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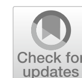
REVIEW



Diagnostics of lysosomal storage diseases by mass spectrometry: a review

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Abstract
Lysosomal storage disorders (LSD) are a group of over 70 rare inherited metabolic disorders that are caused mostly by the deficiency of specific lysosomal enzymes. Lack of these enzymes leads to the interference with cellular functions due to excessive accumulation of undegraded substrate in cells and tissues. Effective treatment of these diseases, if it is available, relies on early and accurate diagnostics. Several traditional methods for diagnostics of LSD are used worldwide; however, new techniques, methods and instruments need to be applied to the diagnostic process to increase its sensitivity, repeatability and reliability. In this review, diagnostic methods based on mass spectrometry and their respective sample preparation steps and eventual separation by liquid chromatography are discussed, emphasizing specific biomarkers of each lysosomal storage disorder subclass. Up-to-date evaluation of research conducted in the areas of diagnostics of lysosomal storage disorders by mass spectrometry is comprehensively summarized in this study.

Keywords Biomarkers · Diagnostics · Lysosomal storage diseases · Mass spectrometry

Introduction

Lysosomal storage diseases (LSD) are a group of metabolic disorders that are caused by the deficiency of specific lysosomal enzymes resulting in the accumulation of undegraded substrate. This process leads to a broad spectrum of clinical manifestations depending on the specific substrate and site of accumulation. LSD can be classified into five groups: mucopolysaccharidoses (MPS), mucolipidoses (ML), oligosaccharidoses, sphingolipidoses and others, where Niemann–Pick disease and neuronal ceroid lipofuscinoses can be included (Parenti et al. 2015; Sun, 2018). Unfortunately, diagnostics, especially of mild cases with more prolonged survival, is often delayed due to clinical symptoms characteristic of other more common conditions (Platt et al. 2018). This issue is one of many reasons for improving diagnostic approaches toward LSD and implementing new analytical methods, focused on the analysis of accumulated specific substrates, e.g., mass spectrometry (MS) and nuclear magnetic resonance (NMR), which provide better and more reliable results. The purpose of this review is to present the successful application of mass spectrometry into the diagnostic process of LSD.

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Mucopolysaccharidoses

Mucopolysaccharidoses are rare inherited disorders caused by the deficit of the lysosomal hydrolases involved in the degradation of mucopolysaccharides, also known as glycosaminoglycans (GAG). Main GAG chains are based on dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) and chondroitin sulfate (CS) that are normally degraded by 11 lysosomal hydrolases via removal of monosaccharides and sulfate groups resulting in the complete degradation of the polysaccharide. There are 13 currently known MPS subtypes (Filocamo et al. 2018). Usual methods used for diagnostics of MPS are dimethylmethylene blue binding assay (DMB), thin-layer chromatography (TLC) and two-dimensional electrophoresis (2-DE). This approach takes advantage of cationic dyes, such as DMB, bind with high specificity to sulfated oligosaccharides. However, mass spectrometry is also successfully used for GAGs measurements. Urine samples usually require chemical hydrolysis (methanolysis) or enzymatic digestion with corresponding enzymes, for example, keratanase II for KS digestion

(Lin et al. 2019). Digestion of complex GAGs into smaller fragments is usually the crucial part of sample preparation before its analysis by liquid chromatography (LC) coupled with MS/MS. Chemical hydrolysis represents unspecific and relatively cost-effective cleavage, while the main advantage of enzymatic digestion lies in the specificity of each enzyme.

LC–MS/MS analysis was reported as an excellent method for diagnostics of all MPS subtypes from urine samples (Auray-Blais et al. 2012; Khan et al. 2018). GAGs in urine samples were enzymatically digested and then quantified by LC–MS/MS. Age and number of patients were different for every MPS (from 2 MPS VII patients (age 0.2–0.3 years) to 34 MPS III patients (age 3.3–66.9 years)). Porous graphitic carbon (Hypercarb, 100 Å × 2.1 mm, 5 µm), appropriate for oligosaccharides separation, was used as a chromatographic column. The mobile phase consisted of 10 mM NH_4HCO_3, and GAG disaccharides were eluted with an acetonitrile gradient. This method is useful for diagnosing the complete spectrum of MPS, and it exhibits higher sensitivity compared with DMB method. Detected disaccharides were quantified in the multiple reaction monitoring (MRM) acquisition modes, using the transition *m/z* values listed in Table 1. Another advantage of LC–MS/MS is based on the utilization of KS for the identification of MPS IVA and MPS IVB (Langereis et al. 2015).

Great specificity and sensitivity of LC–MS/MS analysis of GAG were confirmed with another analysis of urinary DS and HS. Samples were firstly methanolyzed by adding 3 N HCl in methanol and 2,2-dimethoxypropane. After the addition of internal standards and reconstitution in ammonium acetate, samples were separated by dC18 column (3.0 × 50 mm). Mobile phase A consisted of 5 mM NH_4OAc containing 0.1% formic acid and B consisted of 5 mM NH_4OAc in methanol. MS analysis was performed in positive ionization mode with a 4000 QTRAP LC–MS/MS system equipped with a TurboSpray ion source (electrospray ionization, ESI). High specificity of MRM allowed specific detection of DS and HS, in which the parent ion and its daughter ion after collision were *m/z* 426.1 → 236.2 for DS and *m/z* 384.2 → 161.9 for HS (Chuang et al. 2014). In this work, 76 urine samples were analyzed—9 MPS I patients, 13 MPS II patients, 7 MPS III patients, 8 MPS VI patients and 39 normal controls. MPS patients received enzyme replace therapy (ERT) in a varied duration (1 month to 8.8 years) and at different ages (0.7–34.9 years).

Table 1 Summary of transition *m/z* values, considered as GAG biomarkers in the urine of mucopolysaccharidoses patients, determined by multiple reaction monitoring (MRM) acquisition mode (Langereis et al. 2015)

GAG	*m/z*
KS	462.1 > 361.0
DS	458.0 > 299.9; 538.0 > 458.0
HS	378.1 > 175.1; 416.1 > 138.0; 458.1 > 97.0; 496.0 > 416.0

GAG, glycosaminoglycans; KS, keratan sulfate; DS, dermatan sulfate; HS, heparan sulfate

The usual sample types for tandem MS analysis are dried blood spots (DBS) or blood (plasma and serum), where MS/MS analysis is used for newborn screening (NBS) and diagnostics of MPS and other LSD (Kubaski et al. 2017; Gelb et al. 2006; Shimada et al. 2014). DBS samples were analyzed to simultaneously determine the activities of enzymes characteristic for MPS (α-iduronate for MPS I (*m/z* of final product 426.1), iduronate-2-sulfatase for MPS II (*m/z* 644.3), α-N-acetylglucosamidase for MPS III B (*m/z* 420.2), N-acetylgalactosamine-6-sulfatase for MPS IVA (*m/z* 685.4), N-acetylgalactosamine-4-sulfatase for MPS VI (*m/z* 657.3) and lysosomal β-glucuronidase for MPS VII (*m/z* 434.3)) using LC–MS/MS. HPLC preparation of sample was performed at the reverse phase (XSelect CSH C18 column, 130 Å, 2.1 × 50 mm, 3.5 µm). Calculated enzyme activity in DBS (µmol/h/L blood) in newborns was 35.88, while in MPS II type it was 16.11, in MPS III B type it was 1.56, in MPS IV A type it was 0.67, in MPS VI type it was 4.37 and in MPS VII type it was 28.46 (Liu et al. 2017). Another potential biomarker for MPS diagnostics is globotriaosylsphingosine (LysoGb3). Concentration of this glycosphingolipid was determined in DBS samples by HPLC–MS/MS. HPLC separation was performed by reverse-phase liquid chromatography using the XBrdige™ BEH C18 column (2.1 × 50 mm, 3.5 µm), and the MS detection was conducted with mass spectrometer set in the positive mode using an electrospray ionization source. Concentration of LysoGb3 was significantly higher in samples from MPS I, MPS II and MPS III. However, patients with MPS IVA and MPS VI showed no elevation of LysoGb3 concentration compared to healthy controls (Baydakova et al. 2020).

Cerebrospinal fluid (CSF) can be used as a potential MPS diagnostic specimen (Tanaka et al. 2018). Zhang et al. used this sample type for diagnostic of MPS I using UPLC–MS/MS. Samples obtained from 22 control pediatric subjects and 7 MPS I patients were analyzed. Methanolyzed samples were chromatographically separated with an Acquity UPLC system with autosampler. Column used for this separation was a BEH Amide column (2.1 × 50 mm; 1.7 µm) under a flow rate of 400 µL/min with a programmed linear gradient of buffer A (10 mmol/L NH_4OAc in $MeCN/H_2O$ (9:1)) and buffer B (10 mmol/L NH_4OAc in $MeCN/H_2O$ (1:9)). Similar to urine samples analysis (Chuang et al. 2014), ion transition mass-to-charge ratio (*m/z*) 426 → 236 for dimers derived from CS and DS was monitored, plus the sodiated molecular ion transition *m/z* 406 → 245 for the HS dimer. Also monitored were transitions *m/z* 412 → 251, characteristic for deuteromethylated HS dimer and transitions *m/z*

432 → 239, corresponding to deuteromethylated CS and DS dimers (Zhang et al. 2011).

Contrary to LC–ESI–MS/MS, MALDI-TOF/TOF analysis is not a convenient approach for the analysis of complex GAG due to the overlapping of matrix signals in the mass range of 0–500 Da, where the signals of disaccharides are observed. Despite this fact, GAGs represent great potential as a biomarkers for MPS diagnostics using LC–MS/MS or MALDI-TOF/TOF analyses (de Ruijter et al. 2012; Gucciardi et al. 2014; Tomatsu et al. 2010).

Mucolipidoses

There are four currently known mucolipidoses which are classified according to the enzyme that is deficient or mutated: sialidosis (ML I) caused by the deficiency of α-N-acetyl neuraminidase, ML II, ML III, both caused by deficiency of N-acetylglucosamine-1-phosphotransferase and ML IV caused by defects of mucolipin-1 (Boudewyn and Walkley 2019; Khan and Tomatsu 2020). ML are conventionally diagnosed by lysosomal enzyme analysis, sequencing of genomic DNA or real-time quantitative PCR test (Wang et al. 2019). TLC is also often used method for diagnostics of MLs. However, application of other more sophisticated and robust analytical methods is needed. As in case of MPS, MRM analysis of urine samples is also useful for MLs diagnostics (Pino et al. 2020). LC–MS/MS analysis was performed using Interchrom Uptisphere NH_2 (5 µm particle size, 120 Å, 50 mm × 2.1 mm) column. Mobile phase consisted of two solutions: A—0.2% formic acid in water and B—0.2% formic acid in acetonitrile. Ninety-seven urine samples of patients with 13 different LSDs (e.g., ML II, ML III, MPS IVB) and 240 control urine samples were analyzed. In MRMs, only one sialyl oligosaccharide in negative ionization mode (*m/z* 1200.1 > 933.5) was detected. Increased excretion of sialylated oligosaccharides could be considered as a marker of ML (Piraud et al. 2017). ML diagnostics from urine samples can also be performed by simple MALDI-TOF analysis, after sample dilution and spotting on MALDI target plate with dihydroxybenzoic acid as matrix. Bonesso et al. analyzed urine samples, which were randomly divided into a training and blind validation set (41 training sets, 53 validation sets—94 total of samples). This analysis was performed on Ultraflex III MALDI-TOF/TOF in both positive and negative ionization mode, and the obtained signals for ML were represented by *m/z* values of 933.5 and 1460.6 Da in positive mode and 1200.4, 1362.5, 1565.5, 1727.6 and 2018.6 Da in negative mode (Bonesso et al. 2014). After the permethylation of free oligosaccharides occurring in the urine of ML I, II and III patients, characteristic set of 11 unique oligosaccharide signals was obtained, while most of them were sialylated (Xia et al. 2013). However, most of the structures were not specific for ML, and they were observed also in other LSD, such as MPS or GM1 gangliosidosis, and the non-quantitative nature of MALDI-TOF/TOF is widely known. On the other hand, MALDI represents relatively easy method for fast determination of oligosaccharide profiles in urine of suspected patients.

Oligosaccharidoses and sphingolipidoses

Oligosaccharidoses are diseases characterized by increased urinary excretion of oligosaccharides due a deficiency in the lysosomal enzymes responsible for degradation of the saccharide chain of glycoproteins. Many subtypes of oligosaccharidosis (such as fucosidosis, α-mannosidosis, GM1 gangliosidosis, Sandhoff disease) are described for the purpose of biochemical screening with one of the most widely used method, TLC (Casado et al. 2014). As in case of MPS, there are other methods available to determine diagnosis of oligosaccharidoses, such as 2-DE or fluorometric enzymatic assays (Fateen et al. 2018). Identification of oligosaccharidoses can be accomplished also by MALDI-TOF/TOF analysis of permethylated urinary samples that can be purified by solid-phase extraction prior to the analysis. The analysis of urinary free oligosaccharides (FOS) by MALDI-TOF/TOF showed great potential for fast and reliable screening of oligosaccharidoses, without the need of LC pre-separation, which represents its main advantage. A plethora of specific signals were obtained using this method (Xia et al. 2013) (listed in Table 2); however, few were observed in the samples of other LSD as well, and thus, they cannot be considered as specific as reliable biomarkers. On the other hand, the excretion ratios differ with varying intensities of single structures and their overall combination could form a unique fingerprint for each oligosaccharidose in single MALDI-TOF/TOF mass spectra.

Tandem LC–MS/MS is also a valuable tool for diagnostics and screening of oligosaccharidoses. Sowell and Wood developed screening method, where urine samples were firstly derivatized with 3-methyl-1-phenyl-2-pyrazolin-5-one (PMP). The ages of the subjects were as follows: 8–17 years for I-Cell, 9–60 years for mannosidosis,

Table 2 Summary of specific glyco-biomarker signals for oligosaccharidoses obtained by MALDI-TOF/TOF analysis of permethylated urine samples (Xia et al. 2013)

Oligosaccharidosis	*m/z*
GM1 gangliosidosis	1171.6; 1375.7; 1620.8; 1824.9; 2274.1
GM2 gangliosidosis	967.4; 1212.6; 1416.7; 1661.8; 1906.9
Aspartylglucosaminuria	629.1; 990.3; 1078.0; 1439.4
Galactosialidosis	1532.8; 1824.9; 2186.1; 2547.3; 2996.5
Fucosidosis	1053.6; 1346.4; 1550.6; 1875.9; 2173.2
α-Mannosidosis	722.4; 926.5; 1130.6; 1334.7; 1538.8; 1742.9; 1947.0; 2151.1

13 years for fucosidosis, 1 year for Pompe and 6 years for galactosialidosis. Chromatography separation was performed using a Phenomenex Luna PFP column (100 mm × 2 mm, 3 µm), and mobile phases consisted of A—0.1% formic acid in water and B—0.1% formic acid in acetonitrile. MS experiments were performed by a Micromass Quattro Micro triple quadrupole mass spectrometer. Firstly, the MS behavior of derivatized oligosaccharides was characterized by choosing structural analogs to optimize MS parameters. PMP derivatization allowed the selected reaction monitoring (SRM) approach to be utilized. It was observed that signal at *m/z* 175 was always present in product ion scans despite the differences in oligosaccharides. SRM transitions were generated and the process took into consideration acetylation, deoxyhexoses and pentoses. Results of this approach showed perturbations relative to control samples, such as acetylated trihexose in mannosidosis or N-acetyl hexose–deoxyhexose in fucosidosis (Sowell and Wood 2011).

MS was used also for diagnostics of GM1 gangliosidosis in mice brains. Homogenized and diluted tissue samples were purified with solid-phase extraction (SPE) by Hypersep Hypercarb SPE cartridges. Samples were subjected to labeling with aniline, and MS analysis was performed on the electrospray-based Thermo LTQ Orbitrap XL mass spectrometer. LC separation was performed by a Glycan BEH Amide HILIC column (1.7 µm, 2.1 × 150 mm) with gradient consisting of solvent A (100 mM ammonium formate) and solvent B (acetonitrile). Many glycan metabolites were detected with this method and considered as biomarkers for GM1 gangliosidosis. Most of these biomarkers were N-acetylhexosamine (HexNAc) and hexose (Hex) oligosaccharide chains, such as $HexNAcHex_2$ (*m/z* 623.2), $HexNAcHex_3$ (*m/z* 785.2), $HexNAcHex_4$ (*m/z* 947.4), $HexNAc_2Hex_3$ (*m/z* 988.4), $HexNAc_2Hex_4$ (*m/z* 1150.4) or $HexNAc_3Hex_4$ (*m/z* 1353.5), and $HexNAc_3Hex_5$ (*m/z* 1515.5). Some of these signals were identified in human brain as well (Lawrence et al. 2019). GM1 gangliosidosis was diagnosed using MALDI-TOF analysis of glycoconjugates from dog CSF. The basic structure of GM1 consists of a ceramide core with an oligosaccharide head, including sialic acid. Furthermore, there are two molecular species of GM1 with a different ceramide. For the GM1 were identified two major signals at *m/z* 1544.87 and 1572.90, which are considered as biomarkers for this disease. This method is easy to use and relatively fast, is useful for diagnostics of canine GM1 and may be utilized for diagnostics of GM1 in other domestic animals, e.g., dogs (Satoh et al. 2011). Concentration of GM1 in CSF from dogs affected with GM1 gangliosidosis was determined in the range of 131–618 nmol/L, while from unaffected dogs the mean value was calculated as 12 nmol/L. However, the invasiveness of this method does not predetermine it for routine screening and diagnostics, and the analysis of free urinary oligosaccharides remains the most preferred method.

Another subgroup of inborn disorders related to saccharide metabolism is sphingolipidoses. This heterogeneous class is the result of enzymatic deficiencies in lysosomal degradation and subsequent tissue deposition of sphingolipids (SL). SL are fragments formed by a ceramide base which can be modified by several sugar groups and together constitute glycoSL (Conway 2016). GlycoSL have raised concentrations in sphingolipidoses; however, these primary storage molecules had low sensitivity and specificity that make them unusable as biomarkers. On the other hand, lysosphingolipids (LSL; *N*-deacetylated lyso-forms of glycoSL) have been investigated as possible biomarkers for detection of sphingolipidoses and have ability to be useful for both diagnostics and monitoring of the treatment (Polo et al. 2017). SL molecules could be identified in plasma membranes and play significant roles in structural integrity and correct biological functions of eukaryotic cells. Various enzymes are involved in their metabolic pathways, including galactosidases, glucosidases, neuraminidase, hexosaminidase, sphingomyelinase, sulfatase and ceramidase (Kumari 2018). The most known diseases of this rare group are Niemann–Pick, Fabry, Krabbe, Gaucher, Tay–Sachs disease and metachromatic leukodystrophy. Widely used diagnostic approach based on direct enzyme or molecular analysis is time-consuming. Chromatographic techniques coupled with MS represent suitable method for highly selective and sensitive biomarker analysis.

Pettazzoni et al. have analyzed LSL in plasma and amniotic fluid and brought a novel instrument for the screening of sphingolipidoses (Pettazzoni et al. 2017). Ninety-eight samples from Niemann–Pick and sphingolipidosis patients, 23 samples from other inborn errors of metabolism patients and 228 negative control samples (aged 0.1–82 years) were analyzed. After a mixed-mode cation exchange SPE (Oasis MCX), samples were analyzed by LC–MS/MS using C8 Interchrom reverse-phase column (Uptisphere 120 Å, 3 µm, 2.1 mm × 50 mm) with mobile phase A: formic acid 0.2% in water and phase B: formic acid 0.2% in acetonitrile. MS analysis was performed by API 4500 QTrap operated in the MRM mode. Summary of used ion modes and MRM values is listed in Table 3. Some respective biomarkers, such as LysoGb3, lysohexosylceramide, lysosphingomyelin or carboxylated lysosphingomyelin, were observed also in healthy individuals, however, in significantly lower amounts. (For example, the levels of LysoHexCer in the patients with Gaucher disease were 280-fold higher than its mean value for healthy population.)

Polo and co-workers have investigated effective and rapid LC–MS/MS method for simultaneous quantification of different LSL forms (Polo et al. 2017). LC separation was performed by reverse-phase using Acquity UPLC and a BEH

Table 3 MS/MS parameters for quantification of lysosphingolipids in plasma and diagnostics of various sphingolipidoses

Name of target molecule		Syndrome	Ion mode	*m/z*		References
				Parent	Daughter	
Lysoglobotriaosylceramide (lysoGb3)	Fabry		Positive	786.5	282.4	Gold et al. (2013), Mirzaian et al. (2017), Pettazzoni et al. (2017), Polo et al. (2017)
Lysohexosylceramide	Gaucher			462.3		Pettazzoni et al. (2017)
Glucosylsphingosine				464.2		Dekker et al. (2011)
Hexosylsphingosine (HexSph)	Krabbe			462.4		W. L. Chuang et al., (2013), Mirzaian et al. (2017), Polo et al. (2017)
Lysosphingomyelin (lysoSM)	Niemann–Pick	Type A/B		465.3	184.0	Mirzaian et al. (2017), Pettazzoni et al. (2017), Polo et al. (2017)
Carboxylated lysosphingomyelin (lysoSM-509)		Type C		509.3		Pettazzoni et al. (2017), Polo et al. (2017)
Lyso-GM2 ganglioside	Tay–Sachs			1118.0		Kodama et al. (2011)
	GM2 gangliosidoses		Negative	1116.6	290.1	Pettazzoni et al. (2017)
Lyso-GM1 ganglioside	GM1 gangliosidoses			1278.6		

C18 column, 2.1 × 50 mm with 1.7 µm particle size. Mass spectrometry detection was run out with a Xevo TQ-S micro detector in positive mode using ESI source. For each target compound, the $[M+H]^+$ species were selected as precursor ions. Piraud's team carried out analysis with a triple quadrupole device (API 3200) in positive ionization mode. Each Gb3 isoform has been analyzed by its transition and quantified according to the signal of the internal standard C17-Gb3 (Piraud et al. 2010). LysoGb3, lysosphingomyelin and hexosylsphingosine have been studied using UPLC–ESI–MS/MS with identical ^{13}C5-encoded standards that offer advantages in mass spectrometric measurements (Mirzaian et al. 2017). The obtained data are summarized in Table 3.

Lyso-GM2 ganglioside (lyso-GM2) has been examined after its extraction from high-performance thin-layer chromatography (HPTLC) silica gel plates (Kodama et al. 2011). Positive-ion MALDI-TOF MS and MS/MS spectra of the lyso-GM2 were acquired by an UltrafleXtreme mass spectrometer for the purpose to confirm abnormality in accumulated structures. The spectrum showed a main $[M+H]^+$ molecular ion signal at m/z 1118. According to this study, lyso-GM2 can be considered as a possible biomarker of Tay–Sachs and Sandhoff disease. Lyso-GM2 ganglioside was not determined in negative controls (Pettazzoni et al. 2017).

Level of psychosine (hexosylsphingosine) in dried blood spots from newborns that were recognized to be at risk of Krabbe disease was researched as well (Chuang et al. 2013). HPLC–MS/MS methodology, based on the combination of two Acquity UPLC Beh HILIC columns (2.1 × 150 mm, 1.7 µm particle size) and an API 5000 triple quadrupole mass spectrometer with TurboSpray ion source, was chosen to detect concentrations of glucosylsphingosine and psychosine. Quantification was performed by comparing the obtained psychosine/internal standard peak area ratio for each sample to those ratios generated by a matrix-based calibration curve. LC–ESI–MS/MS detected glucosphingosine by its chromatographic elution achieved on a BEH C18 column (1.0 × 50 mm, 1.7 µm) and a tandem quadrupole mass spectrometer (TQD) operating in positive mode (Dekker et al. 2011). A triple-quadrupole MS/MS system (API 5000), operated in positive-ion mode, was also used for measurement of hexosylsphingosine in dried blood spots for a possible improvement to newborn screening for Krabbe disease (Conway, 2016).

Other LSD

Other LSD subtypes include Pompe disease, Niemann–Pick disease type C (NPC) and neuronal ceroid lipofuscinoses (NCLs).

NCL is a family of genetically separate neurodegenerative LSD (PPT1/*CLN1*, TPP1/*CLN2, CLN3, CLN5, CLN6, CLN7, CLN8, DNAJC5, MFSD8, CTSD* and possibly even other genes are involved). In a study by Käkelä et al., phospholipids were analyzed by online LC–ESI–MS analysis and decreased overall quantities of all classes, except lysobisphosphatidic acid, were observed in infantile NCL (Käkelä et al. 2003). On the other hand, in juvenile NCL phospholipid species composition was almost identical to that of the controls. The most dramatic changes occurred in phosphatidylserines (PS) as its major species, i.e., 40:6 (*m/z* 834 in negative ion mode), represented over 40% of all PS in the control brains, but was nearly absent (0.1 and 3.5%) in the infantile NCL brain. Levels of some other phospholipid molecular species were altered as well, as summarized in Table 4. For separation of extracted phospholipids, an Interchrom LiChrospher DIOL column (250 × 1 mm, 5 µm particle size), with both

Table 4 Summary of altered levels of phospholipid species in brain of infantile neuronal ceroid lipofuscinosis patients, determined by LC–ESI (Käkelä et al. 2003)

Phospholipid class	Increased molecular species (*m/z*)	Decreased molecular species (*m/z*)
PS	38:4 (810), 38:3 (812), 40:3 (840), 34:1 (760), 36:1(788), 36:2 (786), 38:2(814)	40:6 (834)
PC	32:1 (732), 34:1 (760)	38:4 (810), 38:6(806), 34:0 (762), 36:1 (788)
PE	Diacyl 36:2 (742), 34:1 (716), 34:2 (714), 38:5 (764) and alkenylacyl 38:5 (748), 36:4 (722)	Diacyl 40:6 (790), 38:4 (766), 40:4 (794)
PI	36:3 (859), 38:5 (883), 38:3 (887)	38:4 (885), 40:6 (909)
LBPA	36:2 (773)	–

For each phospholipid class, representative both increased and decreased species and their *m/z* values are listed

PS, phosphatidylserines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols; LBPA, lysobisphosphatidic acids

polar and hydrophobic properties, was used and the mass spectra were acquired in both ionization modes by the Esquire ESI ion-trap mass spectrometer. However, analysis was performed on the postmortem samples of cerebral cortex that poses markedly limiting factor, and this approach is not useful for the early diagnostics of NCL.

Another utilization of mass spectrometry in molecular characterization of NCL was published in 2008, where *CLN6* mouse knockout models, at the age of 52 weeks, exhibited ninefold and fourfold increased levels of GM3 (*m/z* 1179) and GM2 (*m/z* 1383) gangliosides in the cerebral hemispheres, when compared with wild-type mice (Jabs et al. 2008). However, there are also numerous other LSD such as glycosphingolipidoses, mucopolysaccharidoses and certain types of glycoproteinoses exhibiting significant secondary accumulation of GM2 and GM3 (Walkley, 2004). According to this fact and the invasive origin of the sample, this approach is not suitable for NCL diagnostics. Due to its genetic complexity, no specific metabolite serves as a widely used and reliable biomarker of NCL yet. Nevertheless, such functional studies using MS-based approaches may provide potential pathways to probe for candidate biomarkers in future.

Most of the recent NCL OMICS-based studies are dedicated to the proteomic approach (Kline et al. 2020). Complex proteomic data confirmed the upregulation of many proteins in juvenile NCL, already associated with neurodegeneration, such as brain-derived neurotrophic factor, neuronal cell adhesion molecule, clusterin, adiponectin, apolipoprotein E, vascular cell adhesion protein 1 and myoglobin (Hersrud et al. 2016). Nowadays, recent developments in proteomics technology offer new opportunities for clinical applications in hospital or specialized laboratories including the identification of novel biomarkers, monitoring of disease, etc. (Apweiler et al. 2009).

Metabolomics usually offers essential data for biomarker discovery; however, identifying unknown metabolites remains challenging. Differentially expressed small-molecule metabolites in cerebrospinal fluid (CSF) of *CLN2* patients were determined by untargeted metabolic profiling by LC–MS in 2018 (Sindelar et al. 2018). In this work, 21 samples were analyzed (11 CLN2 disease cases and 10 negative controls). Metabolites, extracted from CSF, were subjected to aqueous normal-phase LC that was performed using Cogent Diamond Hydride column (4 µm, 100 Å, 150 mm × 2.1 mm), coupled with an Agilent 6230 TOF mass spectrometer with a dual electrospray ionization source. By this comprehensive approach, six unique metabolites were determined in significant correlation with the disease severity in the term of their reduction. They were described as N-acetylaspartyl glutamate (*m/z* 303.8 and its adduct at *m/z* 401.0), glycero-3-phosphoinositol (*m/z* 333.1), N-acetylneuraminic acid and its dimer (*m/z* 310.1 and 601.2), N-acetylalanine (*m/z* 130.1), N-acetylserine and N-acetylthreonine. Reduction of these levels have not been detected in any other disease and thus offer a unique biomarker signature for CLN2 disease diagnosis and prognosis.

Mass spectrometry played an essential role also in the newborn screening of NCL type 2, based on the determination of TPP1 activity in DBS (Liu et al. 2017). In this study, a new substrate for TPP1 (*m/z* 639.4) was prepared and, after its incubation with DBS punch, action of TPP1 enzyme resulted in benzylamide bond cleavage releasing the product (*m/z* 350.2). After the addition of internal standard (*m/z* 359.3), the product's levels were determined, with sample separation at reverse-phase HPLC (XSelect CSH C18 column, 130 Å, 3.5 µm, 2.1 mm × 50 mm), by MRM format on a Xevo TQMS tandem quadrupole mass spectrometer. Calculated mean TPP1 activity (µmol/h/L blood) in newborns was 35.9, while in NCL type 2 it was 1.3. Synthetic compounds were designed to mimic the natural substrates, and such assays can be run in multiplex formats for the detection of other LSD as well (Barcenas et al. 2014).

Diagnostic possibilities for Pompe disease by MS were discussed in our previous studies (Pakanová et al. 2016, 2019) and in work of other scientific groups (Sista et al. 2011). Due to the disruption in glycogen degradation, oligohexose chains are accumulated in the urine of Pompe patient and can be easily detected by MALDI-TOF MS. Diluted urine can be directly spotted onto MALDI target plate, premixed with dihydroxybenzoic acid as a matrix and analyzed in positive-ion mode. Due to the effective ionization of oligohexose units in positive MALDI ion source, no sample derivatization (permethylation, labeling of the reducing end, etc.) is required. However, the main disadvantage of MALDI-TOF MS includes no information about the linkage between the monosaccharide units, and thus, it should be used in combination of other analytical methods, such as NMR spectroscopy, that can precisely determine the linkages between monosaccharide units as well as the quantity of selected biomarkers.

New strategies in LSD diagnostics

The development of multiplex assays, combining the analyses of more lysosomal enzymes using a single MS/MS instrument, represents the new challenge due to the widespread interest in expanding newborn screening. In 2013, the first large-scale pilot study of three LSD (Fabry, MPS I and Pompe disease), using flow injection MS/MS, showed the feasibility of this method (Scott et al. 2013). In 2016, a six-plex flow injection MS/MS assay for six LSD (Fabry, Gaucher, Krabbe, MPS I, Niemann–Pick A/B and Pompe disease) was successfully performed on 43,000 newborns (Elliott et al. 2016), where all product and deuterated internal standard MRM peaks were integrated. This approach used a single assay cocktail with a highly optimized buffer for all enzymes that led to shortening the pre-MS/MS workup by elimination of the solid-phase extraction step. During 2010 in Austria, specimens from DBS of 34,736 newborn babies were subjected to multiplex assay (Gaucher, Pompe, Fabry and Niemann–Pick disease A/B) as part of the national routine newborn screening program, using positive SRM mode in API 2000 ESI–MS/MS, equipped with TurboSpray ion source (Mechtler et al. 2012). For NBS of α-mannosidosis and fucosidosis, multiplex tandem MS-based enzyme assay using DBS was also implemented. α-Mannosidosis assay was combined with screening assay for Fabry, Pompe, Gaucher, Niemann–Pick, MPS I and Krabbe disease. Fucosidosis assay was combined with screening assay of five other MPS. DBS was placed for incubation with the cocktail of various substrates and internal standards, samples were subjected to combined LC–MS/MS, and the activities of specific enzymes were determined. These assays were considered as great for NBS and diagnostics of fucosidosis, α-mannosidosis and other LSD as well (Kumar et al. 2019).

Digital microfluidics (DMF) represents a new, promising approach for newborn screening. A multiplex assay has been developed on the digital microfluidics platform for four LSD: MPS I and VII, Pompe and Fabry disease. However, these results are preliminary. Nevertheless, there is reason to be encouraged by the ease with which the device handles these assays and the considerable reduction in time to result compared with current end-point assays. The key characteristics that make digital microfluidic technology attractive include its cost effectivity, low sample volume required, possibility of automation, portability and broad sample compatibility (Mechtler et al. 2012).

There is a plethora of scientific papers comparing mass spectrometry with other analytical and diagnostic methods. Some of the most valuable advantages of mass spectrometry are high sensitivity, low requirements for sample amount, high accuracy, high specificity and full automation of the assay. However, there are some drawbacks of these methods, such as high start-up cost and requirements for personnel, time consuming data acquisition, etc. (Gelb et al. 2006; Israr et al. 2020). Compared to MS, DMF has a key advantage in miniaturization, simplicity, lower cost of the device and reduced time to generate results. However, DMF is difficult to couple to external detectors and pumps, and it is not universal and requires the use of fluorescent reporters (Millington et al. 2018; Wang et al. 2015). Due to these significant disadvantages, the progress in development provides more potential of mass spectrometry toward LSD diagnostics.

Conclusion

MS-based diagnostics of LSD offers a robust, highly reliable and specific method to determine the structures and quantities of respective biomarkers, mostly represented by the accumulated substrates. Up to this date, several studies dedicated to analysis of possible or already approved biomarkers of LSD have been published; however, due to the relatively high cost of MS analyses, some laboratories still prefer traditional diagnostic methods. Future trends of automatization and high-throughput approaches should include MS-based diagnostics as it offers precise information about the presence or severity of disease.

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Declarations

Conflict of interest The paper is not currently being considered for publication elsewhere. The authors declare that they have no conflicts of interest.

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