Minireview

Newborn screening and diagnosis of mucopolysaccharidoses

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abstract

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Mucopolysaccharidoses (MPS) are caused by deficiency of lysosomal enzyme activities needed to degrade glycosaminoglycans (GAGs), which are long unbranched polysaccharides consisting of repeating disaccharides. GAGs include: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), and hyaluronan. Their catabolism may be blocked singly or in combination depending on the specific enzyme deficiency. There are 11 known enzyme deficiencies, resulting in seven distinct forms of MPS with a collective incidence of higher than 1 in 25,000 live births. Accumulation of undegraded metabolites in lysosomes gives rise to distinct clinical syndromes. Generally, the clinical conditions progress if untreated, leading to developmental delay, systemic skeletal deformities, and early death. MPS disorders are potentially treatable with enzyme replacement therapy or hematopoietic stem cell transplantation. For maximum benefit of available therapies, early detection and intervention are critical. We recently developed a novel high-throughput multiplex method to assay DS, HS, and KS simultaneously in blood samples by using high performance liquid chromatography/tandem mass spectrometry for MPS. The overall performance metrics of HS and DS values on MPS I, II, and VII patients vs. healthy controls at newborns were as follows using a given set of cut-off values: sensitivity, 100%; specificity, 98.5–99.4%; positive predictive value, 54.5–75%; false positive rate, 0.62–1.54%; and false negative rate, 0%. These findings show that the combined measurements of these three GAGs are sensitive and specific for detecting all types of MPS with acceptable false negative/positive rates. In addition, this method will also be used for monitoring therapeutic efficacy. We review the history of GAG assay and application to diagnosis for MPS.

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ARTICLE INFO

1. Introduction
2. Historical review for GAG assay

ABBREVIATIONS:
CS, chondroitin sulfate; CF, cystic fibrosis; CNS, central nervous system; CPC, cetylpyridinium chloride; DBS, dried blood spot; DMB, dimethylmethylene blue; DS, dermatan sulfate; ERT, enzyme replacement therapy; ESI-MS/MS, electrospray ionization tandem mass spectrometry; GAGs, glycosaminoglycans; GALNS, N-acetylgalactosamine-6-sulfate sulfatase; GUS, β-glucuronidase; HPLC, high performance liquid chromatography; HS, heparan sulfate; HSCT, hematopoietic stem cell transplantation; KS, keratan sulfate; LC-MS/MS, high performance liquid chromatography tandem mass spectrometry; MPS, mucopolysaccharidoses; NBS, newborn screening; SRT, substrate reduction therapy.

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Contents

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of repetitive disaccharide units containing D-glucuronic acid (GlcA) and monitoring therapies. The backbone of the CS chain consists of GAGs, which are long unbranched polysaccharides consisting of repeating disaccharides [1]. GAGs include: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), and hyaluronan. Their catabolism is inhibited singly or in combination depending on the specific enzyme deficiency. Lysosomal accumulation of GAG molecules results in cell, tissue, and organ dysfunction. In MPS, the undegraded or partially degraded GAGs are stored in lysosomes and/or secreted into the blood stream and subsequently excreted in urine. There are 11 known enzyme deficiencies that give rise to seven distinct MPS.

The MPS share many clinical features, although to variable degrees. Most MPS patients are asymptomatic at birth, with subsequent onset of clinical signs and symptoms. These clinical features include a chronic and progressive disease course, multiple organ system involvement, organomegaly, dysostosis multiplex, and abnormal facies. Hearing, vision, and cardiopulmonary functions are affected. Profound neurological impairment is characteristic of MPS I (Hurler syndrome), the severe form of MPS II (Hunter syndrome), and all subtypes of MPS III (Sanfilippo syndrome). MPS IV (Morquio syndrome) and MPS VI (Maroteaux–Lamy syndrome) have characteristic bone lesions without central nervous system (CNS) involvement. There is clinical similarity between different enzyme deficiencies and, conversely, a wide spectrum of clinical severity within any one enzyme deficiency [1]. In all cases, except for a few mild cases, the disease is ultimately fatal with an average life expectancy of one to two decades if untreated. MPS can be found worldwide. Although the overall incidence of MPS is estimated as higher than 1:25,000 live births, the incidence of a particular type of MPS varies (Table 1) [2–8]. Scott et al. (2013) showed that in a pilot study of 110,000 dried blood spot (DBS), the incidence of MPS I is 1 in 35,700 [9].

We estimate that approximately 200 newborns affected with MPS are born annually in the United States. The stepwise degradation of GAGs requires four exoglycosidases, five sulfatases, and one nonhydrolytic transferase. One endoglycosidase also participates in the degradation [10]. Understanding the degradation of GAGs is relevant to establishing new screening methods and monitoring therapies. The backbone of the CS chain consists of repetitive disaccharide units containing β-glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) residues, whereas DS is a stereoisomeric variant of CS with varying proportions of β-iduronic acid (IdoA) in place of GlcA. CS and DS are involved in cell adhesion and neuritogenesis [11]. The HS-GAG chains are linear polysaccharides composed of alternating N-acetylated or N-sulfated glucosamine units (N-acetylgalactosamine, GlcNAc, or N-sulfoglucosamine, GlcNS) and uronic acids (GlcA or IdoA). HS is involved in diverse biologic functions including activation of growth factors [11]. The backbone of the KS chain consists of repetitive disaccharide units containing lactosamine, which is composed of galactose (Gal) and GlcNAc residues. KS is involved in cell motility, embryonic implantation, wound healing, corneal transparency, extracellular matrix of cartilage, and neuronal regeneration [12].

Therapies for MPS have been developed experimentally and clinically. These include hematopoietic stem cell transplantation (HSCT), enzyme replacement therapy (ERT), gene therapy, and substrate reduction therapy (SRT), all of which lead to the partial restoration of the enzyme activity or inhibition of GAG synthesis. HSCT is not entirely effective and has a relatively high mortality rate, primarily from graft versus host disease [13,14]. Treating MPS with ERT relies on the cellular uptake of the enzyme by receptor-mediated endocytosis. ERT has been quite successful in animal models, leading to application in human patients [15–19]. ERT is FDA approved for use in patients with MPS I [20], MPS II [21,22], and MPS VI [23–26]. Patients treated with ERT demonstrate clinical improvement of somatic manifestations and quality of life. Trials for MPS IVA and other types of MPS are under way. While ERT holds much promise for the treatment of MPS, current experience with MPS animal models indicates that it is unlikely that therapeutic amounts of enzyme: 1) cross the blood brain barrier to correct the CNS pathology and 2) reach the bone cells to correct the skeletal pathology. Experimental gene therapies and SRT are under way in vivo [27–29].

### Table 1

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Deficient</th>
<th>Incidence</th>
<th>Primary storage material</th>
</tr>
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<tbody>
<tr>
<td>MPS I</td>
<td>α-Iduronidase</td>
<td>1/35,700</td>
<td>DS, HS</td>
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<tr>
<td>MPS II</td>
<td>Iduronate-2-sulfatase</td>
<td>1/53,000–200,000</td>
<td>DS, HS</td>
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<td>Heparan-N-sulfatase</td>
<td>1/5,300–370,000</td>
<td>HS</td>
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<td>α-N-acetylgalactosaminidase</td>
<td>1/7,600–1,428,000</td>
<td>HS, KS</td>
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<td>MPS IIIC</td>
<td>Acetyl-CoA–glucosaminide acetyltransferase</td>
<td>1/7,600–1,428,000</td>
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<td>MPS IIID</td>
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<td>1/207,000–2,000,000</td>
<td>DS, HS</td>
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1. Introduction

Mucopolysaccharidoses (MPS) are caused by deficiency of lysosomal enzyme activities needed to degrade glycosaminoglycans (GAGs), which are long unbranched polysaccharides consisting of repeating disaccharides [1]. GAGs include: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), and hyaluronan. Their catabolism is inhibited singly or in combination depending on the specific enzyme deficiency. Lysosomal accumulation of GAG molecules results in cell, tissue, and organ dysfunction. In MPS, the undegraded or partially degraded GAGs are stored in lysosomes and/or secreted into the blood stream and subsequently excreted in urine. There are 11 known enzyme deficiencies that give rise to seven distinct MPS.

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The most commonly used methods for diagnosis of MPS are dye-spectrometric methods such as dimethylmethylen blue (DMB) [30–32] and alician blue [33–35] on urine samples. The direct DMB method was automated for detection of MPS diseases [32]. However, these methods cannot be applied to blood specimens without prior protease, nuclease, or hyaluronidase digestion and are impractical for screening newborn urine since collection is difficult and storage of large volumes of urine samples is costly. These spectrometric assays are also not adequate for screening: 1) proteins in the blood specimen block binding of the dyes to GAG, 2) the staining dyes are prone to decomposition leading to a low signal to noise ratio, and 3) the dye methods cannot predict MPS subtypes. HPLC is a sensitive and specific method but not appropriate for mass screening since it is time-consuming and expensive [36–40]. Although sandwich ELISA methods to measure specific GAGs such as HS and KS have been developed to detect MPS [41–43], a method to detect DS is not available.

A suitable method for newborn screening (NBS) must be inexpensive and should be performed on DBS from a Guthrie card. Two other potential methods have been proposed [44]. One is an immune-capture method for detecting each deficient lysosomal protein from patients with MPS I, MPS II, MPS IIIA, and MPS VI [45,46], and the other one is a direct method, assaying individual enzyme activities for MPS I, MPS II, MPS IIIB, MPS IVA, MPS VI, and MPS VII patients [47–55]. These approaches, which rely on individual antibodies or enzyme activities for first-tier screening, are still being developed to detect all types of MPS. We have developed multiplex assays for three MPS-associated metabolites that make NBS possible. Since cost-performance is an important key to applicability, highly efficient and inexpensive screening methodologies must be formulated and developed. Otherwise, the cost of screening for each type of MPS would be unacceptable, as the incidence ranges from approximately 1:100,000 births to 1:2,000,000 births. However, screening for MPS as a group with a combined incidence of higher than 1:25,000 births would be comparable with other existing screening programs for genetic disorders such as phenylketonuria (1:14,000) and galactosemia (1:50,000). Therefore, strategies that enable the simultaneous detection of a group of MPS by LC–MS/MS method are feasible for NBS [1]. Most NBS methods measure elevated substrates in organic acidemias, urea cycle disorders, and cystic fibrosis (CF). A two-tier strategy is universally used for CF [59–62].

The method described here for MPS has another advantage in that it can be used to monitor therapeutic effect [63,64] and to predict clinical severity.

The goal of this review is to describe the potential of tandem mass spectrometry assay of GAGs for NBS and diagnosis of MPS.

2. Historical review for GAG assay

2.1. Total urine GAG assay

The measurement of total GAG excretion in urine is widely used as a biomarker for MPS [65–67]. Urine GAG analysis is a useful initial screening test for MPS and may be also helpful for monitoring treatment efficacy. Measurement of total urine GAGs can be performed quantitatively and qualitatively. Both are recommended as part of the evaluation of patients for MPS.

In 1971, Berman et al. proposed a spot test for MPS (termed the Ames MPS paper spot) based on Azure A dye staining of GAGs in urine spotted on paper [68]. In 1985, Huang et al. measured the uronic acid content of the precipitated GAGs from MPS patients and age-matched normal controls by using spectrophotometric analysis of samples treated with cetylpyridinium chloride (CPC) [69]. In healthy individuals, uronic acid/creatinine ratios decreased until age 20 years old and then remained low. Most patients with MPS IV had higher uronic acid/creatinine ratios than age-matched controls. This same group also suggested that the Ames MPS paper spot test was a potential approach to screen MPS patients from a high risk group with suspected clinical features [70].

At the same timing, 1,9-dimethylmethylen blue (DMB) has been used to quantitatively detect GAGs in urine from older children [71,72]. The DMB method also measures the total amount of GAG in urine. In 1989, Whiteley et al. showed that this testing could be used as a potential screen for any MPS disorder [73–75] as the test measures levels of all GAG species on the urine spot. In 1991, JG de Jong et al. proposed that the DMB method is more reliable than the Ames MPS paper spot [76].

In 2000, Orii’s group reported that two MPS II patients were diagnosed by screening urine spot samples from approximately 130,000 six-month-old infants in a Japanese population using the DMB method [77, personal communication from T. Oriii]. This was the first practical demonstration that GAG measurements can be used to screen for MPS. The limitations of the method are 1) some substances that respond to DMB are found in a diaper, leading to around 3% of a false positive result and GAG/creatinine ratio is not stable until 4 days old, suggesting that we can apply this method to the urine samples from the 5th day newborns [77,78].

Other dye-based procedures such as the toluidine blue spot test [79] and the alician blue spot test [80] have been evaluated for qualitative screening. Overall, spot tests suffer from a high incidence of false negative results [80] and cannot be applied to blood spot.

Elevated urinary GAG is normally specific for an MPS disorder; however, many patients have borderline or only slightly elevated urinary GAGs, especially those with MPS IV, resulting in false negatives in a screen [41,42,73,75,81,82]. Total urine GAG levels are also unlikely to reflect the clinical severity or predict the specific clinical signs and symptoms that might arise.

Thus, a traditional method for urine GAG is still used for a screening, when the subject is suspected as a clinically high risk group; however, it is not suitable for a general mass screening of newborns. The lack of adequate sensitivity, specificity, and reliability of these screening tests underscores the need for improved methods of GAG detection for MPS.

2.2. Assay for specific GAGs

Several methods have been described for detecting and monitoring specific GAGs in MPS.

2.2.1. Chromatography

In 1968, Orii described the measurement of urine GAG in normal male children by using CPC fractionation and column chromatography on Dowex 1X2, showing the compositions of each specific GAG in urine [83].

In 1980, Kimura et al. described the chemical structure of DS in the urine of a patient with MPS II through the analysis of disaccharide units. Urine was digested by chondroitinase ABC and the resultant disaccharides were separated on a Dowex 1 column [84]. In 1984, the same group fractionated urinary heparan sulfates (“HS”) from two siblings with MPS IIIB by chromatography on Dowex 1 and Sephadex G-50 columns [85].

In 1985, Hopwood and Elliott used Dowex 1 column separation to show that urine from MPS IIIB, IVA, and VI patients contained elevated levels of sulphated N-acetylated hexosamines compared with urine from normal individuals [86]. In 1995, K Murata et al. digested heterogeneous sulphated HS isomers with specific HS-lyases, heparitinase I, and heparinase, and separated the resultant unsaturated disaccharides (ΔDI-SHS) by HPLC. The pattern of ΔDI-SHS obtained from that urine of a patient with MPS I indicated accumulation of unique urinary HS isomers in this MPS [87].

Enzymatic and chemical analyses of the structures of HS in the urine by patients with MPS III and II revealed that their non-reducing ends differ from each other and reflect the enzyme deficiency of the syndromes [88]. In addition to providing a new tool for the differential diagnosis of the MPS, these results bring new insights into the specificity of the heparitinases from Flavobacterium heparinum.
In 1998, Byers et al. used a combination of anion-exchange chromatography and 30–40% gradient polycrylamide gel electrophoresis (gradient-PAGE) to purify and characterize urinary GAG from various MPS [65]. The urinary GAG from the different MPS displayed distinct patterns on gradient-PAGE, and further confirmation of MPS types and subtypes was demonstrated by an electrophoretic shift in the banding pattern after digestion with the appropriate MPS enzyme. They reported that each MPS disorder accumulates a unique spectrum of GAGs, with a non-reducing terminus that is specific for the deficient enzyme that causes each particular MPS disorder. The absolute correlation of the non-reducing terminal structure with a particular MPS and the availability of recombinant lysosomal enzymes provide the means for a rapid and accurate diagnosis of individual MPS.

Thus, qualitative urine-based testing methods can separate the various GAG species and thereby provide a starting path for differential diagnosis. GAGs are first isolated from the urine and then separated by thin layer chromatography or electrophoresis [69,70,89–92]. A GAG specific stain is used to visualize the GAGs and the relative position on the gel or plate is used to identify GAG types [93]. KS, the specific diagnostic GAG in patients with MPS IVA, I VB, VI, and VII patients and correlate with clinical severity [43]. The results showed that blood and urine HS levels are elevated in MPS I, II, III, and subtypes was demonstrated by an electrophoretic shift in the banding pattern of HS and DS, and KS after digestion, the samples are injected into the LC–MS/MS and results are compared with control samples (Fig. 2).

The LC–MS/MS method not only shows sensitivity and specificity for detecting all subtypes of MPS but also is able to monitor therapeutic efficacy in MPS patients and animal models [15,63,98–103]. This new method has an advantage of being both GAG-specific and quantitative. In 2011, Auray-Blais et al. described that methanolysis can be used to prepare HS and DS for LC–MS/MS analysis from GAGs in urine of MPS patients [104,105]. Methanolysis has not yet been used to measure KS.

In 2012, Lawrence et al. showed that another new method with the enzyme digestion can detect DS and HS by analysis of non-reducing ends of urinary GAGs by LC–MS/MS. Currently, this method does not provide a measure of non-reducing ends for KS [106]. Overall, establishment of the method to measure the disaccharides rather than heterogenous oligosaccharides make it feasible to interpret individual GAG values, leading to rapid and accurate diagnosis, prognosis, and monitoring therapies for MPS.

3. Update of LC–MS/MS method with enzyme digestion

3.1. Equipment

At present, the following LC–MS/MS instruments have been confirmed to provide good resolution for disaccharide analysis by multiple laboratories; Alliance 2795XE HPLC system/Quattro micro tandem quadrupole (Waters Corp, Milford, MA, USA); Ultra performance liquid chromatography (UPLC) Acquity system/Xevo TQ-S (Waters Corp, Milford, MA, USA); HP1100 system (Agilent Technologies, Palo Alto, CA, USA); API–4000 or API–5000 (AB Sciex, Foster City, CA, USA); Acquity HPLC system/Quattro Premier XE (Waters Corp, Milford, MA, USA); and 1260 infinity LC/6460 Triple Quad (Agilent Technologies, Palo Alto, CA, USA) [56–58, 95–98, 101,103–106, personal communication from Dr. K. Kida].

3.2. Standards

\[ \Delta \text{DiHS–6S (HS)}; \Delta \text{DiHS–NS (HS)}; \Delta \text{DiHS–OS (HS)}; \Delta \text{Di–4S (DS)}; \text{mono-sulfated KS} = \text{Galβ1–4GlcNAc(6S)}; \text{di-sulfated KS} = \text{Galβ1–4GlcNAc(6S)}; \text{KS I (bovine cornea) digested with keratanase II} \] to yield \( \text{Galβ1–4GlcNAc(6S)}; \\) \( \Delta \text{Di–4S (DS)} \) have all been used to produce standard curves for each specific GAG (Fig. 3). A common internal standard is chondrosine. The KS I standard is the most difficult to quantify. We assessed efficiency of KS I digestion by keratanase II using gel permeation chromatography. We found that 60% of KS I was digested with keratanase II and that the ratio of \( \text{Galβ1–4GlcNAc(6S)} \) to \( \text{Galβ1–4GlcNAc(6S)} \) was 42:58 (data not shown).

3.3. Disaccharide determination derived from GAGs

3.3.1. Recoveries

Extraction efficiencies of KS-derived and DS/HS-derived disaccharides from control samples are over 87% and almost 100%, respectively. These methods have been applied successfully to extract KS– and DS/HS-derived disaccharides from human serum [56,57].
3.3.2. Chromatography and selectivity

Chromatograms of extracts obtained from a control serum sample consist of Galβ1-4GlcNAc(6S), Gal(6S)β1-4GlcNAc(6S), ΔDiHS-0S, ΔDiHS-NS, and ΔDi-4S. All disaccharides are eluted in less than 5 min (see Figs. 3, 4) [56,57].

3.3.3. Calibration curves

Calibration parameters of disaccharides derived from DS, HS, and KS in human serum were assessed. Calibration curves for Galβ1-4GlcNAc(6S), Gal(6S)β1-4GlcNAc(6S), ΔDiHS-0S, ΔDiHS-NS, and ΔDi-4S obtained on five separate days are linear over the concentration ranges of 0.14 to 7.1 μg/ml, 0.06 to 2.9 μg/ml, 10 to 1000 ng/ml, 5 to 500 ng/ml, and 10 to 1000 ng/ml, respectively. The correlation coefficients of determination (r) are not less than 0.99 [56,57].

3.3.4. Precision

The results of intra- and inter-assay precision for ΔDiHS-0S, ΔDiHS-NS, and ΔDi-4S in control serum are as follows. The disaccharide (ΔDi-4S) is detected by the same ions (m/z 462/97) as those of ΔDiHS-0S. Thus, the total concentration of disaccharides expressed as ΔDi-4S includes both ΔDi-4S and ΔDiHS-0S. The intra-assay precision values/coefficient of variation (CV) determined from analysis of ΔDiHS-0S, ΔDiHS-NS, and ΔDi-4S for control serum are less than 6.7, 7.9, and 15.8%, respectively. The inter-assay precision values/CVs for these disaccharides in control serum are less than 5.2, 6.8, and 14.8%, respectively. The intra-assay precision values/CVs for Galβ1-4GlcNAc(6S) and Gal(6S)β1-4GlcNAc(6S) in control serum were less than 8.2 and 5.3% and inter-assay precision values/CVs were less than 6.6 and 5.7%. These results demonstrate the reproducibility and accuracy of the method [56,57].

3.4. Analysis of plasma and serum samples in MPS patients

Each type of MPS has a different pattern of elevated GAG levels in serum or plasma. We described concentrations of DS-, HS-, and KS-derived disaccharides and their composition ratios in human control and MPS patients [56–58].
Diagnosed MPS patients

Plasma samples were digested by heparitinase and chondroitinase B to produce disaccharides of DS and HS. Digested samples were assayed by LC–MS/MS. For the DS and HS assay, 120 samples from patients (MPS I, 22; MPS II, 27; MPS IIIA, 5; MPS IIIB, 4; MPS IIIC, 2; MPS IVA, 42; MPS VI, 4; MPS VII, 6) (age; 0–66 years) and 112 control samples (age; 0–62 years) were analyzed. The results were as follows.

- All MPS I, II, IIIA, IIIB, IIIC, and VI patients had significant elevations of plasma DS + HS (ave. 3024; 2763; 2610; 2933; 1605; 2700 ng/ml, respectively; min. 829 ng/ml, max. 23,800 ng/ml), compared with controls (ave. 448 ng/ml, min. 107 ng/ml, max. 776 ng/ml, p < 0.0001) (Figs. 4, 5). Specificity and sensitivity are 100%, if the cut-off value is 800 ng/ml between control and MPS samples [58].

- All MPS VI patients have significant elevation of plasma DS (ave. 2225 ng/ml, min. 1100 ng/ml; max. 4400 ng/ml), compared with controls (ave. 253 ng/ml, min. <1 ng/ml; max. 610 ng/ml; p < 0.0001) [58].

- Forty-four blood specimens from MPS IVA patients (ages 2–65 years; mean 14.3 years) were analyzed to confirm that the KS concentration is a suitable marker for early diagnosis and longitudinal assessment of disease severity. Blood specimens were obtained from patients categorized phenotypically as severe (n = 33), and attenuated (n = 11). Plasma samples were digested by keratanase II to produce disaccharides of KS [95,96].

Digested samples were assayed by LC–MS/MS. Plasma KS levels varied with age and clinical severity. Plasma KS levels in healthy controls
Severe attenuation of vehicle control, and monitored DS (weekly infusions of human 3.4.2.1. MPS VII mice treated by ERT.

3.4.2. Monitoring therapies can be applied to screening for MPS I, II, III, IV, VI, and VII patients. Suggest that measurement of DS, HS, and KS levels by LC-MS/MS makes it feasible to assay serum KS in mice [15]. We treated MPS IVA mice with recombinant N-acetylgalactosamine-6-sulfate sulfatase (GALNS) and a sulfatase-modifier factor 1 modified form of GALNS, for 12 weekly infusions. Serum KS levels were significantly reduced by 3 weeks compared with untreated mice and continued to decline over the 12 week duration of treatment. After 12 weeks of infusion, the average levels of serum KS were similar to those of normal mice [15].

3.4.2.2. MPS IVA mouse treated by ERT. Mouse serum KS is not measurable by standard methods due to limited KS synthesis in rodents. The sensitivity of LC-MS/MS makes it feasible to assay serum KS in mice [15]. We treated MPS IVA mice with enzyme tagged with a short peptide consisting of acidic amino acids (D6- or D8-GUS) than the untagged enzyme (GUS). The same trend was seen for serum HS concentrations. The final serum HS in native GUS treated mice was higher than HS levels in tagged-GUS treated mice. Thus, this LC-MS/MS method shows efficacy of treatment with different enzyme formulations confirming the utility of this method for measuring biomarkers associated with MPS [63,107].

3.5. Analysis of DBS samples in newborn controls and MPS patients

3.5.1. Newborn screening of MPS and its significance

NBS is recognized internationally as an essential, preventive public health program for 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. Currently, NBS represents the largest source of samples subjected to biochemical genetic testing worldwide. In the United States, the panel of NBS disorders varies from state to state, and decisions for adding or deleting tests involve many complex social, ethical, and political issues. Usually, disorders selected for NBS are tied to issues such as disorder prevalence, sensitivity, treatment availability, outcome, and overall cost effectiveness. Presently, NBS programs utilizing improvements in MS/MS screening procedures, screen for almost 50 disorders at birth and the number will continue to increase.

In the United States, NBS is performed for nearly 4.2 million neonates every year [108]. Almost 200 of these neonates are likely to be affected with MPS. Early detection of MPS would enable genetic counseling of the parents and early treatment. At present NBS for MPS I has been conducted by the enzyme assay method and three patients were found [9]; however, NBS for other types of MPS has not started yet. Thus, a presumptive diagnosis of most MPS patients is made much later when children present with clinical signs and symptoms. Establishing standard methodologies to screen MPS at an early clinical stage should improve the clinical course significantly. Early detection allows medical intervention, which may ultimately slow progression of the disease and reduce morbidity and mortality [109]. There are several important factors supporting a decision to include MPS into NBS programs:

- Number of patients: Incidence of MPS collectively is higher than 1 out of 25,000 births and annually nearly 200 newborns are expected to be diagnosed with MPS in the United States.
- Clinical course: The onset of severe forms of these diseases occurs prior to one year of age, while the diagnosis is typically delayed.

Serum DS was elevated in untreated mutant mice over levels found in heterozygous control mice. Serum DS concentrations in untreated mice increased with age and reached over 7000 ng/ml by age 7 months. Serum HS concentrations in untreated mutant mice increased with age and reached around 4000 ng/ml by age 7 months. Serum HS in untreated mutant mice was significantly elevated at age 3 months compared with heterozygous control mice that had a relatively constant low level of HS over 7 months.

After 12 weekly infusions, all forms of GUS lowered serum DS and HS levels markedly. The concentration of serum DS was lower in mice treated with enzyme tagged with a short peptide consisting of acidic amino acids (D6- or D8-GUS) than the untagged enzyme (GUS). The same trend was seen for serum HS concentrations. The final serum HS in native GUS treated mice was higher than HS levels in tagged-GUS treated mice. Thus, this LC-MS/MS method shows efficacy of treatment with different enzyme formulations confirming the utility of this method for measuring biomarkers associated with MPS [63,107].

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3.5.3. Analysis of DBS samples in newborn controls and MPS patients

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until between two and four years of age for most types of MPS. Delay in diagnosis may lead to irreversible damage to bone and brain, resulting to early death.

- Availability of therapies: ERT, HSCT, and/or SRT treatments are available for several types of MPS. Clinical and experimental studies on MPS patients and animal models show that early diagnosis and early treatment provide better outcomes for these disorders.

- Natural history: The natural history of most types of MPS is under investigation and our understanding of the clinical features and their progression is evolving. Efficient and accurate NBS will prevent birth defects and will provide the affected infants a better quality of life.

Thus, we propose here a novel NBS system for MPS screened by LC–MS/MS.

To confirm effectiveness of simultaneous DS, HS, and KS assay by LC–MS/MS on DBS, we have analyzed DBS samples from newborn normal controls and newborns with MPS.

3.5.2. Normal healthy newborns

We measured ΔDiHS-0S, ΔDiHS-NS, ΔDi-4S, and KS extracted from DBS of healthy normal newborns. We classified DBS samples into 6 groups by birth weight (<1500 g, 1500–1999 g, 2000–2499 g, 2500–2999 g, 3000–3499 g, >3500 g). Each group was comprised of 10 samples. We did not observe any significant difference in overall GAG composition by birth weight (Fig. 7).

![Fig. 7. Distribution of each GAG level in DBS of normal newborns by birth weight.](image)

Average of ΔDiHS-0S, ΔDiHS-NS, and ΔDi-4S was 347 ng/ml (range: 282–502), 85 ng/ml (range: 57–161), respectively. The average of KS was 1185 ng/ml (range: 684–1511). Yellow: ΔDiHS-0S, green: ΔDiHS-NS, pink: ΔDi-4S, blue line: KS. Equipment; HP1100 system/API-4000.

Table 2
HS and DS levels from dried blood spot in six MPS newborns and controls (ng/ml).

<table>
<thead>
<tr>
<th>Case</th>
<th>ΔDiHS-0S</th>
<th>ΔDiHS-NS</th>
<th>ΔDi-4S</th>
<th>Total HS/DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1 (MPS I)</td>
<td>277</td>
<td>158</td>
<td>1090</td>
<td>1525</td>
</tr>
<tr>
<td>Case 2 (MPS I)</td>
<td>285</td>
<td>100</td>
<td>1200</td>
<td>1665</td>
</tr>
<tr>
<td>Case 3 (MPS I)</td>
<td>280</td>
<td>110</td>
<td>2266</td>
<td>2656</td>
</tr>
<tr>
<td>Case 4 (MPS I)</td>
<td>4503</td>
<td>6700</td>
<td>6722</td>
<td>78425</td>
</tr>
<tr>
<td>Case 5 (MPS VII)</td>
<td>408</td>
<td>406</td>
<td>3417</td>
<td>4231</td>
</tr>
<tr>
<td>Case 6 (MPS II)</td>
<td>240</td>
<td>137</td>
<td>836</td>
<td>1210</td>
</tr>
<tr>
<td>Control (n = 326)</td>
<td>117 ± 23</td>
<td>67 ± 28</td>
<td>370 ± 109</td>
<td>553 ± 146</td>
</tr>
</tbody>
</table>

Table 3
Sensitivity and specificity.

<table>
<thead>
<tr>
<th>Cut off point (ng/spot)</th>
<th>ΔDiHS-0S</th>
<th>ΔDiHS-NS</th>
<th>ΔDi-4S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>98.5</td>
<td>98.5</td>
<td>99.7</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>54.5</td>
<td>54.5</td>
<td>85.7</td>
</tr>
<tr>
<td>False positive rate</td>
<td>1.5</td>
<td>1.5</td>
<td>0.3</td>
</tr>
<tr>
<td>False negative rate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

![Fig. 8. Frequency distribution of A) ΔDiHS-NS, B) ΔDi-4S, and C) ΔDiHS-0S for DBS from 326 controls to 6 MPS cases. Values for 6 cases are marked with arrows. Bars represent controls with frequency.](image)
3.5.3. Newborn MPS

When the elevation of GAGs is first detectable in human MPS patients is unknown. To assess whether our LC–MS/MS method can distinguish MPS newborns from the normal control newborns, we compared DS and HS levels in DBS samples from six neonates that were subsequently determined to have MPS (four MPS I, one MPS II, and one MPS VII) and control newborns (n = 326) in a double blind manner. All six cases (Table 2) had marked elevations of DS and HS (above 1500 ng/ml) compared with the values of normal control newborns (mean, 553 ± 146 ng/ml). DS and HS levels in another DBS sample from a one year-old infant with MPS I (Case 3) remained high since this infant went untreated. This infant was a fraternal twin and DS and HS levels were clearly distinguishable between the unaffected and affected fraternal twin (800 ng/ml vs. 2660 ng/ml).

The overall performance metrics of ΔDiHS-0S (HS), ΔDiHS-NS (HS), and ΔDi-4S (DS) values were as follows using a given set of cut-off values (Table 3, Fig. 8): sensitivity, 100%; specificity, 98.5–99.4%; positive predictive value, 54.5–75%; false positive rate, 0.62–1.54%; and false negative rate, 0% in any of these biomarkers. In 2012, Paracchini et al. reported the results of NBS for CF by assaying the immunoreactive trypsinogen level with the cut off of >99th centile. The results showed that among a total of 717,172 newborns screened, 7354 newborns were found positive to NBS. After the second screening by sweat test, 7234 newborns (98.4%: false positive) were negative or not confirmed.

In conclusion, MS/MS methodology for the analysis of disaccharides of GAG has become popular and should be available in diagnostic and clinical settings soon. With increased demand for MS/MS assays and the wider availability of instrumentation, MS/MS methodologies are likely to have a large impact in screening, diagnosis, and monitoring of MPS in the future.

Acknowledgments

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