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

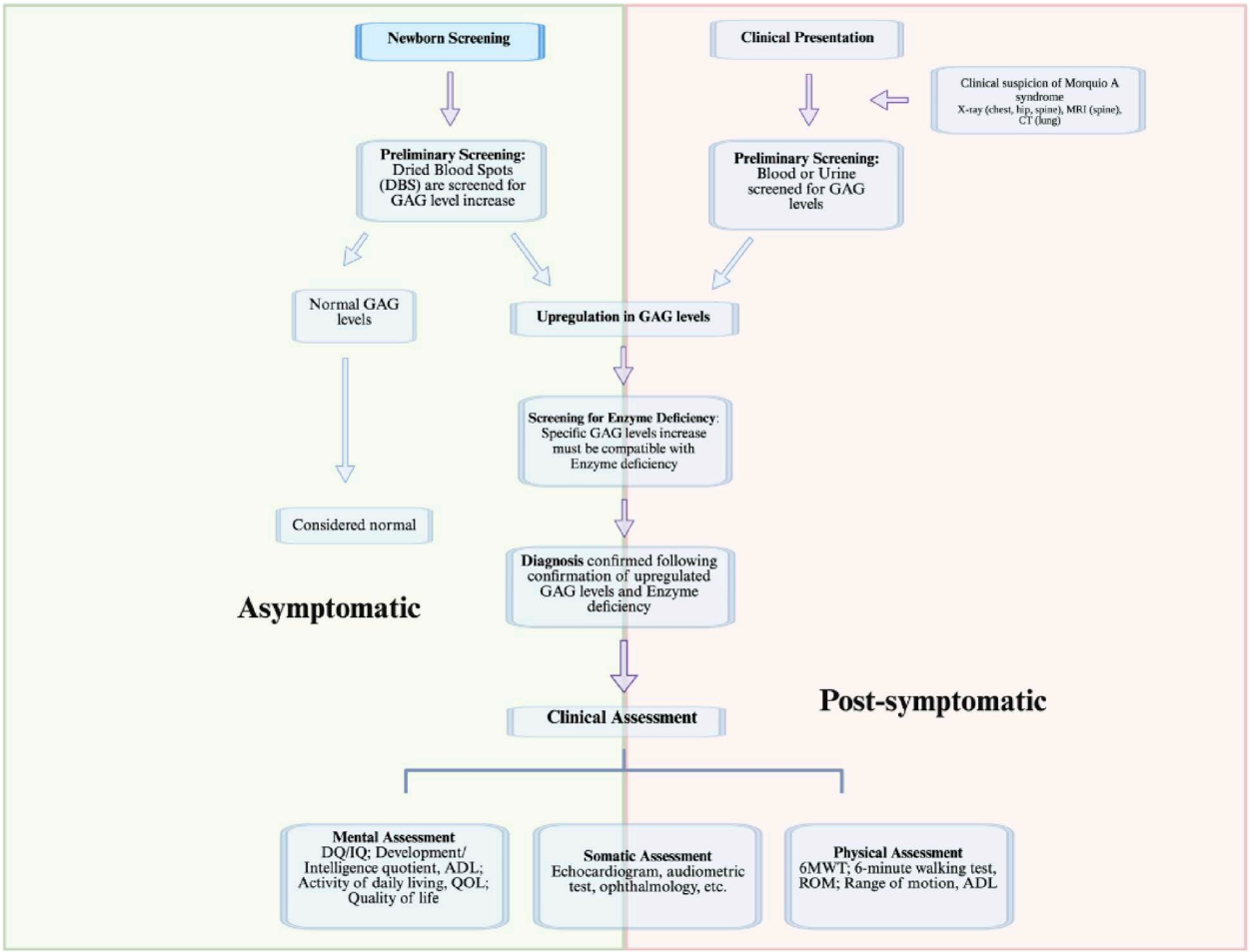

Fig. 1. Flow chart of asymptomatic and post-symptomatic MPS patients.

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

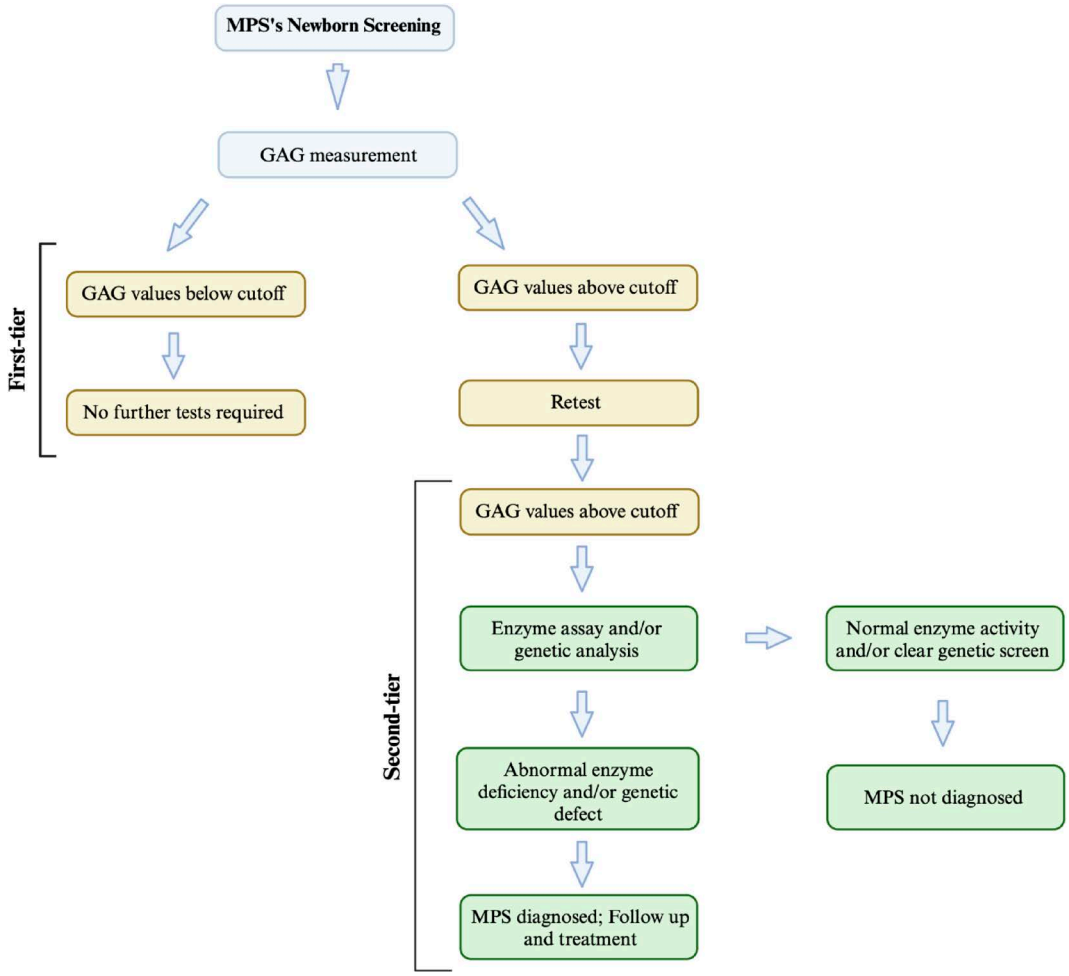

Fig. 2. Schematic presentation of the proposed two-tier newborn DBS screening. The first tier is performed using GAG analysis. When GAG levels are above cut-off values, the second tier is performed using enzyme activity to confirm MPS.

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

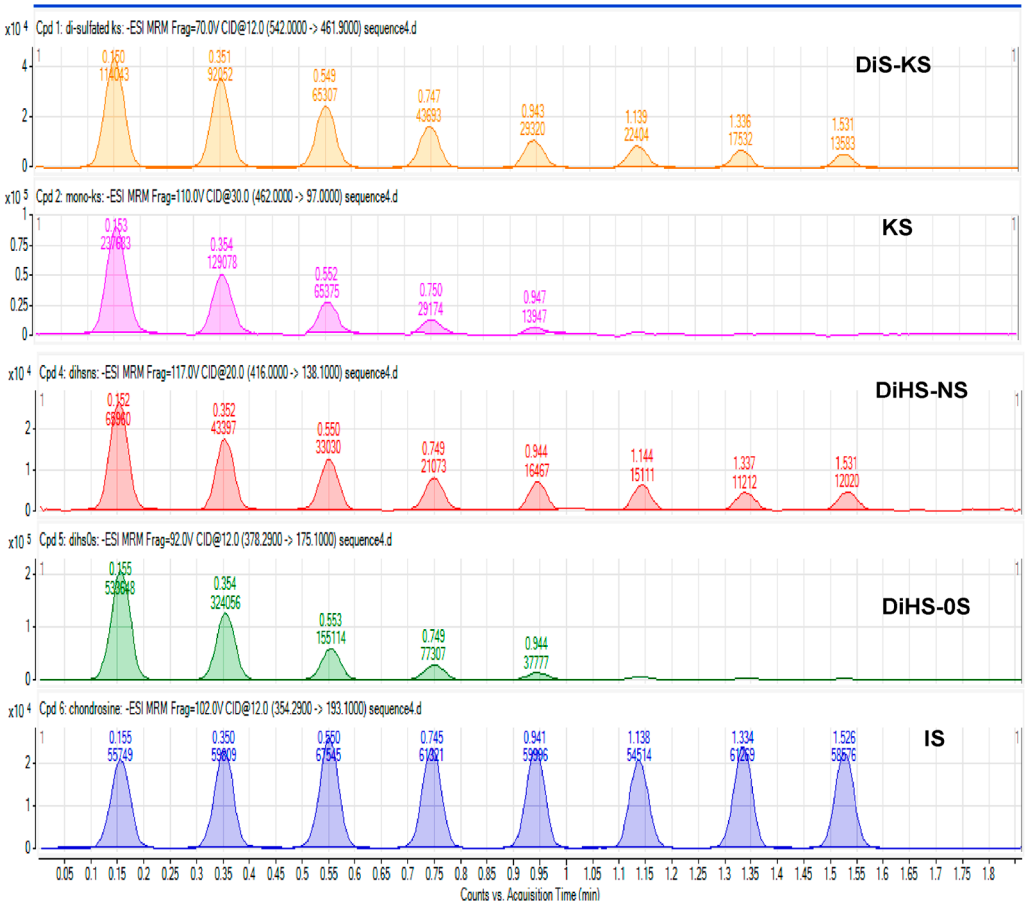

Fig. 3. Chromatogram of standard (serial dilution from high to low). Two keratan sulfates (mono-sulfated and di-sulfated keratan sulfate), two heparan sulfates (DiHS-0S and DiHS-NS), and chondrosine internal standard.

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

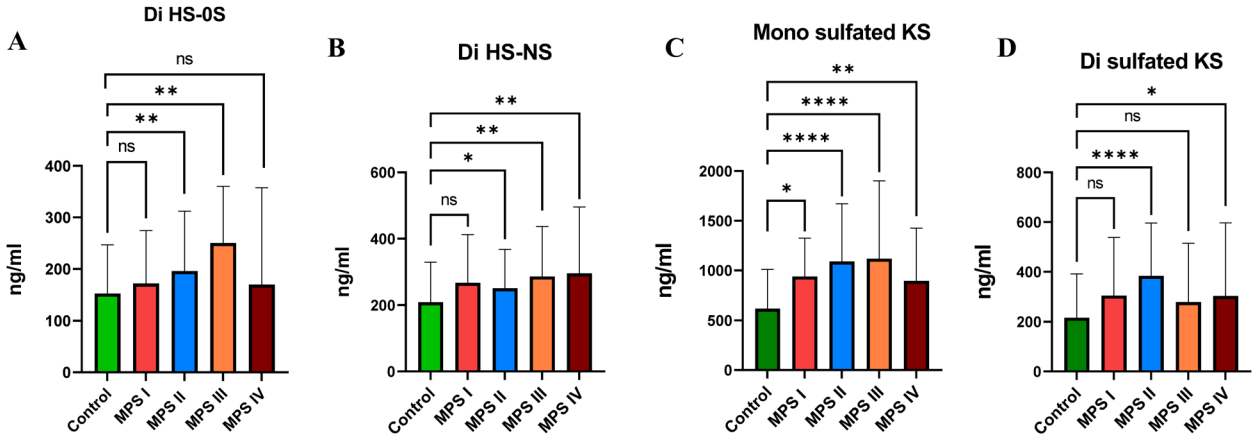

Fig. 4. Di HS-0S (A), Di HS-NS (B), mono-sulfated KS (C), and di-sulfated KS (D) in control, MPS I, II, III, and IV. One-way ANOVA with multiple comparisons. p*＜0.05, **＜0.01, **＜0.0001.**

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-

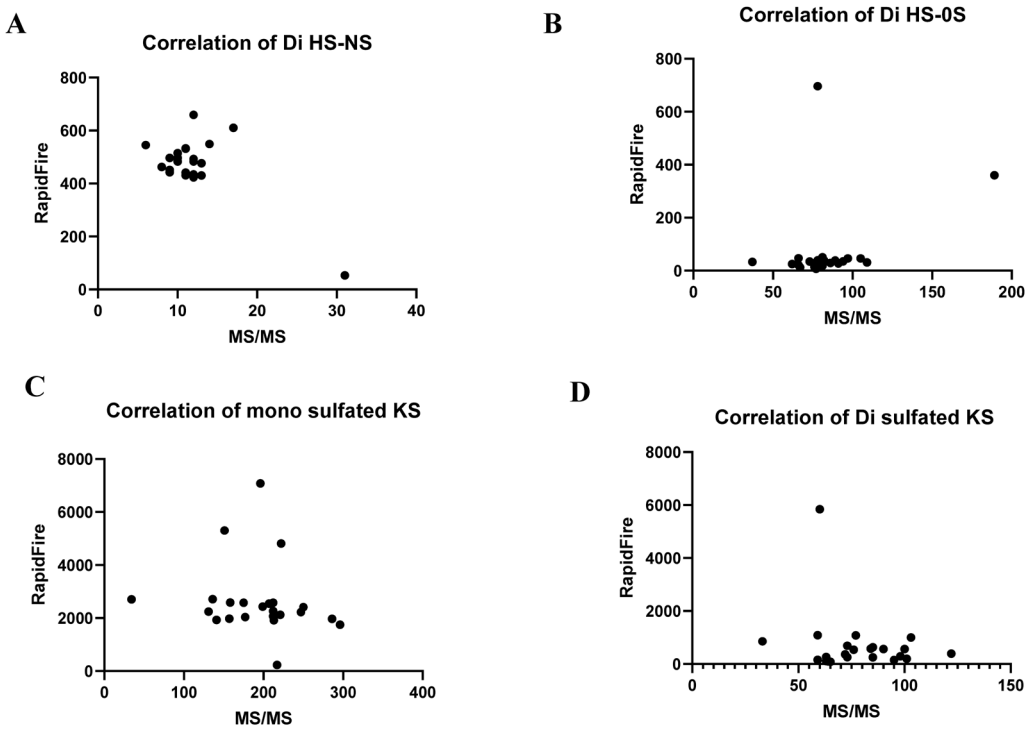

Fig. 5. Correlation between RapidFire and MS/MS for Di HS-NS (A), Di HS-0S (B), mono-sulfated KS (C), and di-sulfated KS (D).

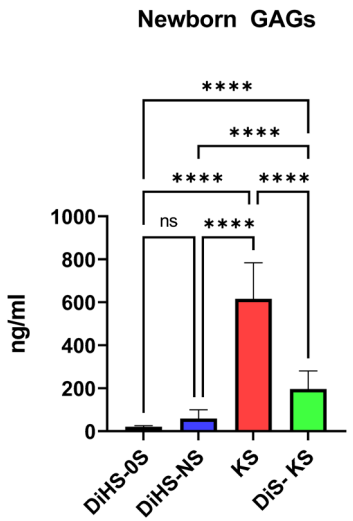

Fig. 6. DiHS-0S, DiHS-NS, mono- and di-sulfated KS (ng/mL) in newborn DBS. Data presented mean± SDEV. Data were analyzed using one-way ANOVA and Tukey's multiple comparisons test. p****< 0.0001

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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