Mucopolysaccharidosis type I (MPS I): Assessment of disease severity, therapeutic options and early diagnosis

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Heparan sulfate and dermatan sulfate derived disaccharides are sensitive markers for newborn screening for mucopolysaccharidoses types I, II and III

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ABSTRACT

Introduction Mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders (LSDs) caused by a defect in the degradation of glycosaminoglycans (GAGs). The accumulation of GAGs in MPS patients results in extensive, severe and progressive disease. Disease modifying therapy is available for three of the MPSs and is being developed for the other types. Early initiation of treatment, before the onset of irreversible tissue damage, clearly provides a favorable disease outcome. However, early diagnosis is difficult due to the rarity of these disorders in combination with the wide variety of clinical symptoms. Newborn screening (NBS) is probably the optimal approach, and several screening techniques for different MPSs have been studied. Here we describe a relatively simple and sensitive method to measure levels of dermatan and heparan sulfate derived disaccharides in dried blood spots (DBS) with HPLC-MS/MS, and show that this reliably separates MPS I, II and MPS III newborns from controls and heterozygotes.

Methods Newborn DBS of 11 MPS I, 1 MPS II, and 6 MPS III patients, with phenotypes ranging from severe to relatively attenuated, were collected and levels of dermatan and heparan sulfate derived disaccharides in these DBS were compared with levels in DBS of newborn MPS I and MPS III heterozygotes and controls.

Results The levels of dermatan and heparan sulfate derived disaccharides were clearly elevated in all newborn DBS of MPS I, II and III patients when compared to controls. In contrast, DBS of MPS I and III heterozygotes showed similar disaccharide levels when compared to control DBS.

Conclusions Our study demonstrates that measurement of heparan and dermatan sulfate derived disaccharides in DBS may be suitable for NBS for MPS I, II and MPS III. We hypothesize that this same approach will also detect MPS VI and VII patients, as heparan sulfate and/or dermatan sulfate are also the primary storage products in these disorders.
INTRODUCTION

Diagnosis and treatment in MPS I and III

Mucopolysaccharidoses (MPSs) are a group of rare, invariably progressive and ultimately fatal lysosomal storage diseases (LSDs), caused by a deficiency of one of the enzymes involved in the degradation of glycosaminoglycans (GAGs). As a result, GAGs accumulate in lysosomes, the extracellular matrix and body fluids. Depending on the specific storage products and the extent of the enzyme deficiency, MPS patients may present with a wide variety of signs and symptoms. This includes musculoskeletal, airway and cardiac manifestations of disease predominantly in MPS I, II, IV, VI and VII. In addition, central nervous system disease is observed in the severe, neuronopathic, phenotypes of MPS, including MPS I, II and VII, while central nervous system disease is predominant in MPS III [1]. During the last decades, several disease modifying treatment options for MPSs have been developed. These include hematopoietic stem cell transplantation (HSCT) for the severe form of MPS I (Hurler phenotype) and intravenous enzyme replacement therapy (ERT) for MPS I, II and VI [2-5]. Intravenous ERT is currently being studied for MPS IV and VII, and trials with intrathecal enzyme delivery have been initiated for MPS I (clinical trials.gov, identifiers NCT00638547, NCT00638547 and NCT00852358), MPS III (subtype A) (clinical trials.gov, identifier NCT01155778) and for the neuronopathic phenotype of MPS II (clinical trials.gov, identifier NCT00920647). Other therapeutic options, including small-molecule therapy and gene therapy, are under investigation for several MPSs [6].

Early initiation of treatment, before the onset of irreversible tissue damage, appears to be critical in obtaining optimal outcomes. HSCT for MPS I should preferably be performed before the age of 2.5 years [7] as long-term disease outcome is correlated with the age of transplantation [8]. Several studies have demonstrated that early start of ERT may significantly improve its efficacy [9-12]. Early diagnosis, however, is difficult due to the rarity of these disorders, as well as to the wide variety of clinical symptoms [13]. MPS III is the only MPS with relatively minor somatic disease, and is characterized by a delay in speech development and behavioural problems, followed by progressive cognitive decline and later by loss of motor skills. There are four subtypes of MPS III (types A to D), each distinguished by a specific enzyme deficiency but with identical symptoms. A diagnostic delay is also common in MPS III [14-15].

Newborn screening methods in LSDs

Newborn screening (NBS) is the key to early identification of MPS patients, thus allowing timely initiation of treatment. Several NBS methods for MPSs have been studied. Chamoles et al. [16] demonstrated the feasibility of the analysis of activity of the enzyme alpha-L-iduronidase, deficient in MPS I, in dried blood spots (DBS) using a fluorometric substrate. Fluorometric enzymatic assays in DBS have been described also for MPS II, IIIA, IIIB, IVA, VI
and VII [17-20]. The disadvantage of these fluorometric assays is that each enzyme needs to be tested separately, because of the use of similar analytes as indicators of activity. Meikle et al. [17] and Fuller et al. [21] developed a multiplexed immune-quantification assay for a total of 14 LSDs. The possibility, however, that some patients may have normal protein levels but an inactive form of the enzyme has been recognized as a potential drawback of this approach [22]. Another method to detect activity of multiple lysosomal enzymes in a one-tier analysis is by quantification of the specific product of each enzyme by electrospray ionization (ESI)-MS/MS [23-25]. Following this approach, a screening method to detect four different LSDs (Gaucher, Pompe, Krabbe and Fabry disease) in one assay has been described recently [26].

Heparan sulfate and dermatan sulfate derived disaccharides as NBS method for MPSs

All MPSs are characterized by intra- and extracellular accumulation and subsequent urinary excretion of derivatives of specific GAGs that cannot be degraded. Analysis of levels of undegraded GAGs in urine is generally used for first line diagnostics. In MPS I and II, heparan and dermatan sulfate derived polysaccharides are excreted in urine, while MPS III and MPS VI are characterized by excretion of only heparan sulfate and only dermatan sulfate derived polysaccharides, respectively. In MPS IV, keratan sulfate accumulates and is excreted in the urine, and in patients with MPS VII a combination of dermatan, heparan and chondroitin sulfate can be detected in urine. Tomatsu et al. [27,28] demonstrated that for MPS I, II, III, and VI the characteristic accumulation of GAGs is represented by increased levels of heparan sulfate and dermatan sulfate derived disaccharides in plasma, measured by LC-MS/MS, and they suggested that this technique might be used for NBS for MPS I [27].

In this study, we describe a simple, rapid, sensitive and specific method, using LC-MS/MS, to analyze levels of heparan sulfate and dermatan sulfate derived disaccharides in newborn DBS. We used this assay to analyze newborn DBS of controls, MPS I, MPS II and MPS III patients and MPS I and MPS III carriers, and show that MPS I, MPS II and MPS III newborns can be easily distinguished from controls and carriers.

METHODS

Newborn blood spots

Newborn DBS from control subjects were acquired from the Dutch National Institute for Public Health and the Environment. Newborn DBS from MPS I, MPS II and MPS III patients, whose diagnoses were made at a later age based on clinical diagnoses and confirmed by enzymatic analysis in leukocytes and/or mutation analysis, were retrieved from storage.
from the Dutch National Institute for Public Health and the Environment (seven MPS I patients, Hurler phenotype, one MPS II patient with the neuronopathic phenotype, and all six MPS III patients) and the Danish Newborn Screening Biobank (two MPS I patients; one Hurler-Scheie and one Scheie phenotype). In addition, duplicate screening cards from two MPS I patients (Hurler phenotype), made for research purposes only, were obtained from two sites in the USA. Newborn DBS of two MPS III carriers and one MPS I carrier, all identified by mutation analysis through sibling studies, were retrieved from the Dutch National Institute for Public Health and the Environment.

All newborn DBS were made between days 3 and 7 after birth. The two US duplicate screening cards were obtained from the patients at the age of 1 and 4 weeks, respectively. The blood spots had been stored for a maximum of 11 years. Patient characteristics and storage conditions are reported in Tables 1 and 2. All DBS were pseudonymised before analysis and during all subsequent data processing.

Informed consent
All parents of MPS II and MPS III patients and parents of all carriers gave their informed consent to use the DBS for this research project. Parents of MPS I patients 1-9 (Table 1) gave informed consent for the use of DBS for this research project. Parents of MPS I patients 10 and 11 (Table 1) did not give dissent for the pseudonymised use of the DBS spots for research purposes at the time of the NBS.

Table 1. MPS I and II patient characteristics and results

<table>
<thead>
<tr>
<th>Age of DBS (years)</th>
<th>Phenotype†</th>
<th>Storage condition of DBS</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>D0A0 (ng/punch)*</th>
<th>D0a4 (ng/punch)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS I Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>0.3</td>
<td>H</td>
<td>rt</td>
<td>p.Q70X</td>
<td>p.Q70X</td>
<td>4.95</td>
</tr>
<tr>
<td>Patient 2</td>
<td>5.2</td>
<td>H</td>
<td>4°C-rt</td>
<td>p.L218P</td>
<td>p.L218P</td>
<td>3.21</td>
</tr>
<tr>
<td>Patient 3</td>
<td>5.2</td>
<td>H</td>
<td>4°C-rt</td>
<td>p.L218P</td>
<td>p.L218P</td>
<td>2.56</td>
</tr>
<tr>
<td>Patient 4</td>
<td>5.3</td>
<td>H</td>
<td>4°C-rt</td>
<td>p.Q70X</td>
<td>p.L218P</td>
<td>4.71</td>
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<td>Patient 5</td>
<td>4.1</td>
<td>H</td>
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<td>p.Q70X</td>
<td>p.L218P</td>
<td>4.80</td>
</tr>
<tr>
<td>Patient 6</td>
<td>4.2</td>
<td>H</td>
<td>4°C-rt</td>
<td>p.W402X</td>
<td>p.Q70X</td>
<td>3.25</td>
</tr>
<tr>
<td>Patient 7</td>
<td>5.9</td>
<td>H</td>
<td>4°C-rt</td>
<td>p.W402X</td>
<td>p.W402X</td>
<td>3.10</td>
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<tr>
<td>Patient 8</td>
<td>2.8</td>
<td>H</td>
<td>4°C-rt</td>
<td>p.A367E</td>
<td>p.1650del117</td>
<td>4.07</td>
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<tr>
<td>Patient 9</td>
<td>0.1</td>
<td>H</td>
<td>rt</td>
<td>p.W402X</td>
<td>p.W402X</td>
<td>2.00</td>
</tr>
<tr>
<td>Patient 10</td>
<td>11.3</td>
<td>H/S</td>
<td>-20°C</td>
<td>p.L490P</td>
<td>p.L490P</td>
<td>2.90</td>
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<tr>
<td>Patient 11</td>
<td>10.3</td>
<td>S</td>
<td>-20°C</td>
<td>p.S633L</td>
<td>n.d.</td>
<td>1.92</td>
</tr>
</tbody>
</table>

MPS II Patient

<table>
<thead>
<tr>
<th>Age of DBS (years)</th>
<th>Phenotype†</th>
<th>Storage condition of DBS</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>D0A0 (ng/punch)*</th>
<th>D0a4 (ng/punch)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>4.9</td>
<td>neuronopathic</td>
<td>4°C-rt</td>
<td>n.a.</td>
<td>n.a.</td>
<td>2.9</td>
</tr>
</tbody>
</table>

4°C-rt: 1 year at 4 °C, thereafter at room temperature. rt: room temperature.
*: values are in nanogram per 1/8 inch diameter punch from DBS. †: Phenotypes based on clinical evaluation, family history and mutation analysis. n.d.: not detected, n.a.: not available.
Ethical approval

This study was approved by the Medical Ethical Committee of the AMC in Amsterdam.

Chemicals

The pET19b plasmid was obtained from Novagen-EMD4Biosciences, USA, and *Pedobacter heparinus* from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Heparin, heparan sulfate and dermatan sulfate were purchased from Sigma, de-O-sulfated heparin from Neoparin Inc., the heparan sulfate and dermatan sulfate disaccharide standards and internal standard from Iduron and the Amicon Ultra 30K centrifugal filter from Millipore. All other reagents were of analytical grade.

Spiked control blood spots

Control blood from an anonymous healthy donor was obtained from the Dutch blood bank, Sanquin. Two ml of control blood was spiked with 20 µl of heparan sulfate and dermatan sulfate each to obtain a final concentration of 2 µg/mL. Unspiked control blood was diluted to the same volume as the spiked blood with distilled water. Both blood samples were spotted on Guthrie cards, dried overnight and stored at 4°C until analysis.

Expression of chondroitinase B, heparinase I, II and II.

Chondroitinase B from *Pedobacter heparinus* is the only GAG lyase known which specifically digests dermatan sulfate and has no activity towards chondroitin sulfate. We cloned chondroitinase B from *Pedobacter heparinus* into pET19b. Expression plasmids (pET15b or pET19b) containing the coding sequence of the mature heparinases were a generous gift from Dr. Ding Xu (University of California, CA, USA).

All enzymes were expressed as His-tagged fusion proteins in *E.coli* (BL21 AI, Invitrogen) in Terrific Broth medium with 8 g/L glycerol at 22°C. The enzymes were purified on HisLink Protein Purification Resin (Promega) according to the manufacturer’s protocol.

The purified enzymes were dialyzed against a buffer containing 50 mM Tris (pH=7.5), 10 mM CaCl$_2$, 200 mM NaCl and 2 mM Dithiothreitol (DTT). Thereafter, 126 g/L glycerol and 2 mg/mL BSA was added and aliquots were snap-frozen in liquid nitrogen and stored at -80°C.

Before each experiment, the activity of the enzymes was tested. Heparinase I and II activity was measured at 30°C in an incubation medium (1 mL final volume) containing 25 mM $4\text{-}(2\text{-hydroxyethyl})\text{-1\text{-piperazineethanesulfonic acid (HEPES)(pH=7.0)}}$, 100 mM NaCl, 1 mM CaCl$_2$, 2 mM DTT and 3-4 mIU enzyme. The reaction was started by addition of 0.2 mg/mL heparin and the increase in absorbance due to the introduction of double bonds was followed in time at a wavelength of 232 nm. The activity was calculated using the Beer-Lambert law with an extinction coefficient of 5200 L.mol$^{-1}$.cm$^{-1}$. 


The activities of heparinase III and chondroitinase B were measured essentially as described above, using de-O-sulfated heparan sulfate or dermatan sulfate as substrate, respectively.

Measurement of heparan sulfate and dermatan sulfate in blood spots

Heparan sulfate and dermatan sulfate were determined according to the methods initially reported by Oguma and co-workers [29] with minor modifications.

The relative abundance of D0A0, D0S0, D0A6+D2A0 and D0S6+D2S0 (nomenclature according to Lawrence et al. [30]) in the heparan sulfate (Sigma-Aldrich) used to spike control samples was 39.5, 26.3, 11.1 and 23.1%, respectively, as determined by HPLC-MS/MS analysis (as described below). Using the relative abundance and the specific molecular weight of each disaccharide in this batch of heparan sulfate, an average MW of 425.48 Da for a disaccharide in this heparan sulfate was calculated. The relative abundance of D0a4 and D0a10 [30] in the dermatan sulfate (Sigma-Aldrich) used to spike control samples was 94 and 6%, respectively, as determined by UPLC-MS/MS analysis. The average MW of the disaccharides in this batch of dermatan sulfate was calculated to be 464.18 Da.

These average MWs were used to calculate the exact concentration of digestible heparan sulfate and dermatan sulfate. The maximum enzymatic digestion of heparan sulfate and dermatan sulfate (as used to spike a control sample and to calculate the recovery) was determined by monitoring the complete digestion at 232 nm (extinction coefficient of 5200 L.mol⁻¹.cm⁻¹) using an excess of heparinase I, II and III or chondroitinase B, respectively. We found that the digestion of these glucosaminoglycans is incomplete, but reproducible; typically 44% of the heparan sulfate and dermatan sulfate standards can be digested. Possibly certain domains within heparan sulfate and dermatan sulfate and/or the shortened products (eg. tetra- and hexamers) are poor substrates for the heparinases and chondroitinase B, respectively.

Dried blood spot punches (1/8 inch diameter) were incubated in 135 µl of 111 mM NH₄Ac and 11 mM Ca(Ac)₂ (pH=7.0) for 10 minutes at room temperature and subsequently sonicated for 15 minutes. Next, heparan sulfate and dermatan sulfate in the blood spot punches were enzymatically digested to disaccharides by addition of 5 mIU of each Heparinase I, II, III and 50 mIU chondroitinase B (combined volume of 12 µL) and 3 µL of a 100mM solution of DTT (2 mM final concentration). The final volume of the reaction mixture was 150 µl. After 2 hours of incubation at 30°C, 15 µL 150 mM EDTA (pH=7.0) and 125 ng internal standard, 4UA-2S-GlcNCOEt-6S (HD009, Iduron), was added and the reaction was stopped and proteins denatured by boiling for 5 minutes. The reaction mixture was centrifuged at 16,000 g for 5 minutes at room temperature. The supernatant was subsequently applied to an Amicon Ultra 30K centrifugal filter (Millipore) and centrifuged at 14,000 g for 15 minutes at 25°C. The filtrate was stored at -20°C until analysis.
The disaccharides were quantified on a Waters Quattro Premier XE (tandem) mass spectrometer (Waters Corporation, Milford, MA, USA) coupled to an Acquity HPLC system (HPLC-MS/MS). The disaccharides were separated on a Thermo Hypercarb HPLC column (100×2.1 mm, 5 µm). The mobile phase consisted of 10 mM NH$_4$HCO$_3$ pH 10 and the disaccharides were eluted with a acetonitrile gradient of 0% to 20% in 2.5 minutes, hold at 20% for the next 2.5 minutes and 2 minutes equilibration with 0% before the next injection; the flow rate was 0.2 mL/minute, and the total run time of 7.1 minutes. Disaccharides were detected and quantified by MRM acquisition mode, using the transitions m/z 378.1>175.1 for D0A0, 416.1>138.0 for D0S0, 458.1>97.0 for D0A6+D2A0, 496.0>416.0 for D0S6+D2S0, 458.0>299.9 for D0a4, 538.0>458.0 for D0a10 and 472.0>97.0 for the 4UA-2S-GlcNCOEt-6S internal standard (HD009, Iduron).

All samples were digested and analyzed in duplicate. As the quantity of heparan sulfate and dermatan sulfate is very low in a 1/8 diameter punch, only D0A0 and D0a4 could be detected and were used as marker for heparan sulfate and dermatan sulfate, respectively. The concentration of D0A0 and D0a4 were calculated using a calibration curve of each of the disaccharides with 4UA-2S-GlcNCOEt-6S (HD009, Iduron) as internal standard.

Furthermore in each experiment, three control blood punches, spiked with heparan sulfate and dermatan sulfate (2 µg/mL each, Sigma-Aldrich) were analyzed to determine the efficiency of extraction of heparan sulfate and dermatan sulfate from the blood spot punches.

Intra-assay variation was calculated by analysing 10 spiked control blood spot punches on the same day. Inter-assay variation was calculated by analysing spiked control blood spot punches, in triplicate, on 10 different days. To determine the linear range of the assay, control blood was spiked with different quantities of heparan sulfate and dermatan sulfate, spotted on Guthrie cards which were dried overnight, stored at 4°C and analyzed as described above.

### Table 2. MPS III patient characteristics and results

<table>
<thead>
<tr>
<th>Age of DBS (years)</th>
<th>Phenotype†</th>
<th>Subtype</th>
<th>Storage condition of DBS</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>D0A0 (ng/punch)*</th>
<th>D0a4 (ng/punch)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>5.8</td>
<td>Severe</td>
<td>A</td>
<td>p.R245H</td>
<td>p.Q380R</td>
<td>2.74</td>
<td>0.18</td>
</tr>
<tr>
<td>Patient 2</td>
<td>5.5</td>
<td>Attenuated</td>
<td>A</td>
<td>p.S298P</td>
<td>p.S298P</td>
<td>3.27</td>
<td>0.14</td>
</tr>
<tr>
<td>Patient 3</td>
<td>5.8</td>
<td>Severe</td>
<td>A</td>
<td>p.R245H</td>
<td>p.V126FS</td>
<td>4.42</td>
<td>0.18</td>
</tr>
<tr>
<td>Patient 4</td>
<td>6.6</td>
<td>Intermediate</td>
<td>C</td>
<td>p.D40VfsX19</td>
<td>p.D40VfsX19</td>
<td>3.78</td>
<td>0.21</td>
</tr>
<tr>
<td>Patient 5</td>
<td>5.9</td>
<td>Attenuated</td>
<td>B</td>
<td>p.R297X</td>
<td>p.S612G</td>
<td>3.83</td>
<td>0.14</td>
</tr>
<tr>
<td>Patient 6</td>
<td>3.3</td>
<td>Severe</td>
<td>A</td>
<td>p.G191R</td>
<td>p.R245H</td>
<td>4.32</td>
<td>0.00</td>
</tr>
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</table>

4°C-rt: 1 year at 4 °C, thereafter at room temperature.
* : values are in nanogram per 1/8 inch diameter punch from DBS. †: Phenotypes based on mutation analysis and/or clinical evaluation [14,15,35-37].
Data analysis
Analyses were performed using the SPSS software for Windows, version 16 (SPSS, Chicago, IL). An independent t-test was used to detect differences in levels of D0A0 and D0a4 between the MPS patients and control samples. We considered a $p$-value of <0.05 as statistically significant.

RESULTS
Assay validation
D0A0, the most abundant disaccharide in heparan sulfate, and D0a4, which makes up 94% of dermatan sulfate, were the only two disaccharides which could be detected in blood spots, as the absolute quantity of heparan sulfate and dermatan sulfate in 1/8 inch diameter blood spot punches is very low.

Control blood spiked with different quantities of heparan sulfate and dermatan sulfate was used to analyze the linear range of this assay. The calibration curves were linear over the range 0-6 ng/blood spot punch. The intra-assay (n=10) and inter-assay (10 measurements in triplicate) coefficient of variations (CV) were 7.5% and 11.4% for D0A0 and 8.3% and 15.2% for D0a4, respectively. To determine recovery of D0A0 and D0a4 from blood spots, both water and control blood were spiked with 2 $\mu$g/mL heparan sulfate and dermatan sulfate each. Half of each sample was spotted on Guthrie cards, dried overnight and processed as described for the blood spots, the other half was stored as liquid.

Equal quantities of D0A0 were measured when spiked water or spiked blood was analyzed. No difference was found in D0A0 recovery when spiked samples were added directly to the reaction mixture or when samples were spotted on Guthrie cards and treated like blood spots (data not shown). The quantity of D0A0 extracted from blood spot punches was similar to the quantity of D0A0 that can be enzymatically cleaved from heparan sulfate using an excess of heparinase I, II and III (see methods).

The recovered quantity of D0a4 was similar to the maximal amount of D0a4 that can be released from dermatan sulfate (see methods) in all control incubations (spiked water spotted on a Guthrie card, spiked water or blood added directly to the reaction mixture, spiked blood incubated overnight at room temperature, addition of a non-spiked control blood spot punch to spiked water, spiked water or blood dried overnight in an eppendorf tube). In contrast, when spiked blood was spotted on a Guthrie card and dried overnight the recovery of D0a4 was only 50% of the expected amount of D0a4. Possibly, some component in blood protects dermatan sulfate from enzymatic digestion when dried on a Guthrie card.
MPS I newborn DBS

To assess whether D0A0 and D0a4 levels can distinguish MPS I-affected newborns from control newborns, we compared newborn DBS from 11 MPS I patients with 59 control newborn DBS. D0A0 and D0a4 levels of control newborn DBS were 0.54 ± 0.20 ng/punch (mean ± SD), and 0.32 ± 0.13 ng/punch (mean ± SD), respectively. Both the D0A0 level (> 1.9 ng/punch; Fig.1) and D0a4 level (> 1.9 ng/punch; Fig.2) in newborn MPS I DBS were

Figure 1. DBS levels of D0A0 for MPS I, II and III patients, MPS I and III carriers and controls. Values are in nanogram per 1/8 inch diameter punch from DBS.

Figure 2. DBS levels of D0a4 for MPS I, II and III patients, MPS I and III carriers and controls. Values are in nanogram per 1/8 inch diameter punch from DBS.
significantly elevated as compared to newborn control DBS (p<0.001). In contrast, the DBS of a newborn carrier of a MPS I mutation showed normal levels of D0A0 and D0a4 (Table 3, Fig.1 and Fig.2).

No differences in D0A0 and D0a4 levels in DBS were observed between MPS I patients with Hurler, Hurler-Scheie or Scheie phenotype (Table 1), although the numbers of patients with attenuated MPS I were very limited.

MPS II newborn DBS

We analyzed if the levels of D0A0 and D0a4 can distinguish the MPS II affected newborn from the control newborns. Both the D0A0 level (2.5 ng/punch) and the D0a4 level (0.9 ng/punch) were found significantly elevated compared to controls DBS (p<0.001).

MPS III newborn DBS

Next, we analyzed whether MPS III-affected newborns can be identified via measurement of DBS D0A0 level. The DBS of all six MPS III patients showed significantly elevated levels of D0A0 (>2.7 ng/punch, Fig.1), as compared to control newborn DBS (p<0.001). Importantly, in contrast to MPS I samples, D0a4 levels in MPS III patients’ DBS were ≤0.21 ng/punch, and did not differ significantly from controls (Fig.2). The levels of D0A0 and D0a4 of the three DBS from newborn MPS III carriers were within the normal range (Table 3, Fig.1 and Fig.2).

No differences were observed between DBS D0A0 levels from patients with different MPS III subtypes and phenotypes.

**DISCUSSION**

In this study, we describe a method to detect newborn MPS I, MPS II and MPS III patients by analysis of disaccharides derived from their primary storage products, heparan sulfate and dermatan sulfate. Patients (MPS I, II and III) could be easily discriminated from control newborns and from heterozygote carriers (MPS I and III). Furthermore, patients with more attenuated disease phenotypes, as demonstrated by clinical evaluation in MPS I (Table 1, patients 10 and 11: Hurler-Scheie and Scheie phenotype, respectively), and by predictive mutations in MPS III (Table 2, patients 2 and 5), also had clearly abnormal GAG derived disaccharides in newborn DBS. This demonstrates the sensitivity of this technique and its potential for NBS.

DBS for NBS programs are often sent to central screening laboratories by mail, therefore it is important that the analytes of interest are stable at room temperature. The GAGs measured in our study appear to be remarkably insensitive to temperature changes, as some of the DBS were stored for over four years at room temperature before analyses were performed (Tables 1 and 2).
We hypothesize that this method can also be applied to NBS for MPS VI and VII, as in these disorders heparan sulfate and/or dermatan sulfate are also the primary storage products. The feasibility of disaccharide analysis to identify newborn MPS VII patients was already established by Tomatsu et al., who reported elevated concentrations of heparan sulfate and dermatan sulfate in plasma of a newborn patient [28].

Recently, LC-MS/MS analysis of keratan sulfate derived disaccharides in plasma and urine of MPS IV patients was described [31,32]. Incubation of DBS with keratanase could be used to analyze keratan sulfate storage, due to MPS IV, in newborn DBS. A combination of measurement of keratan sulfate derived disaccharides with the analysis of heparan and dermatan sulfate derived disaccharides would allow a technique for multiplex detection of all MPSs. This is currently under investigation.

Once abnormal levels of GAG derived disaccharides in DBS are observed, diagnosis of MPS needs to be corroborated by appropriate enzymatic testing, preferable in isolated lymphocytes, as a second-tier diagnostic strategy.

The levels of the different disaccharides in DBS of newborn MPS I and MPS III patients do not appear to be predictive for disease severity (Tables 1 and 2). As decisions on the optimal treatment strategy in MPS I depend on the phenotype, biomarkers that may predict disease severity are needed. The serum heparin cofactor II–thrombin (HCII-T) complex [33] and/or the dermatan sulfate:chondroitin sulfate ratio in urine [34] may be such markers.

In summary, our study demonstrates that detection of heparan and dermatan sulfate derived disaccharides in DBS may be used as a sensitive technique for NBS for MPS I, II and III. We hypothesize that this assay may also detect MPS VI and VII patients in DBS. Further large scale studies, including pilot studies within NBS programs, are needed to establish the potentials of this approach.

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Table 3. MPS carrier characteristics and results

<table>
<thead>
<tr>
<th>Age of DBS (years)</th>
<th>Storage condition of DBS</th>
<th>Mutation</th>
<th>D0A0 (ng/punch)*</th>
<th>D0a4 (ng/punch)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS I carrier</td>
<td>4°C-rt</td>
<td>p.P533R</td>
<td>0.57</td>
<td>0.52</td>
</tr>
<tr>
<td>MPS III carrier</td>
<td>4°C-rt</td>
<td>p.S298P</td>
<td>0.41</td>
<td>0.40</td>
</tr>
<tr>
<td>MPS III carrier</td>
<td>4°C-rt</td>
<td>p.R245H</td>
<td>0.60</td>
<td>0.31</td>
</tr>
<tr>
<td>MPS III carrier</td>
<td>4°C-rt</td>
<td>p.R245H</td>
<td>0.76</td>
<td>0.47</td>
</tr>
</tbody>
</table>

4°C-rt: 1 year at 4 °C, thereafter at room temperature.
*: values are in nanogram per 1/8 inch diameter punch from DBS.
REFERENCES


