Application of MALDI-TOF Mass Spectrometry for Non-invasive Diagnostics of Mucopolysaccharidosis IIIA

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Abstract

Mucopolysaccharidosis IIIA (MPS IIIA) is a lysosomal storage disorder (LSD) caused by deficiency of lysosomal *N*-sulphoglucosamine sulphohydrolase, which is one of four enzymes involved in heparan sulfate degradation. Traditional methods used for MPS IIIA diagnostics usually constitute of selective screening, based on the analysis of urinary glycosaminoglycans, further enzymatic assays in leukocytes, and mutation analysis. Nowadays, some LSDs, including mucopolysaccharidoses, can be precisely diagnosed by mass spectrometry-based techniques. Up to this date, there are no comprehensive studies of MPS IIIA diagnostics by MALDI-TOF analysis of free oligosaccharides in urine published. In the presented work, MALDI-TOF/TOF analysis of permethylated oligosaccharides was performed to obtain the set of glyco-biomarkers that together form the specific fingerprint of this disease. Early and accurate diagnostics of MPS IIIA is crucial to stabilize the progressive cellular damage and improve the overall well-being of patients.

Keywords

Mucopolysaccharidosis IIIA, Sanfilippo syndrome A, MPS IIIA, MALDI-TOF/TOF

Introduction

Mucopolysaccharidosis type IIIA (MPS IIIA) is a rare, autosomal-recessive lysosomal storage disorder (LSD), caused by a deficiency of *N*-sulphoglucosamine sulphohydrolase, coded by *SGSH* gene. This enzyme is involved in the stepwise degradation of the glycosaminoglycan (GAG) heparan sulfate in lysosomes [1]. The clinical manifestations described in MPS IIIA patients include language delay, coarse facial features, abnormal behavior, hepatomegaly, and epilepsy [2, 3].

Usually, the first suspicion for MPS is based on the medical assessment and then the selective screening, based on the investigation of GAGs in urine, is performed [4]. To assay and quantify GAGs, spectrometric methods, using dyes that bind with high specificity, are widely used. The most commonly used assay utilizes dimethylmethylene blue (DMMB); however, false-negative results have been reported for urine samples from mildly affected MPS I, III, and IV patients, and a false-positive rate of around 5% is common [5]. Alcian blue (AB), a tetravalent cationic dye, that interacts with sulfated GAGs, is another but less commonly used screening test for MPS [5, 6]. Quantitative determination of hexuronic acid based on carbazol [7] can be also used for MPS screening, however, this test does not detect keratan sulfate. It is also important to mention that the reference ranges (GAG normalized to creatinine) are strongly age-dependent and DMMB and AB are not sensitive and specific enough to measure GAGs in blood or tissue samples [4]. Total quantitative GAG excretion may be increased in other connective tissue disorders, *e.g.* rickets, rheumatoid arthritis, and disseminated lupus erythematosus [8]. Another approach toward MPS diagnostics is molecular genetic analysis, which is accomplished by using of polymerase chain reaction between coding exons and flanking intronic regions of the gene of interest [9].

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Nowadays, MPSs and other LSDs can be diagnosed also by approaches based on mass spectrometry (MS), often combined with liquid chromatography (LC) which offer high sensitivity and specificity. Some LSDs were detected by measuring of activities of their corresponding enzymes (galactocerebroside-β-galactosidase for the analysis of Krabbe disease; α-galactosidase A for the analysis of Fabry disease) in dried blood spots using tandem mass spectrometry (MS/MS) [10]. MS/MS analysis of dried blood spots is also useful for enzymatic-based newborn screening of every MPS type except MPS IX [11–13]. MS analysis in combination with liquid chromatography (LC-MS) was used for GAG detection in urine and blood samples [14–16]. Prior to the analysis, GAGs are cleaved into disaccharides (by enzymatic hydrolysis or methanolysis) in order to degrade them to become more suitable analytes for the LC-MS/MS quantification [16–18]; however, enzymatic GAG hydrolysis does not detect chondroitin sulfate, while the methanolysis method is less suitable for the determination of keratan sulfate.

During the last decade, effective approaches based on the analysis of free oligosaccharides in urine were applied in MPS diagnostics. Saville *et al*. established a method of oligosaccharide derivatization by 1-phenyl-3-methyl-5-pyrazolone and their subsequent analysis by electrospray ionization (LC-ESI-MS/MS). For the study of MPS IIIA subtype, the relative intensity of the characteristic fragment, monosulfated disaccharide, which consisted of uronic acid and hexosamine, was determined by this approach [19, 20].

Contrary to LC-ESI-MS/MS, MALDI MS profiling of free urinary oligosaccharides offers an easy and reliable way to determine the characteristic fingerprints for various diseases. MALDI-TOF analysis of oligosaccharides from urine samples was used for diagnostics of LSDs, where these oligosaccharides were identified and evaluated as reliable biomarkers for various LSDs [21]. However, there are some studies, where mass spectrometry, especially LC-MS/MS, was used for diagnostic purposes of MPS IIIA. This method was used for measurements of sulfamidase activity in dried blood spots samples of newborns and MPS IIIA patients. Defect of this enzyme leads to the accumulation of heparan sulfate and measurement of its activity is useful for diagnosis of MPS IIIA. LC-MS was also used for determination of concentration of amino acids that represent potential biomarkers for MPS IIIA and other MPS III types. This type of analysis revealed profound metabolic impairments in patients with MPS III and provided better understanding of the pathological mechanism of these disorders [22, 23]. Based on our knowledge; there are no comprehensive studies of MPS IIIA diagnostics by MALDI-TOF published up to this date. In this work, MALDI-TOF/TOF analysis of permethylated free urinary oligosaccharides was applied to obtain and identify a set of oligosaccharide biomarkers for MPS IIIA. The overall aim of this work was to obtain a characteristic fingerprint consisting of a set of relevant biomarkers for MPS IIIA in urine using MALDI-TOF (MS) and MALDI-TOF/TOF (MS/MS). Obtained results can lead to acquisition of new biomarkers for MPS IIIA and eventually lead to the improvement of diagnostic approach of this disorder using MALDI-TOF/TOF analysis of permethylated urine samples.

Case Report

Urine samples of two MPS IIIA patients were obtained from the National Institute of Children's Diseases, Bratislava, Slovakia, following standard operating procedures. Written informed consent was given by every subject and the study protocol was reviewed and confirmed by the hospital ethics committee. At the time of sample collection, Patient I was 11 years old female and Patient II was 6 years old male. As a negative control, urines from two age- and gender- matching healthy individuals (Negative control I and Negative control II) were used.

Patient I suffered from delayed psychomotor development, accompanied by speech delay and behavioral disorders. The patient's clinical manifestation included epileptic seizures, limb deformities, scoliosis, and tonsil and adenoid hypertrophy. Electrophoretic analysis of GAGs in urine detected a dominant level of heparan sulfate, a significant level of chondroitin sulfate, and the presence of a trace amount of heparitin sulfate. By sequencing of *SGSH* gene, a likely pathogenic homozygous variant c.1448C>T (p.Pro483Leu), a homozygous variant of unknown clinical significance c.1322G>A (p.Arg.441Gln), and heterozygous intron variant of unknown significance c.664-2A>T were identified. At the age of 5 years, MPS IIIA was diagnosed.

Patient II suffered from delayed psychomotor development, accompanied by hypotonic syndrome, frequent upper respiratory tract infections, recurrent gastroenteritis, speech delay, limb deformities, and progressively receding manifestations of aggression and hyperactivity. Electrophoretic analysis of GAGs in urine detected a dominant level of heparan sulfate, a significant level of chondroitin sulfate, and the presence of a trace amount of heparitin sulfate. By sequencing of *SGSH* gene, pathogenic homozygous variant c.1080delC (p.Val361Serfs*52) was identified. At the age of 4.5 years, MPS IIIA was diagnosed.

Methods

The initial step of sample preparation was the dissolution of 50 µL of urine in 500 µL of LC-MS water. Samples were lyophilized overnight and permethylated according to the literature [24]. The main advantage of permethylation of urinary oligosaccharides

lies in increased signal intensities, increased stability in the ion source and furthermore, no labeling of reducing end is required. Dried samples were dissolved in 10 µL of a solution consisting of methanol and water (50:50; v/v). 1 µL of the sample was spotted onto a ground steel MALDI plate and premixed 1 µL of DHB matrix solution (20 mg/mL 2,5-dihydroxybenzoic acid in 30% acetonitrile + 0.1% TFA, with the addition of 1 mM NaOH to unify the adduct formation in mass spectra). MALDI-TOF analysis was performed in reflectron positive ionization mode using UltrafleXtreme II mass spectrometer *(Bruker Daltonics, Germany)* with spectra processing parameters set as follows: *m/z* range 1,000-3,000, and signal-to-noise (S/N) threshold 4. Data were processed by software programs *flexAnalysis 3.4 (Bruker Daltonics, Germany)* and *GlycoWorkbench* [25].

Results

The overall aim of this work was to obtain a characteristic fingerprint consisting of a set of relevant biomarkers for MPS IIIA in urine using MALDI-TOF (MS) and MALDI-TOF/TOF (MS/MS). MS spectra of permethylated oligosaccharides from the urine of MPS IIIA patients (Patient I and II), compared to negative controls (Neg. Ctrl. I and II), are shown in Figure 1. In total, the set of 16 oligosaccharidic structures with unique *m/z* values were identified as common for MPS IIIA (summarized in Table 1). Representative MALDI-TOF/TOF fragmentation spectra, confirming the composition of neutral, oligohexose, or sialylated glycan structures, are shown in Figure 2.

According to the literature, most of the signals observed in the urine of MPS IIIA patients are common for various LSDs [12, 21, 26]. Signals at *m/z* values of 1293.6, 1497.7, 1701.7, 1905.9, 2110.0, and 2314.0 correspond with a set of oligohexose structures that could originate from an external source (food) or internal glycogen degradation; their increased levels were observed in Pompe patients [27]. Some other signals (*m/z* 1171.5, 1240.6, 1375.6, 1532.7, 1736.7, 1824.8, 1981.9, 2185.9, and 2547.1) are common also for other MPS subtypes (MPS I, II, IVA or IVB), however, the spectra of urinary oligosaccharide profiles of other MPSs does not contain signals of oligohexoses in such significant intensities. Another set of signals (*m/z* 1240.6, 1532.7, 1736.7, 1824.8, 1981.9, 2185.9, 2547.1, and 2792.3) is common for mucolipidoses (ML I, II, or III) and three signals of asialylated (neutral) structures, based on the combination of hexoses and *N*-acetylhexosamines (*m/z* 1171.5, 1375.6 and 1824.8), were observed also in the urine of GM1 gangliosidosis patients. All structures, published previously as biomarkers of galactosialidosis [12, 21, 26], were observed in MPS IIIA urines as well (*m/z* 1240.6, 1532.7, 1736.7, 1981.9, 2185.9, 2547.1 and 2792.3) and most of them were also detected in the urines of patients suffering from sialidosis [21].

In the urinary oligosaccharide profile of both MPS IIIA patients, the most abundant structures were identified as i.) NeuAc2Hex1HexNAc1 (*m/z* 1240.6, relative intensities in Patient I and II samples of 11.15 and 10.56% respectively); ii.) NeuAc1Hex3HexNAc2 (*m/z* 1532.7, relative intensities in Patient I and II samples of 6.50 and 5.27% respectively) and iii.) NeuAc2Hex5HexNAc3 (*m/z* 2547.1, relative intensities in Patient I and II samples of 5.61 and 5.24%, respectively). Relative intensities of single structures were calculated from all obtained signals in MS spectra as an average from three measurements for each sample (triplicates). High intensities of *m/z* 1240.6 and 1532.7 were observed in other MPS subtypes [21, 26]; however, the significantly high relative intensity of the signal at *m/z* 2547.1 differs MPS IIIA from other MPSs. This signal is also a part of the biomarker panels for other LSDs, such as ML II or sialidosis, but in the case of MPS IIIA, it has significantly higher relative intensity compared to its intensities in samples from other LSDs [21, 26]. Oligohexose-based structures and structures consisting of NeuAc2Hex1HexNAc1 (*m/z* 1240.6) were usually observed also in the samples of negative controls; however, these molecules are originated mostly from external sources, such as a diet rich in saccharides. Eight specific oligosaccharide structures were observed in significantly increased levels in MPS IIIA samples: *m/z* 1240.6, increased 1.6- and 2.2- fold; *m/z* 1532.7, increased 18.1- and 17.6- fold; *m/z* 1736.7, increased 4.3- and 10.2- fold; *m/z* 1824.8, increased 3.8- and 2.3- fold; *m/z* 1981.9, increased 2.0- and 2.8- fold; *m/z* 2185.9, increased 3.8- and 3.6- fold; *m/z* 2547.1, increased 10.2- and 20.2- fold; and *m/z* 2729.3, increased 2.4- and 4.3- fold.

Based on the described pattern of urinary oligosaccharides, diagnostics of MPS IIIA by MALDI-TOF mass spectrometry might be a useful and reliable tool to distinguish this disorder from other LSDs, particularly when its differentiation from other MPSs can be challenging. Compared to other LSDs, signals identified in this work are different, or they have different relative intensities. For example, dominant signals for MPS IVB are *m/z* 1171.6 and 1826.0 [26]. Characteristic biomarkers for GM1 gangliosidosis are *m/z* 1150.4, 1353.5 and 1515.5 [28]. For fucosidosis, predominant ion signals of biomarkers for this disorder are *m/z* 504.2 and 1079.4. In case of Sandhoff disease, the most significant biomarkers are referred to signals with *m/z* values of 771.2, 1136.3 and 1339.4 [29]. None of these dominant signals for Sandhoff disease, GM1 ganglisodosis and fucosidosis were identified in significant intensities in MPS IIIA sample. In our case, the three most dominant signals were at *m/z* 1240.1, 1532.7 and 2547.1.

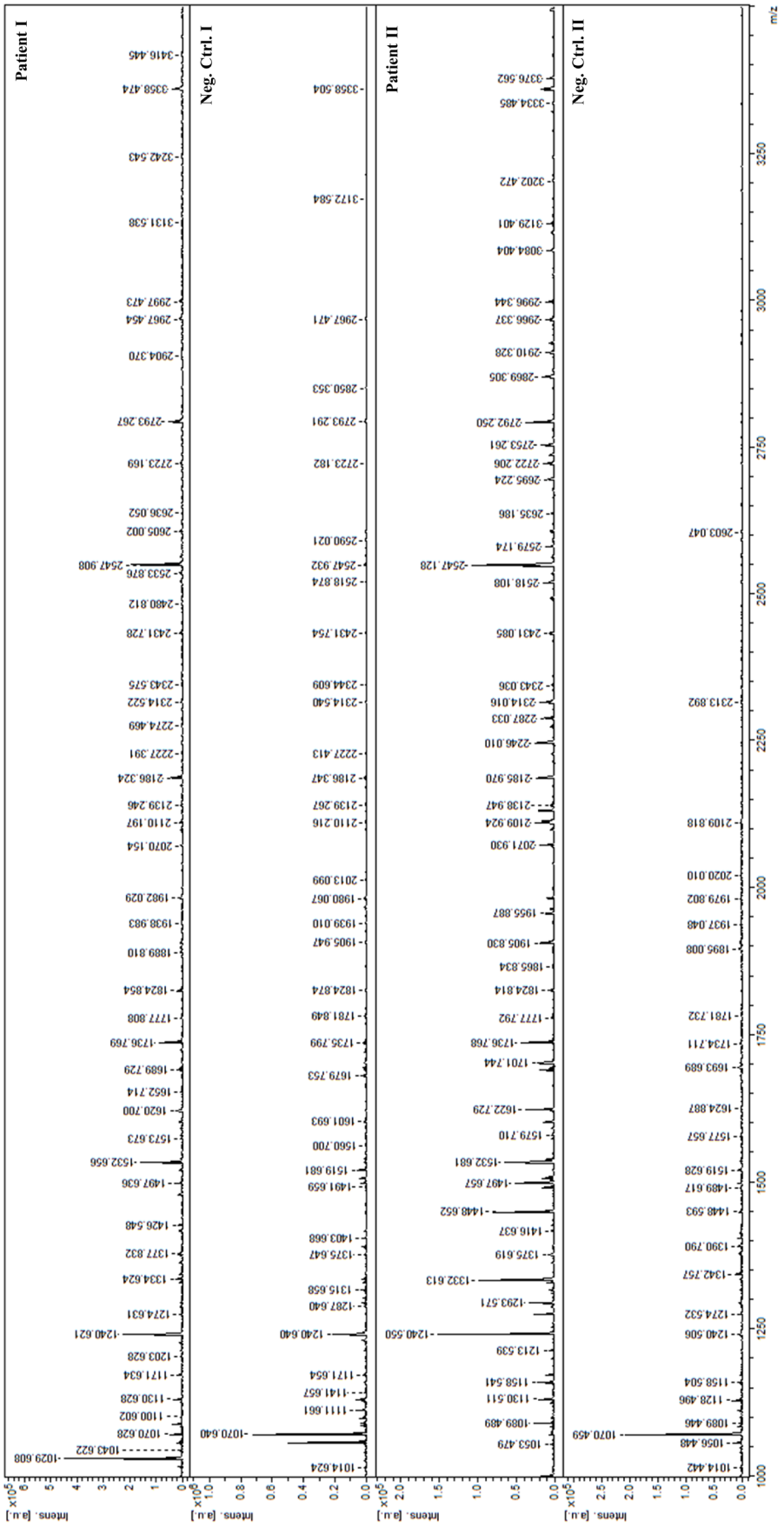

Figure 1. MALDI-TOF spectra of permethylated free oligosaccharides from urine samples of MPS IIIA patients (Patient I and II) and age-matching negative controls (Neg. Ctrl. I and II). Identified structures of interest are marked as 1-16 and listed in Tab. 1.

Table 1. List of urinary oligosaccharides identified in controls and the patients with MPS IIIA. The composition of all annotated structures was identified by MALDI-TOF/TOF analysis and compared with literature [12, 21, 26].

#	m/z	Structure	Relative intensity (%) Patient I	Relative intensity (%) Neg. Ctrl. I	Relative intensity (%) Patient II	Relative intensity (%) Neg. Ctrl. II	Common also for other LSDs
1	1171.5	Hex3HexNAc2	1.20	0.75	1.10	1.02	MPS IVB, GM1 gangliosidosis
2	1240.6	NeuAc2Hex1HexNAc1	11.15	7.15	10.56	4.76	MPS IVA, ML I and II, galactosialidosis, sialidosis, Schindler disease
3	1293.6	Hex6	0.39	1.33	2.10	0.70	food (external source), Pompe disease
4	1375.6	Hex4HexNAc2	0.94	0.43	0.76	0.50	MPS IVB, GM1 gangliosidosis
5	1497.7	Hex7	1.22	2.27	3.33	0.96	food (external source), Pompe disease
6	1532.7	NeuAc1Hex3HexNAc2	6.50	0.36	5.27	0.30	MPS I, II and IVA, ML II and III, galactosialidosis, sialidosis
7	1701.7	Hex8	0.21	1.57	1.40	0.48	food (external source), Pompe disease
8	1736.7	NeuAc1Hex4HexNAc2	3.29	0.76	3.15	0.31	MPS I, II and IVA, ML II and III, galactosialidosis, sialidosis
9	1824.8	Hex5HexNAc3	1.13	0.30	0.68	0.29	MPS I, II and IVB, ML II and III, GM1 gangliosidosis
10	1905.9	Hex9	0.28	1.14	1.20	0.42	food (external source), Pompe disease
11	1981.9	NeuAc1Hex4HexNAc3	0.73	0.36	0.76	0.27	MPS I, II and IVA, ML II and III, galactosialidosis, sialidosis
12	2110.0	Hex10	0.35	1.03	1.17	0.37	food (external source), Pompe disease
13	2185.9	NeuAc1Hex5HexNAc3	1.55	0.41	1.35	0.37	MPS I, II and IVA, ML II and III, galactosialidosis, sialidosis
14	2314.0	Hex11	0.23	0.77	0.79	0.18	food (external source), Pompe disease
15	2547.1	NeuAc2Hex5HexNAc3	5.61	0.55	5.24	0.26	MPS I, II and IVA, ML II and III, galactosialidosis, sialidosis
16	2792.3	NeuAc2Hex5HexNAc4	1.23	0.51	1.55	0.36	galactosialidosis, ML II and III

ND – not detectable. # - number of identified oligosaccharide in the sample.

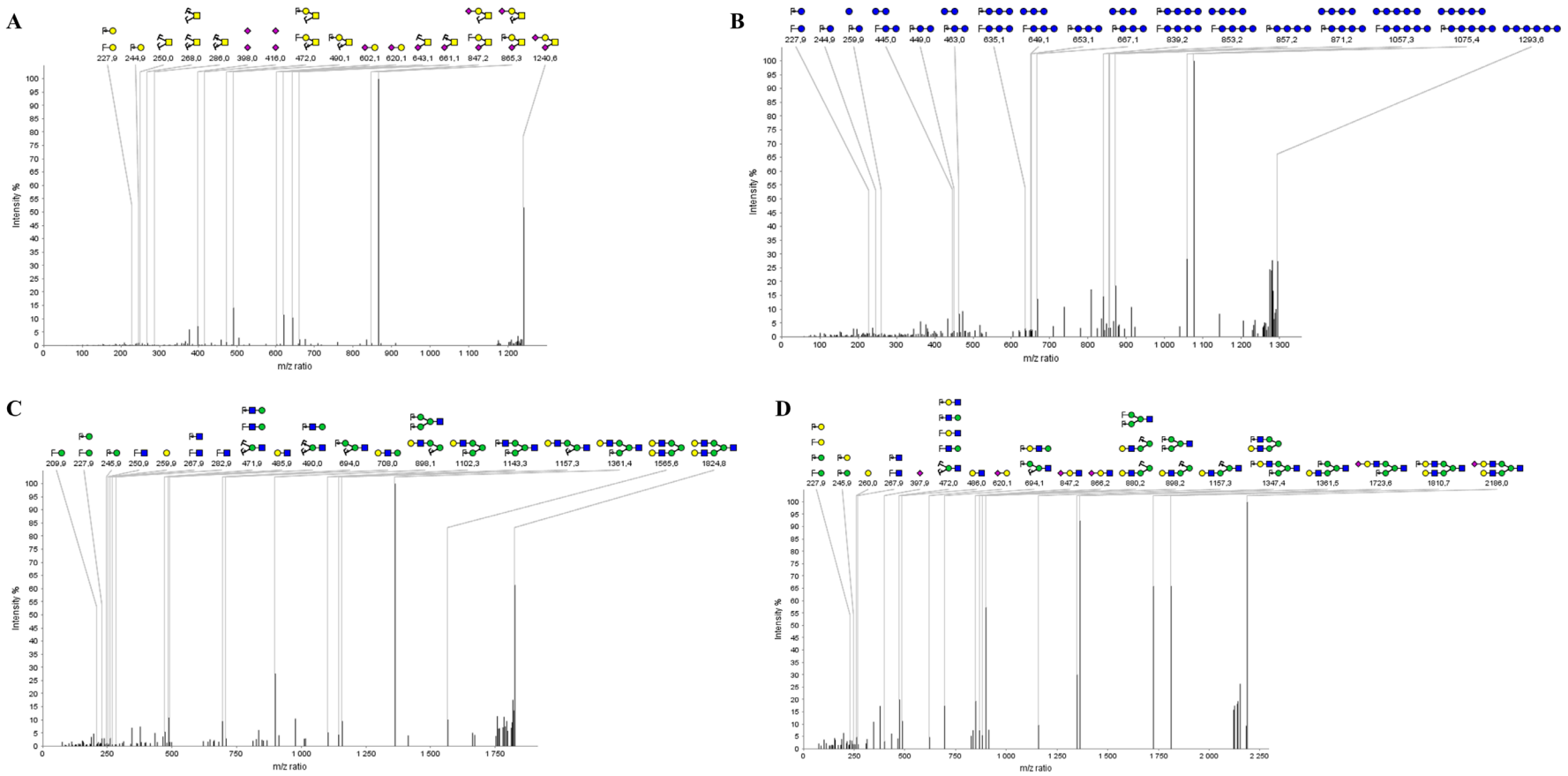

Figure 2. MALDI-TOF/TOF fragmentation spectra of representative neutral and sialylated oligosaccharides identified in the urines of MPS IIIA patients. A – MALDI-TOF/TOF spectrum of the signal at *m/z* value of 1240.6, confirming the NeuAc2Hex1HexNAc1 tetrasaccharide structure. B - MALDI-TOF/TOF spectrum of the signal at *m/z* value of 1293.6, assigned as Hex6. C - MALDI-TOF/TOF spectrum of the signal at *m/z* value of 1824.8, confirming the Hex5HexNAc3 structure. D - MALDI-TOF/TOF spectrum of the signal at *m/z* value of 2185.9, confirming the monosialylated NeuAc1Hex5HexNAc3 structure. *N*-Acetylhexozamines (HexNAc): blue square – *N*-Acetylglucosamine (GlcNAc), yellow square – *N*-Acetylgalactosamine (GalNAc); hexoses (Hex): green circle – mannose (Man), yellow circle – galactose (Gal), blue circle – glucose (Glc); sialic acids: purple diamond – *N*-Acetylneuraminic acid (NeuAc).

Discussion

By the application of MALDI-TOF mass spectrometry, the characteristic set of 16 urinary oligosaccharides was identified in the urine of MPS IIIA patients (Tab. 1), from which eight structures were present in increased levels in both MPS IIIA patients. Because of the limited number of patients included in this study (n=2) and the fact that MPS IIIA is a rare disease, it was not possible to perform a statistical analysis of obtained results. All of these signals were present in negative controls; however, in distinctly lower intensities. Furthermore, some of the identified oligosaccharides are not specific for MPS IIIA – these signals were previously published as biomarkers for other LSDs as well. However, their overall combination consisted of relative intensities and m/z values represent a unique and specific fingerprint that could constitute a reliable set of non-invasive biomarkers for MPS IIIA.

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Contribution of individual authors

FP acquisition of data, data interpretation, writing - original draft. ZP analysis and data interpretation, acquisition of data, writing – original draft. MN critical revision, writing – review and editing. FK technical procedures, writing – review and editing. AŠ critical revision, acquisition of clinical data. AH acquisition of clinical data. PB conception and design, writing – review and editing. SK final approval.

Declaration of conflict of interests

The Authors declare that there is no conflict of interest.

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