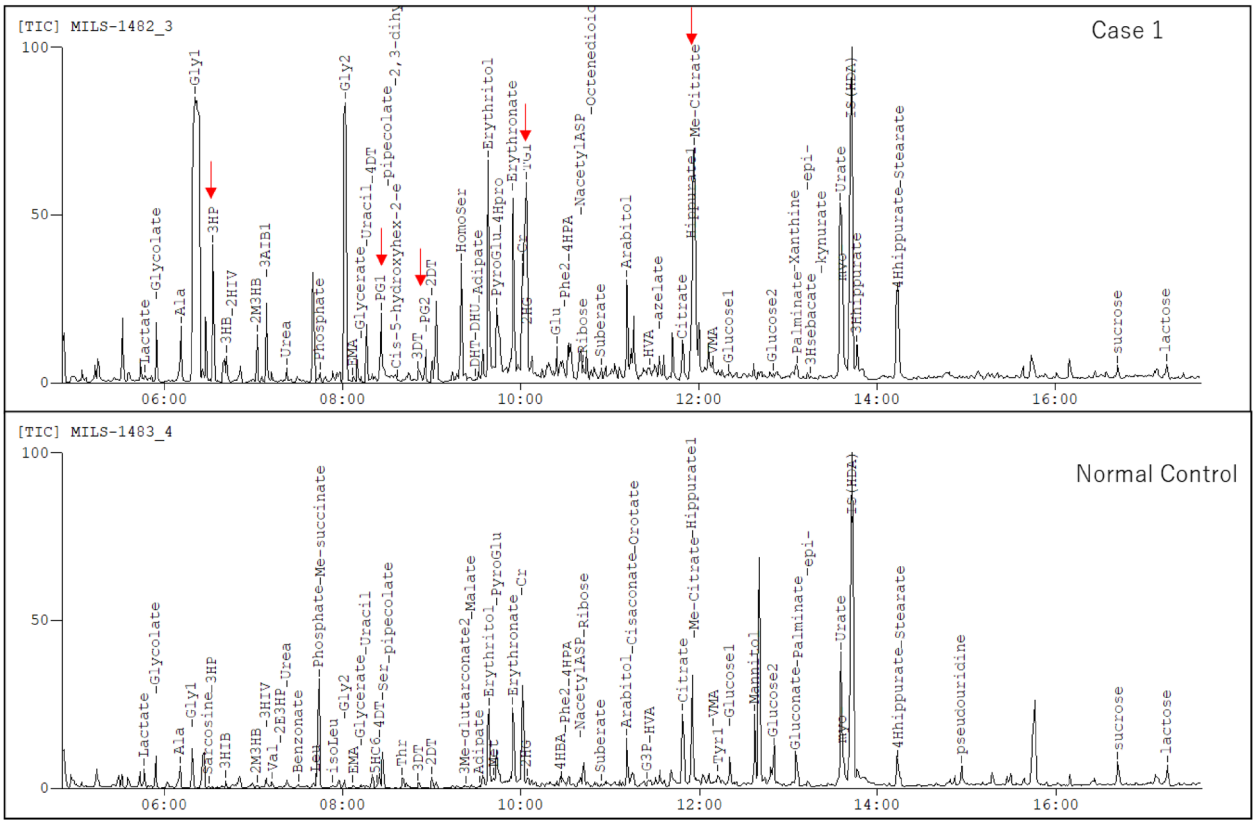

Fig. 1. Case 1 (2 years 5 months, male) GCMS data analysis result.
Upper half showing the TIC chromatogram of urinary metabolites from a patient with Propionic acidemia (PA) & lower half showing the normal control.
Biomakers of Propionic acidemia are marked with arrows-3HP: 3-hydroxyprpionic acid, PG1: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

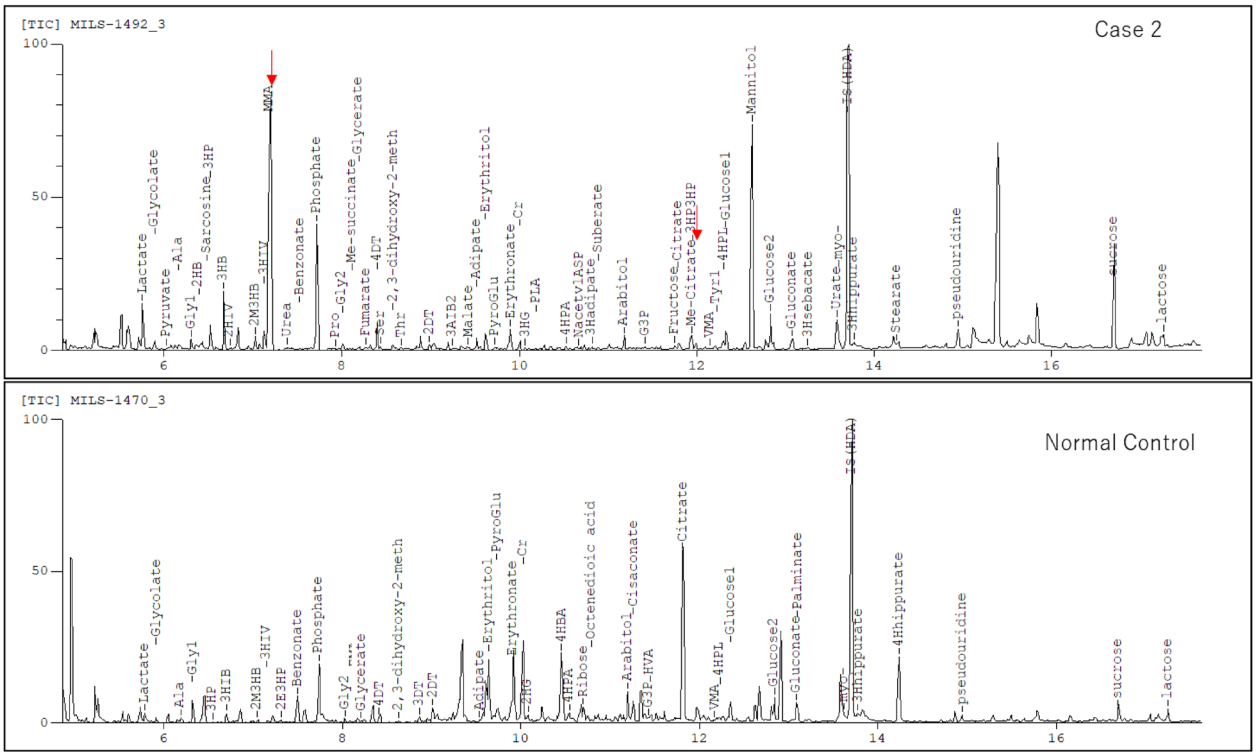

Fig. 2. Case 2 (5 years, male) GCMS data analysis result.
Upper half showing the TIC chromatogram of urinary metabolites from a patient with Methylmalonic acidemia (MMA) & lower half showing the normal control TIC chromatogram.
Biomakers of Methylmalonic acidemia were marked with arrows. MMA: methylmalonic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

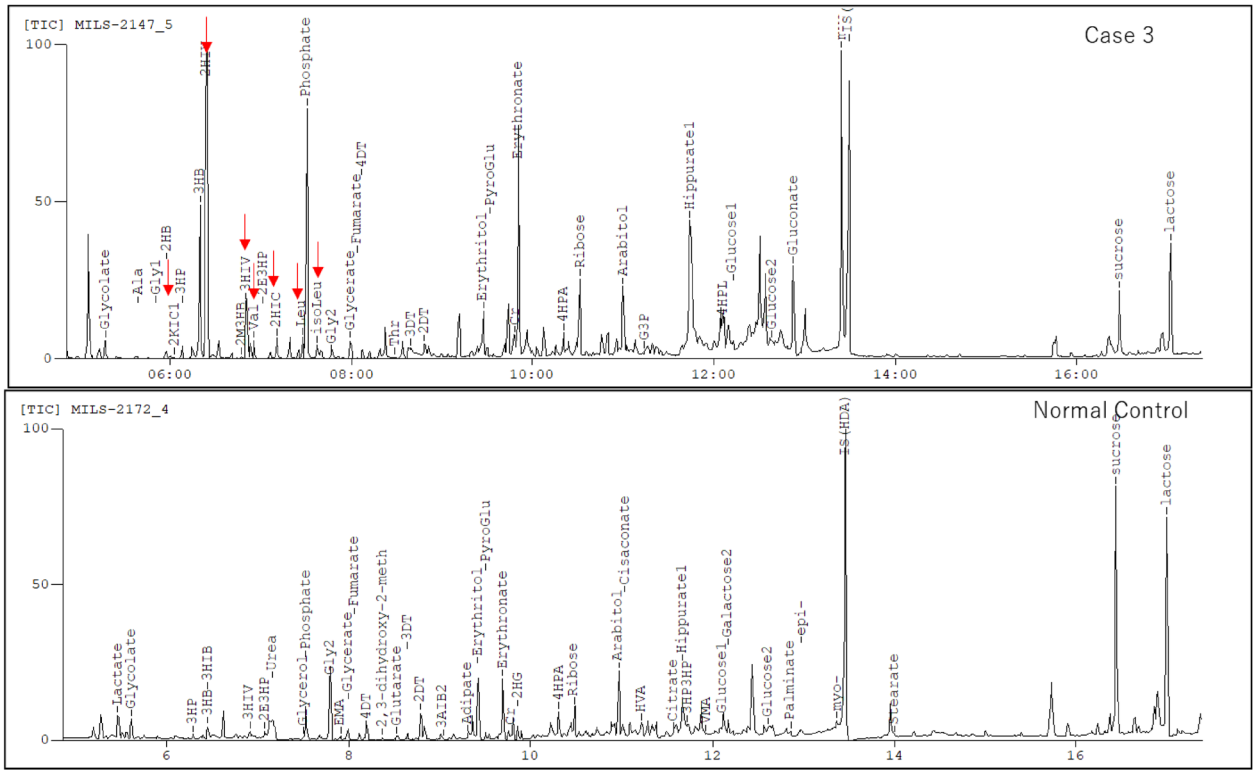

Fig. 3. Case 3 (1 year, female) GCMS data analysis result.
Upper half showing the TIC chromatogram of urinary metabolites from a patient with Maple Syrup Urine Disease (MSUD) & lower half showing the normal control TIC chromatogram.
Biomakers of MSUD were marked with arrows. 2KIC: 2-ketoisocaproic acid,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

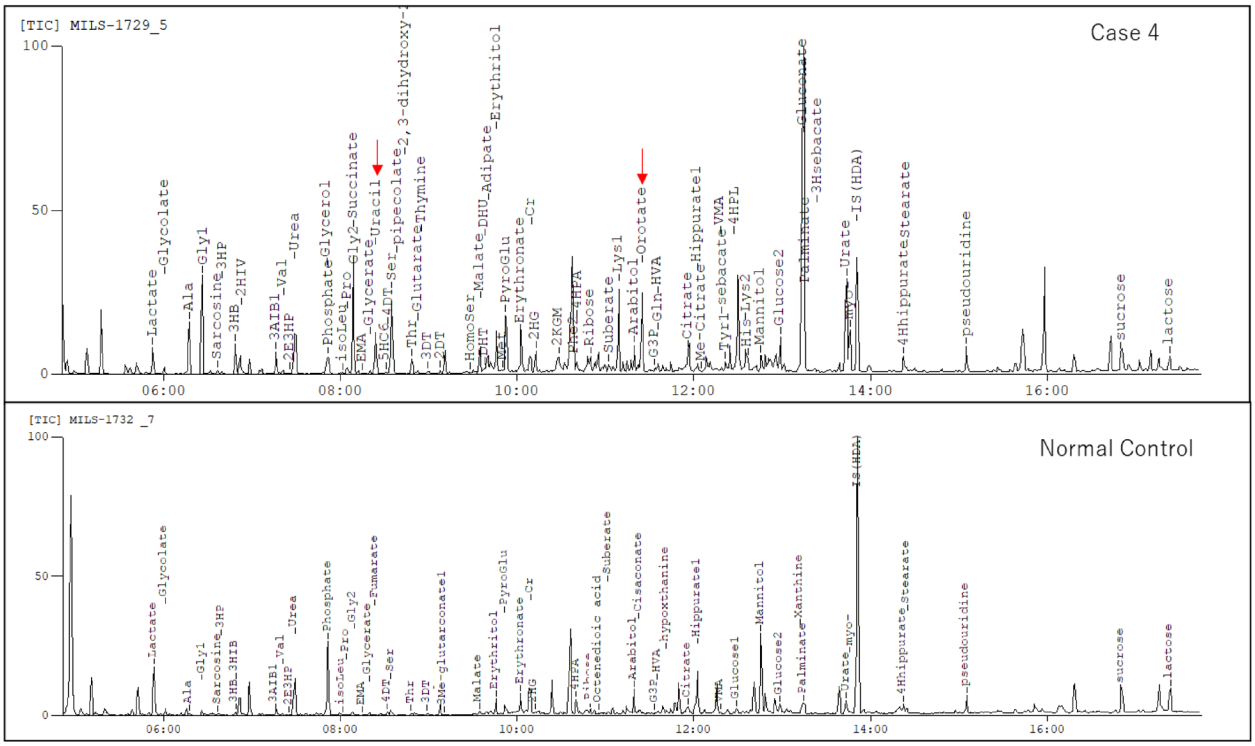

Fig. 4. Case 4 (3 years 6 months, female) GCMS data analysis result.
Upper half showing the TIC chromatogram of urinary metabolites from a patient suspected as Urea Cycle Disorder (UCD), lower half showing the normal control TIC chromatogram.
Biomakers of urea cycle disorder were markerd with arrows.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

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Conflict of Interest

None.

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Research Paper

Newborn screening for mucopolysaccharidoses: Measurement of GAGs by automated high-throughput mass spectrometry/RapidFire

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Abstract Newborn screening (NBS) is recognized internationally as an essential, preventive public health program for the early identification of disorders in newborns that can affect their long-term health. Early detection, diagnosis, and treatment of certain genetic, metabolic, or infectious congenital disorders can lead to significant reductions in disease severity, associated disabilities, and death. Presently, NBS programs using MS/MS screening procedures screen more than 50 disorders at birth, and the number will continue to increase. The present article focuses on newborn screening of mucopolysaccharidoses (MPS) using a RapidFire high-throughput tandem mass spectrometry system (HT-MS/MS). MPS are a group of lysosomal storage disorders caused by a deficiency of enzymes that catalyze the degradation of glycosaminoglycans (GAG). Early detection, diagnosis, and treatment of MPS can significantly reduce disease severity, associated disabilities, and death. We hypothesized that HT-MS/MS can detect most MPS in a single determination covering broad screening purposes. Dried blood spots (DBS) were collected from patients with different types of MPS diseases with a broad spectrum of clinical severity, and age-matched control samples were analyzed to determine cut-off values. The test analyzed heparan sulfate (Di HS-0S and Di HS-NS) and keratan sulfate (mono-sulfated KS and di-sulfated KS) to screen MPS type I, II, III, and IVA. Additionally, 5000 de-identified newborn DBS were assessed for validation. We established a novel HT-MS/MS MPS screening method, demonstrating its usefulness in newborn screening and monitoring of MPS diseases. However, based on the limitations of HT-MS/MS, the cut-off values in this HT-MS/MS screening require further validation, not only in terms of false positives but also in terms of false negatives. The measurements of all the GAGs combined with MS/MS may lead to the highly specific and sensitive detection of most MPS.

Key words: newborn screening, MPS, MS/MS, high-throughput tandem mass spectrometry

1. Introduction

Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders caused by a deficiency of enzymes catalyzing the degradation of glycosaminoglycans (GAGs) with an incidence of 1 in 33,000 live births[1)]. MPS are autosomal recessive disorders except for MPS II, which is X-linked. GAGs are sulfated polysaccharides comprising repeating disaccharides, uronic acid (or galactose) and hexosamines, including chondroitin sulfate (CS), dermatan

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sulfate (DS), heparan sulfate (HS), and keratan sulfate (KS). Hyaluronan is an exception in the GAG family because it is a non-sulfated polysaccharide. Lysosomal enzymes are crucial for the stepwise degradation of GAGs, which is necessary for the normal function of tissues and the extracellular matrix (ECM). The deficiency of one or more lysosomal enzyme(s) results in the accumulation of undegraded GAGs, causing cells, tissue, and organ dysfunction. Accumulated GAGs of various tissues and their ECM are secreted into the blood circulation and then excreted in the urine. Prenatal lysosomal GAG storage has been demonstrated in patients with MPS and animal models. Initial clinical signs and symptoms in newborn patients with MPSs include sacral dimple, gibbus, and abnormal vertebral shape in X-ray images[2]. Skeletal abnormalities represent the earliest clinical observations in MPS VII mice. MPS can be detected in the amniotic fluid of the fetus (MPS I, II, III, IVA, and VII)[3–5] and placenta (MPS II and VI)[6] by measuring GAG accumulation. Human fetuses of MPS I, II, III, and IV have storage vacuoles in major organs as early as 18–30 weeks gestation[3,4]. Newborn mice with MPS I, II, IVA, or VII also have storage vacuoles. Histological analysis of the growth plate, articular cartilage, and cortical bone showed early pathology and progressive bone lesions[7]. Earlier reports indicated that accumulation of GAGs has already started before birth and that therapy should start at a newborn stage to prevent irreversible damage, especially in bone and brain[8–10]. A flowchart of the diagnosis for asymptomatic or post-symptomatic MPS is shown in Fig. 1.

Newborn screening has been extensively applied to lysosomal storage disorders (LSDs). In 2016, two LSDs (Pompe and MPS I) were added to the Recommended Uniform Screening Program (RUSP) by the Advisory Committee on Heritable Disorders in Newborns and Children in the United States[11]. In 2022, MPS II was added to RUSP in the United States[12]. Currently, 64% of the states in the USA universally screen for Pompe disease, 60% screen for MPS I, 18% screen for Krabbe disease, 12% screen for Fabry disease, 10% screen for Gaucher disease, and 4% screen for

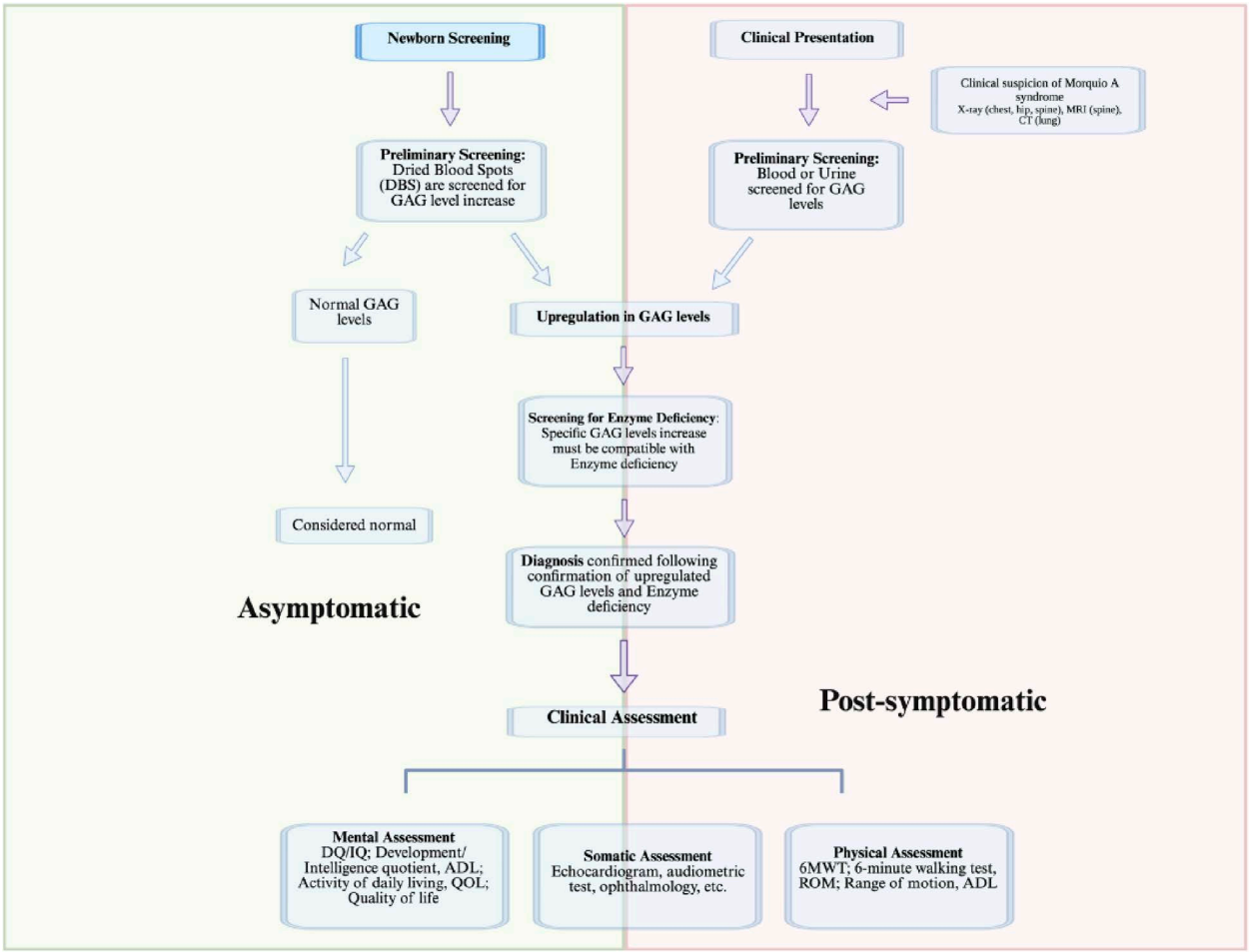

Fig. 1. Flow chart of asymptomatic and post-symptomatic MPS patients.

MPS II and acid sphingomyelinase deficiency (ASMD)[13]. Several countries are also universally screening or running pilot studies for LSDs, such as Austria[14], Belgium[15], China[16], Denmark[17], Hungary[18], Italy[19,20], Japan[21–23], Mexico[24], Taiwan[25–27], and the Netherlands[28,29]. Newborn screening for MPS disorders typically involves first-tier measurement of the activity of the relevant lysosomal enzyme, which can lead to false positives due to pseudo deficiencies[30]. DNA sequencing analysis can also be inconclusive due to unknown or partially penetrant pathogenic variants[30]. However, GAG analysis in DBS is more potent than DNA sequencing in reducing false positives[30]. This suggests that newborn screening for MPS could be carried out by first-tier GAG analysis followed by second-tier enzymatic activity assays. Kubaski et al. and Stapleton et al. have demonstrated the potential of a two-tier study for diagnosing MPS patients using traditional MS/MS[31,32]. Mass spectrometry is a sensitive, specific, and accurate method widely used for measuring all species of GAGs in a biological system, including blood, urine, DBS, and tissue for prognosis, diagnosis, monitoring, and screening purposes[33]. Due to its wide range of applications, MS/MS is considered the most suitable analytical tool for basic or clinical research in the biomedical field and is the method of choice for GAG analysis. However, the MS/MS method requires 5–10 min to analyze each sample; therefore, there is a limit on the number of samples (30,000 to 50,000 samples per year). The schematic presentation of newborn screening is summarized in Fig. 2. We have developed a novel heparan sulfate assay by using a RapidFire automated high-throughput tandem mass spectrometry system (HT-MS/MS) (Agilent Technologies, Inc., Santa Clara, CA) for MPS[8]. The RapidFire HT-MS/MS method requires only 7–10 s per sample compared to the MS/MS method. The GAG assay is performed as the first-tier newborn screening for MPS, and subsequently, an enzyme assay is performed

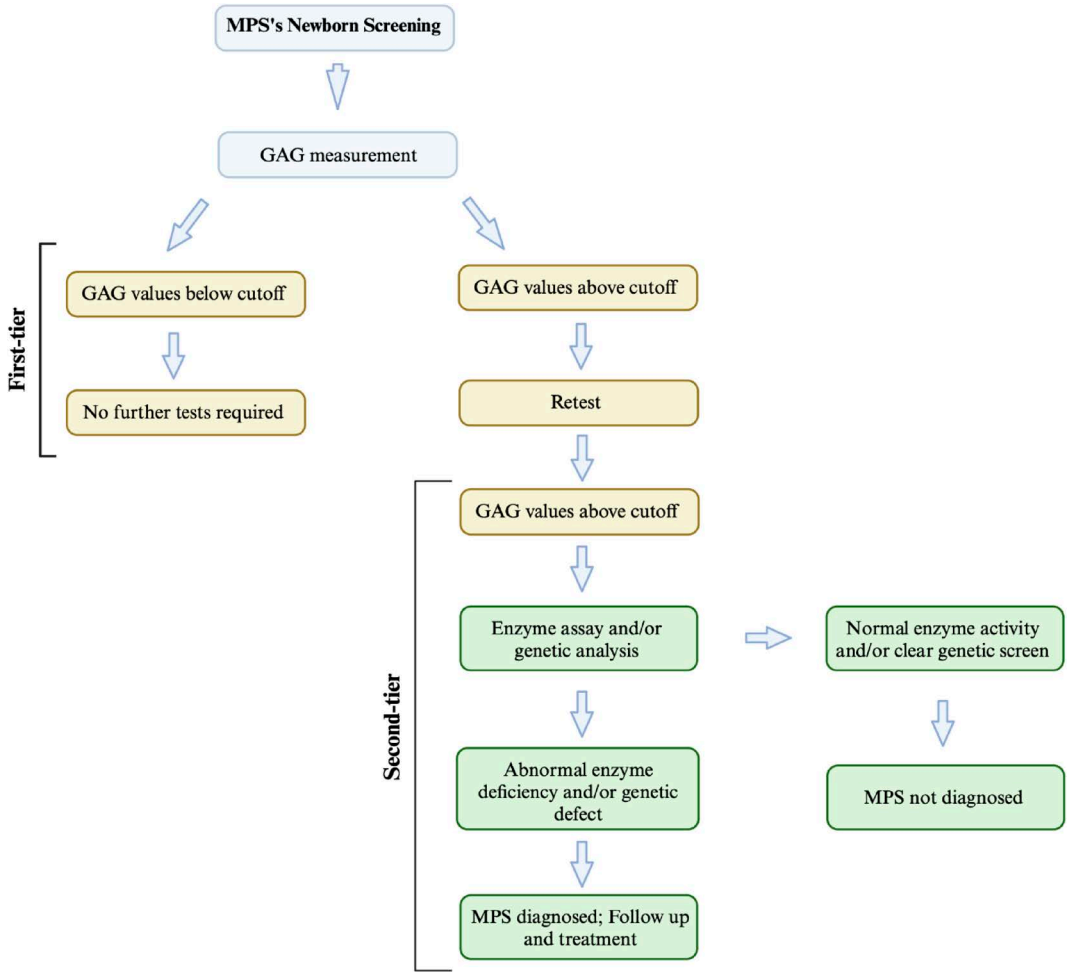

Fig. 2. Schematic presentation of the proposed two-tier newborn DBS screening. The first tier is performed using GAG analysis. When GAG levels are above cut-off values, the second tier is performed using enzyme activity to confirm MPS.

for the second-tier confirmation of the diagnosis. As an alternative approach, the objective of this study was to conduct a pilot study of newborn screening for MPS by measuring GAGs in DBS using the RapidFire HT-MS/MS. We hypothesized that the elevated levels of GAGs are valuable as a first-tier screen to identify a high-risk group with an MPS and that they can be confirmed in a second-tier screen using a specific enzyme or genetic assays.

The present study utilized DBS from different types of MPS and age-matched control samples to determine cut-off values. Additionally, 5,000 de-identified newborn DBS were assessed for validation.

2. Materials and Methods

2.1. Materials

2.1.1. Standards and Enzymes

The study used unsaturated disaccharides [ΔDiHS-NS, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy α-L-threo-hex-4-enopyranosyluronicacid)-D-glucose; ΔDiHS-0S, 2-acetamido-2-deoxy-4-*O*- (4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-D-glucose; mono-sulfated KS [Galβ1−4GlcNAc(6S)] and di-sulfated KS [Gal(6S)β1−4GlcNAc(6S)] obtained from Seikagaku Corporation (Tokyo, Japan) and used to make standard curves. Chondrosine was used as an internal standard (IS). Stock solutions ΔDiHS-NS (1000 ng/mL), ΔDiHS-0S (1000 ng/mL), mono- and di-sulfated KS (10000 ng/ml), and IS (5 mg/mL) were prepared separately in Milli-Q water. Standard working solutions of ΔDiHS-NS, ΔDiHS-0S (7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 ng/mL), and mono- and di-sulfated KS (80, 160, 310, 630, 1250, 2500, 5000, and 10000 ng/mL) each mixed with IS solution (5 μg/mL) were prepared. Samples were digested with heparitinase and keratanase II, resulting in two heparan sulfates (ΔDiHS-NS and ΔDiHS-0S) and two keratan sulfates (mono-sulfated KS and di-sulfated KS). These enzymes were obtained from Seikagaku Corporation (Tokyo, Japan).

2.2.2. Reagents and solvents

Acetonitrile, optima, acetone optima, and ammonium hydroxide optima (all HPLC or MS/MS grade) were from ThermoFisher Scientific, Waltham, MA. AcroPrep™ Advance 96-Well Filter Plates that have Ultrafiltration Omega 10K membrane filters (PALL Corporation, NY, USA), Hypercarb column (2.0 mm i.d., 50 mm, 5-μm; ThermoFisher Scientific, Waltham, MA).

2.2.3. Subjects

The DBS samples from 192 MPS patients and 104 control samples were used. MPS I (15), MPS II (105), MPS III (31), and MPS IVA (41). In addition, 5000 de-identified newborn DBS were obtained from collaborators (Shimane University, Japan). The study was performed under the IRB-approved protocol at Nemours Children's Health.

2.2.4. Sample preparation

Two disks of DBS spots were obtained by DBS puncher (PerkinElmer®, Waltham, MA) into an AcroPrep™ Advance 96-Well Filter Plate. 100 μL of 0.1% BSA solution was added to each well, incubated for 15 min, and centrifuged for 15 min at 14.4 × *g* using 96-well plates. The filtrate solution was discarded. A cocktail mixture of 30 μL with heparitinase, keratanase II (each 1 mU/sample), and IS solution (5 μg/mL) was added, followed by 170 μL of 50 mM Tris-hydrochloric acid buffer (pH 7.0) to each well. The plate was incubated overnight at 37°C, then centrifuged for 15 min at 14.4 × *g*, and the supernatant was collected into 96-well plates. The processed samples were injected into a RapidFire high-throughput system with a 6400 series MS/MS.

2.2.5. Apparatus

The study utilized a RapidFire high-throughput system with a 6400 series MS/MS (Agilent Technologies, Inc., Santa Clara, CA) to analyze a sample of graphitic carbon. The system interfaced with a mass spectrometer, Agilent Jet Stream Technology, and was operated in the negative ion mode. (Drying gas temperature of 350°C, drying gas flow of 11 L/min, nebulizer pressure of 40 PSI, sheath gas temperature of 200°C, sheath gas flow of 11 L/min, capillary voltage of 4000 V, and nozzle voltage of 500 V). The RapidFire microscale solid-phase extraction cartridge D was used to aspirate 10 μL of the sample per well [100 milliseconds (ms)], loaded (2000 ms), and coeluted into MS in a 2000 ms elution cycle with organic phase containing 25% acetone, 25% acetonitrile, and 50% 100 mM ammonia. The aqueous phase was 100 mM ammonia. Chondrosine was used as an internal standard (IS). The ΔDiHS-0S, ΔDiHS-NS, mono-sulfated KS, di-sulfated KS, and internal standard were measured using a 6460 Triple Quad MS/MS with QQQ quantitative analysis software (Agilent Technologies, Inc., Santa Clara, CA) in negative ESI mode following multiple-reaction monitoring (MRM). Specific precursor and

product ions, *m/z* 378.2, 174.3; 416.0, 137.7; 462, 97; 542, 462, and 354.3, 193.1, respectively, were used to quantify each disaccharide.

2.2.6. Statistical analysis

The statistical analysis was performed as t-tests (and non-parametric tests) using GraphPad Prism 9.0 (GraphPad, San Diego, CA). All data were expressed as means and standard deviations (SD). The statistical significance of the difference was considered as $p<0.05$.

3. Results

3.1. Analysis of control and MPS samples

Our group has published several articles for newborn screening using a liquid chromatography-tandem mass spectrometer (LC-MS/MS)[9,31,32,34–36]. In the present study, we have analyzed DBS from controls and different MPS patients using the RapidFire high-throughput system. The chromatogram of the standard on RapidFire is shown in Fig. 3.

We have analyzed age-matched control and MPS samples, as shown in Table 1. The MPS samples showed higher GAG levels than the control samples. Among all MPSs, MPS II exhibited the highest GAG levels compared to the other MPSs. However, the ratio of KS is similar in the control group and all MPS groups (Table 1).

Table 1 shows that DiHS-0S and DiHS-NS were significantly higher than the age-matched control (5–9.9 and 15–19.9 years, respectively) in MPS II. In addition, mono-sulfated KS was significantly higher than the age-matched control, which could be due to a secondary elevation as described in the previous studies[37–43]. Rowan et al. showed that HS (but neither CS nor DS) inhibited the activity of the N-acetylgalactosamine-6-sulfate sulfatase (GALNS) enzyme (responsible for degrading KS) in mice, indicating that secondary elevation of KS levels seen in MPS II patients could be caused by inhibition of the GALNS enzyme by the elevated HS[44]. The DiHS-0S, DiHS-NS, and mono-sulfated KS levels were significantly higher in MPS III compared with age-matched controls

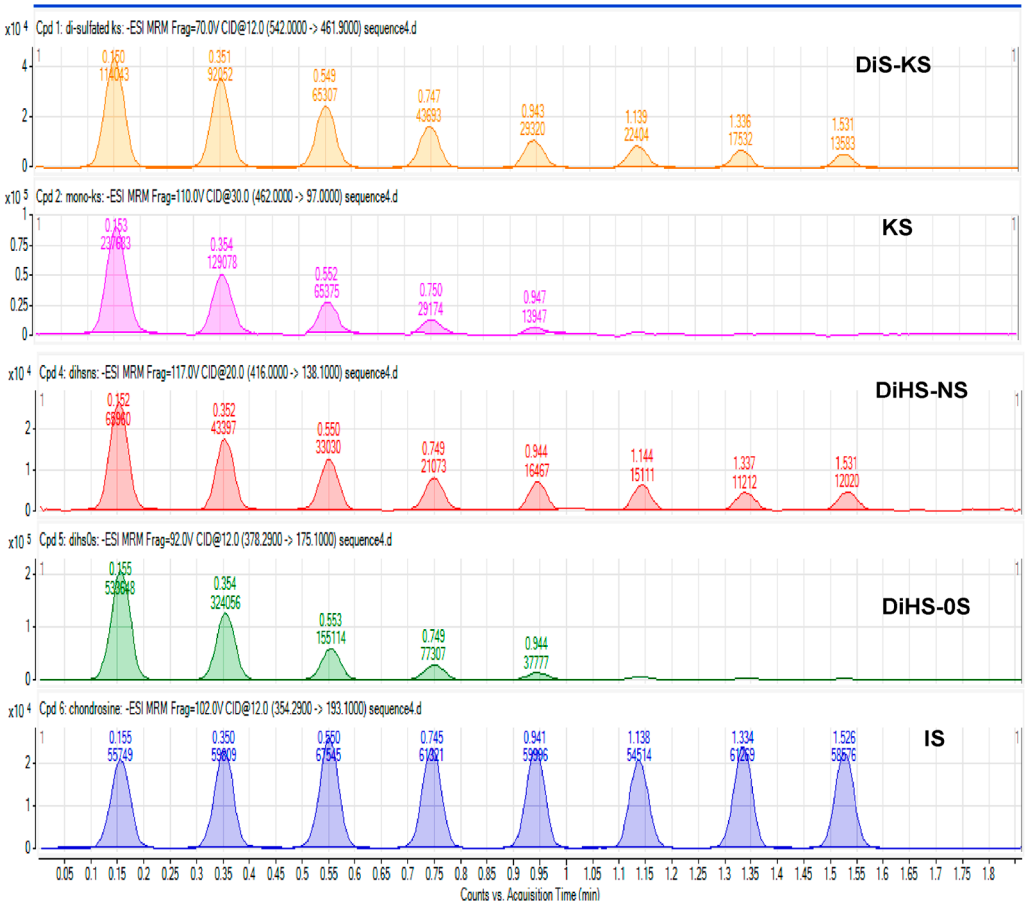

Fig. 3. Chromatogram of standard (serial dilution from high to low). Two keratan sulfates (mono-sulfated and di-sulfated keratan sulfate), two heparan sulfates (DiHS-0S and DiHS-NS), and chondrosine internal standard.

Table 1. Age matched control and MPS (Type 1, II, III and IV) GAG

MPS Type	Age (year)	Number	DiHS-0S	DiHS-NS	Mono-sulfated KS	Di-sulfated KS	Di-sulfated KS/total KS
MPS I	0−2.9	4	186±88	349±161	1015±339	486±226	0.31±0.05
	3−4.9	3	258±130	266±83	1075±375	362±186	0.24±0.07
	5−9.9	2	110−368	147−175	541−689	86−229	0.14−0.25
	10−14.9	2	80−93	214−375	369−672	103−130	0.16−0.22
	15−19.9	N/A	N/A	N/A	N/A	N/A	N/A
	20 and above	4	129±29	264±214	1140±502*	291±317*	0.16±0.14
MPS II	0−2.9	12	276±142	206±83	746±189	236±94	0.24±0.09
	3−4.9	14	174±94	292±129	1056±579	394±224*	0.29±0.16
	5−9.9	23	212±129**	244±124	1172±787**	404±283**	0.26±0.09
	10−14.9	29	170±75	252±110	1198±500***	406±189***	0.26±0.08
	15−19.9	13	164±83	264±124*	1182±649**	425±258*	0.26±0.09
	20 and above	14	187±127	231±130	997±479*	399±100**	0.31±0.09
MPS III	0−2.9	3	351±175	397±105	1063±668	378±386	0.24±0.11
	3−4.9	N/A	N/A	N/A	N/A	N/A	N/A
	5−9.9	6	189±119	192±139	983±512	342±386	0.23±0.12
	10−14.9	8	254±36***	235±137	1061±807	219±170	0.17±0.08
	15−19.9	7	235±125	324±180*	991±1048	198±97	0.23±0.11
	20 and above	7	242±129	307±152	1324±887*	302±195	0.19±0.12
MPS IV	0−2.9	1	114	241	477	349	0.42
	3−4.9	5	119±95	249±235	1190±583	198±141	0.18±0.11
	5−9.9	15	107±77	184±99	631±236	287±257	0.29±0.13
	10−14.9	7	169±153	356±174*	953±483	283±177	0.23±0.11
	15−19.9	6	96±36	237±130	803±416	197±162	0.23±0.15
	20 and above	7	266±230	484±304*	865±484	359±355	0.25±0.14
Control	0−2.9	23	213±109	240±131	726±515	250±219	0.3±0.19
	3−4.9	11	136±51	204±71	687±223	208±122	0.2±0.19
	5−9.9	33	129±71	206±122	599±307	203±167	0.2±0.12
	10−14.9	27	138±85	190±83	552±283	209±153	0.3±0.09
	15−19.9	6	122±49	138±66	427±358	123±91	0.3±0.21
	20 and above	4	149±108	139±64	438±187	156±68	0.3±0.05

All data represents mean±SDEV. *Represent $p<0.05$, **represent $p<0.01$, and ***represent $p<0.0001$ compared to age matched control. Data were analyzed with one way ANOVA; Kruskal−Wallis multiple comparision.

(10−14.9, 15−19.9, and 20 and above years, respectively). In the case of MPS IV, both mono-sulfated and di-sulfated KS were higher than those of the age-matched control, but the difference was not significant. In control, all the GAGs declined with age, as shown in Table 1. Identification of MPS from the control group has been demonstrated in Fig. 4. The heparan sulfate levels (Di HS-0S and Di HS-NS) were significantly higher in MPS II and III, as evident in Fig. 4A and B. Mono-sulfated and di-sulfated KS levels in MPS IV (Fig. 4C and D). KS level is elevated in other types of MPS, as seen in Fig. 4C and D. Several hypotheses have been proposed to explain the elevation of blood KS in patients with different types of MPS, as reported by Tomatsu et al.[37].

3.2. Analysis of Newborn DBS

After controlling for and analyzing the known MPS DBS, we applied 5,000 de-identified newborn DBS samples for GAG analysis using RapidFire. The cut-off values were determined from the control DBS samples of newborns under one year old, as we did not have the known control newborn DBS samples. The cut-off values for four GAGs were set over two standard deviations: 410, 419, 1631, and 527 ng/mL for DiHS-0S, DiHS-NS, mono-sulfated KS, and

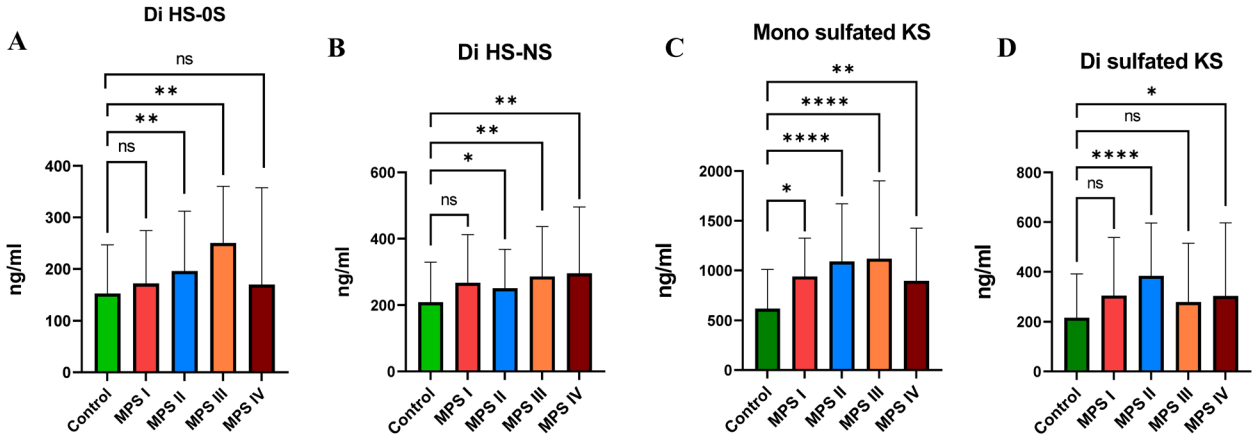

Fig. 4. Di HS-0S (A), Di HS-NS (B), mono-sulfated KS (C), and di-sulfated KS (D) in control, MPS I, II, III, and IV. One-way ANOVA with multiple comparisons. p*＜0.05, **＜0.01, **＜0.0001.**

di-sulfated KS, respectively. We detected 22 samples with at least two GAGs above the cut-off values in Table 2.

Sample number 1 detected all four GAGs significantly higher than the cut-off values. Sample 2−22 detected Di HS-NS and mono-sulfated KS higher than their cut-off values. Di-sulfated KS in some samples was higher than the cut-off value. The two-tier identification was valid for these samples; however, we wanted to repeat the analysis on LC-MS/MS to verify whether these samples were indeed MPS with higher GAGs. All samples with higher GAGs in RapidFire were found to be normal GAG on MS/MS, and their cut-off values were similar to those described earlier, with a few samples (for DiHS-0S and di-sulfated KS) slightly higher than cut-off values[31]. We also tested one known MPS II newborn patient as a positive control by MS/MS and RapidFire, which showed 189 and 360 ng/mL DiHS-0S, respectively. This confirms that the RapidFire value is higher than the MS/MS value. Thus, all 22 samples with higher GAG were false positives. Therefore, since extensive newborn screening by RapidFire will result in some false positives with higher GAGs, we should first confirm these samples on LC-MS/MS and then verify them with enzyme activity.

3.3. Correlation between RapidFire and MS/MS

We wanted to see if there is a correlation between RapidFire and MS/MS. The DBS samples with higher GAG data, as measured by RapidFire, were also analyzed using the MS/MS system, which separates disaccharides through a chromatographic process. We found a statistically significant, strong negative linear relationship for Di HS-NS between the two variables (Figure 5). As one variable increases, the other tends to decrease in value. This relationship accounts for approximately 46.21% of the variance in one variable. However, in the Di HS-0S (Fig. 5B), mono and di-sulfated KS (Fig. 5C and D), there was a nonsignificant weak negative monotonic relationship (Spearman r=−0.3966).

The averages of the remaining newborn samples are shown in Fig. 6. The mean values of DiHS-0S, DiHS-NS, mono-sulfated, and di-sulfated KS were 20, 59, 617, and 196 ng/mL, respectively. Mono- and di-sulfated KS were significantly higher than DiHS-0S and DiHS-NS. Mono-sulfated KS was also significantly higher than di-sulfated KS (Fig. 6).

4. Discussion and Conclusion

Newborn screening is a preventive public health program that enables the early identification of disorders in newborns that can impact their long-term health. The advantage of developing NBS for MPS by measuring elevated GAGs reflects initial screening, which is sensitive and cost-effective for these rare disorders. GAGs are considered biomarkers for MPS. In the case of MPS I and II, DS and HS are mostly elevated. Elevation of HS in MPS III, CS and KS in MPS IV, CS, and DS in MPS VI and VII, hyaluronan in MPS IX, and DS in MPS X have been associated as described by Tomatsu et al.[37]. However, GAGs are also elevated with conditions beyond MPS, such as mucolipidosis[40,45], arthritis[46], diabetes[47], cancer[48], respiratory and renal disorders, fatty acid metabolism disorders, viral infections, vomiting disorders, liver disorders, epilepsy, hypoglycemia, myopathy, developmental disorders, hyperCKemia, heart disease, acidosis, and encephalopathy as

Table 2. Comparision of GAG analysis by MS/MS and RapidFire

Serial Number	Sample ID	DiHS-0S (ng/mL)		DiHS-NS (ng/mL)		Mono-sulfated KS (ng/mL)		Di-sulfated KS (ng/mL)	
		MS/MS	RapidFire	MS/MS	RapidFire	MS/MS	RapidFire	MS/MS	RapidFire
1	K00914	78	696	12	659	196	7074	60	5842
2	K04791	67	12	10	483	141	1931	59	158
3	K04838	66	21	9	496	222	4812	103	1001
4	K04839	66	47	8	462	175	2578	90	562
5	K04842	81	18	10	496	247	2226	72	362
6	K04843	62	25	9	442	177	2035	77	1086
7	K04844	73	34	11	532	151	5300	98	290
8	K04848	77	7	12	483	212	2262	95	147
9	K04849	78	39	9	451	212	2575	76	538
10	K04854	97	46	12	492	158	2584	101	198
11	K04855	82	38	12	434	199	2428	73	255
12	K04861	76	14	11	531	131	2246	63	264
13	K04863	91	27	14	549	136	2707	85	631
14	K04880	105	46	13	430	213	1916	122	391
15	K04913	94	35	10	514	221	2120	63	107
16	K05044	37	33	6	545	34	2701	33	855
17	K05046	86	29	11	431	207	2544	65	83
18	K05069	89	38	13	476	250	2412	100	565
19	K05130	79	38	11	441	296	1749	85	253
20	K05160	78	26	12	423	157	1978	64	94
21	K05175	109	31	17	610	286	1971	84	575
22	K05191	81	51	12	493	212	2072	73	693
NB	MPS II	189	360	31	53	217	228	59	1089

22 newborn DBS samples which had at lest two GAG values higher than cut-off values were run on MS/MS.
The data from two different devices were compared. One known MPS newborn DBS was also compared.
The higher GAG values from RapidFire when run on on MS/MS were found to be normal.

documented by Paige et al.[49], giving false-positives results that require second-tier screen using a specific enzyme or genetic assays. In the present study, we established a novel HT-MS/MS MPS screening demonstrating its usefulness in newborn screening for determining MPS patients. We obtained DBS samples from MPS patients (MPS I: 15, MPS II: 105, MPS III: 31, and MPS IVA: 41) and control samples (n=104). We have analyzed age-matched control and MPS samples (Table 1). The MPS samples showed higher GAG levels than the control samples. Among all MPSs, MPS II exhibits the highest GAG levels compared to the other MPSs. After conducting the control and known MPS DBS analysis, we analyzed 5,000 de-identified newborn DBS samples for GAG analysis using RapidFire. Cut-off values were established, as evident in the overall performance metrics of different disaccharide values and differences in various MPS versus age-matched controls. We detected 22 samples with at least two GAGs above the cut-off values (Table 2). Sample number 1 detected all four GAGs at extremely high levels compared to the cut-off values. Sample 2−22 detected Di HS-NS and mono-sulfated KS higher than their cut-off values. Di-sulfated KS in some samples was higher than the cut-off value. Before the second-tier identification, we analyzed these samples using LC-MS/MS to verify whether they were true MPS with higher GAGs. All samples showed normal GAG values under MS/MS analysis, and their cut-off values were similar to those described earlier[31]. As positive control, we also tested one MPS II newborn patient using MS/MS and RapidFire, which showed 189 and 360 ng/mL DiHS-0S, respectively. This confirms that the RapidFire value is higher than the MS/MS value. Therefore, we decided to check the high GAG values on MS/MS first to ensure these samples are true MPS (the values are still higher than the cut-off values) and then confirm the MPS using enzyme activity or another parameter. The average newborn sam-

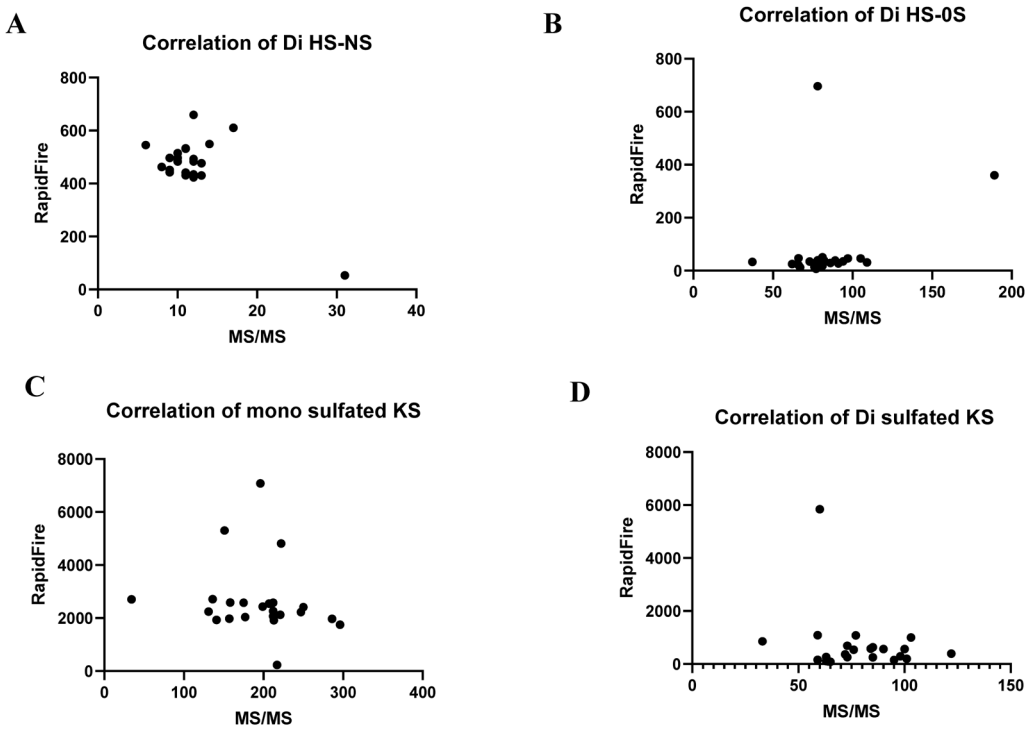

Fig. 5. Correlation between RapidFire and MS/MS for Di HS-NS (A), Di HS-0S (B), mono-sulfated KS (C), and di-sulfated KS (D).

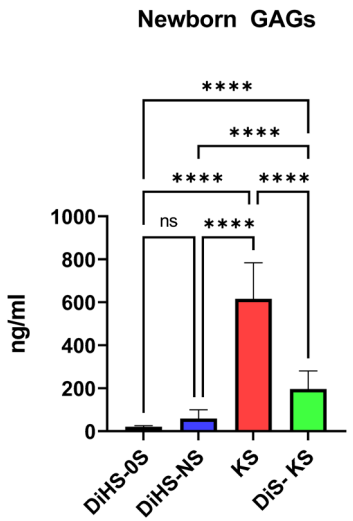

Fig. 6. DiHS-0S, DiHS-NS, mono- and di-sulfated KS (ng/mL) in newborn DBS. Data presented mean± SDEV. Data were analyzed using one-way ANOVA and Tukey's multiple comparisons test. p****< 0.0001

ples had low DiHS-0S (20 ng/mL) and DiHS-NS (59 ng/mL); however, mono-sulfated KS (617 ng/mL) and di-sulfated KS (196 ng/mL) were significantly higher than the HS levels (Fig. 6). In addition, mono-sulfated KS was significantly higher than di-sulfated KS.

Based on known MPS GAGs, newborn screening can detect patients with severe forms of MPS. MPS patients are asymptomatic at birth, and symptoms typically present within the first three years of life and rapidly progress afterward[50–53]. Therefore, therapy should start at a newborn stage to prevent irreversible damage, especially to the bone and brain. Shimada et al. used RapidFire to validate HS in blood from control and MPS patients and demonstrated the correlation of levels of disaccharides derived from HS in blood between MS/MS and HT-MS/MS[8]. We also found a statistically significant, strong negative linear relationship between the two variables DiHS-NS. As one variable increases, the other tends to decrease in value. This relationship accounts for approximately 46.21% of the variance in one variable. However, in the DiHS-0S, mono, and di-sulfated KS, there was a nonsignificant weak negative monotonic relationship (Spearman r=－0.3966). Therefore, we cannot confidently conclude that a monotonic relationship exists between these two variables in the larger population. The observed correlation could reasonably be due to random chances.

In conclusion, elevated levels of GAGs will be valuable as a first-tier screen to identify a high-risk group with an MPS, and they can be confirmed in a second-tier screen using a specific enzyme or genetic assays.

Advantages and Limitations

The advantage of using RapidFire is that it takes 7−10 s per sample to enable screening of more than one million samples per year, compared to MS/MS, which takes 5−10 min per sample and is unsuitable for a large number of screenings. A limitation of this study is that the control samples were obtained from patients who presented to the clinic with conditions other than MPS and whose blood was collected for DBS. Therefore, some control samples may overlap with MPS. Another limitation of RapidFire is that disaccharides with identical molecular weights cannot be distinguished from each other. One of the disadvantages of this study was that we did not have normal newborn DBS values, and cut-off values were determined from control DBS samples of children under one year old.

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