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ORIGINAL ARTICLE

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Analysis of urinary oligosaccharide excretion patterns by UHPLC/HRAM mass spectrometry for screening of lysosomal storage disorders

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Abstract

Oligosaccharidoses, sphingolipidoses and mucolipidoses are lysosomal storage disorders (LSDs) in which defective breakdown of glycan-side chains of glycosylated proteins and glycolipids leads to the accumulation of incompletely degraded oligosaccharides within lysosomes. In metabolic laboratories, these disorders are commonly diagnosed by thin-layer chromatography (TLC) but more recently also mass spectrometry-based approaches have been published. To expand the possibilities to screen for these diseases, we developed an ultra-high-performance liquid chromatography (UHPLC) with a high-resolution accurate mass (HRAM) mass spectrometry (MS) screening platform, together with an open-source iterative bioinformatics pipeline. This pipeline generates comprehensive biomarker profiles and allows for extensive quality control (QC) monitoring. Using this platform, we were able to identify α-mannosidosis, β-mannosidosis, α-*N*-acetylgalactosaminidase deficiency, sialidosis, galactosialidosis, fucosidosis, aspartylglucosaminuria, GM1 gangliosidosis, GM2 gangliosidosis (M. Sandhoff) and mucolipidosis II/III in patient samples. Aberrant urinary oligosaccharide excretions were also detected for other disorders, including NGLY1 congenital disorder of deglycosylation, sialic acid storage disease, MPS type IV B and GSD II (Pompe disease). For the latter disorder, we identified heptahexose (Hex7), as a potential urinary biomarker, in addition to glucose tetrasaccharide (Glc4), for the diagnosis and monitoring of young onset cases of Pompe disease. Occasionally, so-called "neonate" biomarker profiles were observed in young patients, which were probably due to nutrition. Our UHPLC/HRAM-MS screening platform can easily be adopted in biochemical laboratories and allows for simple and robust screening and straightforward interpretation of the screening results to detect disorders in which aberrant oligosaccharides accumulate.

KEYWORDS

bioinformatics pipeline, lysosomal storage disorders, oligosaccharidoses, semi-quantitative automated analysis, UHPLC/HRAM mass spectrometry

1 | INTRODUCTION

Oligosaccharidoses are lysosomal storage disorders (LSDs), which are characterized by the accumulation of carbohydrate side chains of glycosylated proteins and glycolipids due to defects in enzymes involved in the sequential breakdown of oligosaccharides.[1] As a group, they share common clinical features, for example, mental retardation, coarse facial features, and organomegaly, but depending on the type of glycan storage material various disease-specific clinical manifestations are present.[2] An overview of LSDs in which anomalous glycans accumulate is given in Table 1, together with corresponding defective enzymes and distinct storage material.

The most commonly used qualitative screening method for oligosaccharidoses is one-dimensional thin-layer chromatography (TLC) by which urinary oligosaccharides are separated on silica gel plates and visualized by sulfuric orcinol staining.[3] Free sialic acids or sialyloligosaccharides can be visualized by using resorcinol reagent[4] and oligosaccharides containing amino acids by ninhydrin staining.[5] Although TLC is relatively inexpensive and easy to perform, interpretation of the results poses several challenges due to (i) limited chromatographic resolution, (ii) age-, drug- and nutrition-induced artifacts, and (iii) the required experience with respect to a correct interpretation of complex patterns.[6] Indeed, European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) diagnostic proficiency testing of oligosaccharidurias revealed relatively low proficiency of participating centers underscoring its diagnostic challenges.[7]

Recently developed novel mass spectrometry (MS)-based methods largely overcome these analytical and interpretative difficulties with (i) electrospray ionization (ESI)-MS coupled to (ultra)-high-performance liquid chromatography (HPLC) and (ii) matrix-assisted laser desorption/ionization (MALDI) coupled to time-of-flight (TOF) MS being adopted in diagnostic biochemical laboratories, often in conjunction with glycan derivatization prior to analysis (see Table S1).[8] In the last decade, the first few tandem-MS screening methods that do not require derivatization for the detection of oligosaccharidurias have been reported.

The first structural characterization of underivatized urinary oligosaccharides was performed by MALDI-TOF/TOF in both positive ion mode (complex oligosaccharides and glycoamino acids) and negative ion mode (sialylated oligosaccharides).[9] This approach was subsequently adapted into a qualitative LC/MS/MS method in which the application of disorder-specific multiple reaction monitoring (MRM) expanded the number of detectable urinary oligosaccharidoses.[10] An additional novel UHPLC–MS/MS method adopted these MRM transitions with the inclusion of the negative MRM transition of glucose tetrasaccharide (Glc4).[11] Similarly to these three approaches, a LC–MS/MS glycomic profiling method was published[12] in which all of the previously published oligosaccharidoses could be detected[10,13,14] but also NGLY1 deficiency and several mucopolysaccharidoses (MPS).

Here, we describe a highly sensitive ultra-high-performance liquid chromatography/high-resolution accurate mass (UHPLC/HRAM) mass spectrometry method for the rapid screening of LSDs in which oligosaccharides accumulate in underivatized urine samples. We adapted previously published biomarkers[9,10] and developed a negative-ESI MS screening method, together with a novel intuitive automated analysis pipeline that (i) calculates biomarker-specific semi-quantitative concentrations, (ii) establishes Z-scores for individual components corrected by age using reference samples, (iii) generates Z-score plots of all biomarkers accompanied by disease-specific values, and (iv) enables extensive batch-specific quality control (QC) monitoring, which allows for fast, intuitive, and reliable interpretation of the screening results.

2 | MATERIALS AND METHODS

2.1 | Reagents and materials

Ammonium acetate (≥98% purity) and acarbose (≥95% purity) were purchased from Sigma-Aldrich. Ammonium hydroxide solution p.a. was obtained from Honeywell, UPLC water (ULC-MS grade) from Biosolve and acetonitrile (hypergrade) from Merck. *N*-acetyl-D-[1,2,3-$^{13}C_3$] neuraminic acid ($^{13}C_3$-NANA) and hexaacetyl-chitohexaose were purchased from Omicron Biochemicals Inc. and Neogen, respectively. The ACQUITY UPLC BEH Amide Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm and ACQUITY UPLC BEH Amide VanGuard Pre-column, 130 Å, 1.7 µm, 2.1 mm × 5 mm were purchased from Waters.

2.2 | Patient material

Urine samples were obtained from patients of which diagnoses were previously confirmed by TLC or NANA

TABLE 1 Overview of disorders included in this study.

	Disease	Gene	Enzyme/protein deficiency	Accumulated products	OMIM #
Oligosaccharidoses	Sialidosis (Mucolipidosis I)	*NEU1*	Neuraminidase	Sialyl-OS	256550
	Galactosialidosis	*CTSA*	Cathepsin A	Sialyl-OS and Galactosyl-OS	256540
	α-Mannosidosis	*MAN2B1*	α-Mannosidase	Mannosyl-OS	248500
	β-Mannosidosis	*MANBA*	β-Mannosidase	Hex-HexNac and derivatives	248510
	α-*N*-Acetylgalactosaminidase deficiency	*NAGA*	α-*N*-acetylgalactosaminidase	HexNAc-Ser, HexNAc-Thr, Glycosphingolipids	609241
	Fucosidosis	*FUCA1*	α-Fucosidase	Fucosyl-OS	230000
	Aspartylglucosaminuria	*AGA*	Aspartylglucosaminidase	GlcNAc-Asn and glycoasparaginyl-OS	208400
Sphingolipidoses	GM1 gangliosidosis	*GLB1*	β-Galactosidase	Galactosyl-OS	230500
	GM2 gangliosidosis (M. Sandhoff)	*HEXB*	Hexosaminidase A and B	GlcNAc-OS	268800
Mucolipidoses	Mucolipidosis II (I-Cell Disease)	*GNPTAB*	UDP-*N*-acetylglucosamine-1-phosphotransferase	Sialyl-OS	252500
	Mucolipidosis III (Pseudo-Hurler)	*GNPTAB or GNPTG*			252600
Mucopolysachharidoses	MPS type IV B (Morquio syndrome)	*GLB1*	β-Galactosidase	Galactosyl-OS	253010
Congenital disorder of deglycosylation	NGLY1 deficiency (CDDG1)	*NGLY1*	*N*-Glycanase I	GlcNAc-Asn and glycoasparaginyl-OS	615273
Other lysosomal storage disorders	Sialic acid storage disease	*SLC17A5*	Sialin (transporter)	NANA	269920 604369
	M. Pompe (GSD type II)	*GAA*	α-1,4-glucosidase	Tetraglucoside and other glucosides	232300

Abbreviations: CDDG1, congenital disorder of deglycosylation 1; GM1, monosialotetrahexosylganglioside; GM2, disialotetrahexosylganglioside; GSD, glycogen storage disorder; MPS, mucopolysaccharidosis; NGLY1, *N*-glycanase 1; NANA, *N*-acetylneuraminic acid; OS, oligosaccharide; OMIM, Online Mendelian Inheritance in Man.

measurement, as well as enzyme and/or genetic testing. The urine of individuals who screened negative for inborn errors of metabolism (residual material) were included as normal control samples in this study. In agreement with national legislation and institutional guidelines, patients or their guardians approved the possible anonymous use of the remainder of their (pseudonymized) samples for method validation purposes. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

2.3 | Sample preparation

Urine samples were thawed in a water bath at 37°C. Next, 30 μL of each sample was transferred into an Eppendorf tube and combined with 270 μL of internal standard solution (20 μmol/L; $^{13}C_3$-NANA, acarbose, and hexaacetyl-chitohexaose in water). These samples were briefly vortexed and centrifuged at 13 000 rpm for 5 min. Following centrifugation, 100 μL supernatant of each sample was transferred into a 2 mL glass vial with an insert for analysis.

2.4 | Ultra-high-performance liquid chromatography

Chromatographic separation of analytes in diagnostic, positive and negative quality control (QC) samples was achieved by injection of 2.5 μL sample onto the ACQUITY UPLC BEH Amide Column. Prior to injection, the needle was rinsed using 10:90 water/acetonitrile +0.1% (v/v) ammonium hydroxide solution to ensure optimal chromatographic peak shape and retention time for *N*-acetyl neuraminic acid (analyte) and $^{13}C_3$-NANA (internal standard). Chromatographic elution was achieved under gradient conditions between buffer A (5 mM ammonium acetate 10:90 water/acetonitrile + 0.1% ammonium hydroxide) and buffer B (5 mM ammonium acetate 70:30 water/acetonitrile + 0.1% (v/v) ammonium hydroxide; pH 9). Elution started with 99% buffer A, followed by a linear decrease to 50% (0–1.5 min) and 50%–5% (2.5–4.0 min). This condition was maintained for 2 min before returning to the starting composition. The temperature of the column was 40°C with a flow rate of 0.45 mL/min.

2.5 | HRAM mass spectrometry analysis and quantification

HRAM mass spectrometry analysis was performed using the Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher), which was operated with a spray voltage of 3.50 kV in negative ionization mode with a resolution of 70 000 (m/Δm, FWHM @ 200 m/z) and a maximum injection time of 200 ms. The sheath gas flow rate and auxiliary gas rate were set at 40 and 15 arbitrary units, respectively, with nitrogen gas. The capillary and auxiliary gas heater temperatures were 350 and 250°C, respectively. The S-lens value was set at 80. Oligosaccharide components were detected in full scan mode with a mass range of 150–2000 *m/z*. Before each run, a mass calibration was performed instead of using a lock mass for the analysis. Quantification of the components was achieved within a mass accuracy of 5 ppm.

2.6 | Bioinformatics pipeline and statistical analysis

Analysis of raw data was performed using TraceFinder software (Thermo Fisher Scientific). The concentration of each component was calculated semi-quantitatively: (peak area analyte/peak area internal standard) × [internal standard]. For each component of interest, one of the three internal standards was used to estimate the semi-quantitative concentration (acarbose for components <800 *m/z* and hexaacetyl-chitohexaose for components >800 *m/z*). NANA was reported quantitatively using $^{13}C_3$-NANA as an internal standard.

Statistical analysis and data visualization were performed using our open-source analysis pipeline that consists of the following steps:

1. The semi-quantitative concentration of each component was normalized to the creatinine concentration, obtained by a separate quantitative measurement (Roche Modular P), to obtain values in mmol/mol creatinine.
2. Results obtained from multiple samples across different experimental runs (i.e., batches) were merged to obtain a large dataset for the determination of (age dependent) reference values for individual components.
3. For each patient and component (Steps 1 and 2), a Z-score was determined using two possible approaches:
   a. Fixed: For components having <100 non-zero measurements, the mean and standard deviation was taken from all measurements (including measurements that are equal to zero). Subsequently, the Z-score was obtained by subtracting the mean and dividing it by the standard deviation.
   b. Regression: For components having more than 100 non-zero measurements, we used the

following (polynomial) regression model that corrects for age:

$$\widehat{y}_i = \widehat{\beta}^{\text{Intercept}} + \sum_{p=1}^{3} \widehat{\beta}_p^{\text{Age}} \left(x_i^{\text{Age}}\right)^p + \widehat{\epsilon}_i \qquad (1)$$

where $\widehat{y}_i$ is the estimated mean for sample i. $\widehat{\beta}^{\text{Intercept}}$ is the intercept coefficient, and $\widehat{\beta}_p^{\text{Age}}$'s are the slopes for each polynomial term. x_i^{Age} indicates the age of sample i and $\widehat{\epsilon}_i$ being the error term. Ordinary least square estimation was used to fit the model parameters. The standard deviation was determined from a more extensive procedure, details of which can be found in the study by Bongaerts et al.[15] The obtained mean and standard deviation from the fitted model was used to calculate the Z-scores for each sample and compound for the analyzed batch. Prior to fitting the model parameters values in the reference batches with $|\text{Z-score}| > 3$ were removed. Note that the latter Z-scores were determined from the mean and standard deviation calculated from all reference samples.

4. QC criteria were monitored for each batch run. The IS and the QC samples had to meet the following four requirements:
   a. An intrabatch coefficient of variation (CV) < 60% for the IS.
   b. Z-scores of IS peak areas (mean per batch across all batches) < 3 standard deviations (SD).
   c. $2 < |Z| < 3$ for Z-scores of IS peak areas was not allowed to occur in more than two successive batches.
   d. Expected biomarkers had to be detected in the positive quality control samples.

   If the QC did not meet these requirements, the batch run was rejected and needed to be analyzed again. If accepted, additional QC plots were generated for monitoring of additional QC parameters, for example, trend analyses of the measurements

5. The Z-scores were plotted together with disease-associated Z-scores obtained from confirmed inherited metabolic disease (IMD) patients that served as disease reference values.

2.7 | Availability of the analysis pipeline

All resources and code to run our UHPLC/HRAM MS screening pipeline are available on GitHub repository: https://github.com/mbongaerts/OLIGO_pipeline

3 | RESULTS

3.1 | Development of an UHPLC/HRAM MS processing method

To develop a semi-quantitative UHPLC/HRAM MS screening platform, we curated mass-to-charge (m/z) ratios of previously published biomarkers (see Table S1). Chromatographic separation of underivatized (pathological) glycans in urine samples was established by hydrophilic interaction liquid chromatography (HILIC), which allowed for the identification of disease-specific elution peaks with sufficient resolution for incorporation into a single 4-minute chromatographic run. We optimized chromatography and MS to detect all oligosaccharides in the negative ionization mode only. An overview of the analytical parameters that were implemented in our HRAM-MS screening platform is provided in Table 2.

3.2 | Open-source diagnostic workflow and analysis pipeline

We developed an open source analysis workflow of which a schematic overview is given in Figure 1. (Age)-dependent Z-scores of all the biomarkers in both patient and positive QC samples were calculated using (previously) marked reference batch files, that is, a reference population consisting of non-IMD patient measurements (see Section 2 for decision criteria, Figure S1 for examples of reference populations and Supplementary Table S2 for the composition of the positive QC samples). In parallel, extensive quality control (QC) parameters were being monitored of which graphical output files are generated (see Figure S2). Trend analysis was performed by monitoring the interassay variation of the IS peak areas (see Figure S2a). Importantly, data processing by our analysis pipeline is an iterative procedure, which allows for optimization and refinement of the normal control and patient reference datasets, resulting in more accurate diagnostics over time.

3.3 | UHPLC/HRAM MS screening pipeline validation

We validated our analysis workflow by investigation of urine samples from known patients diagnosed with fucosidosis ($n = 4$), GM1 gangliosidosis ($n = 9$), GM2 gangliosidosis ($n = 8$), NAGA deficiency ($n = 3$), aspartylglucosaminuria ($n = 4$), α-mannosidosis ($n = 8$), β-mannosidosis ($n = 4$), sialidosis ($n = 6$), galactosialidosis ($n = 3$), mucolipidosis (ML) type II/III ($n = 10$), and sialic acid storage disease

TABLE 2 Oligosaccharide-species detected by UPLC/HRAM mass spectrometry in patient urine samples.

IEM	Rt (min)	Component	m/z	Adduct	Chemical formula	Oligosaccharides	ID	Reference
Fucosidosis ($n = 4$)	2.66	366_fuco (sec)	366.1400	[M-H]-	C14H25NO10	GlcNAc-Fuc	3	Ramsay et al.[16]
	3.39	480_fuco	480.1835	[M-H]-	C18H31N3O12	Asn-GlcNAc-Fuc	3	Bonesso et al.[9]
	3.37	1055_fuco	1055.3784	[M-H]-	C40H68N2O30	GlcNAc-Man-Man-GlcNAc(-Fuc)-Gal	3	Bonesso et al.[9]
GM1 Gangliosidosis ($n = 9$) MPS IV B ($n = 3$)	3.27	945_GM1	945.2972	[M + Cl]-	C34H58N2O26	GlcNAc-Man-Man-GlcNAc-Gal	3	Bonesso et al.[9]
	3.29	909_GM1	909.3205	[M-H]-	C34H58N2O26	GlcNAc-Man-Man-GlcNAc-Gal	3	Bonesso et al.[9]
	3.38	1071_GM1	1071.3812	[M-H]-	C40H68N2O31	GlcNAc-Man-Man-GlcNAc-Gal-Gal	3	Bonesso et al.[9]
	3.39	1274_GM1	1274.4527	[M-H]-	C48H81N3O36	GlcNAc-Man–Man–Man-GlcNAc-GlcNAc-Gal	3	Bonesso et al.[9]
	3.45	1436_GM1	1436.5055	[M-H]-	C54H91N3O41	GlcNAc-Man(-Man-GlcNAc-Gal)-Man-GlcNAc-Gal	3	Bonesso et al.[9]
	3.45	1472_GM1	1472.4822	[M + Cl]-	C54H91N3O41	GlcNAc-Man(-Man-GlcNAc-Gal)-Man-GlcNAc-Gal	3	Bonesso et al.[9]
GM2 Gangliosidosis ($n = 8$)	3.19	747_GM2 (sec)	747.2677	[M-H]-	C28H48N2O21	GlcNAc-Man-Man-GlcNAc	3	Bonesso et al.[9]
	3.19	783_GM2 (sec)	783.2444	[M + Cl]-	C28H48N2O21	GlcNAc-Man-Man-GlcNAc	3	Bonesso et al.[9]
	3.33	1112_GM2	1112.3999	[M-H]-	C42H71N3O31	GlcNAc-Man(-Man-GlcNAc)-Man-GlcNAc	3	Bonesso et al.[9]
	3.33	1148_GM2	1148.3766	[M + Cl]-	C42H71N3O31	GlcNAc-Man(-Man-GlcNAc)-Man-GlcNAc	3	Bonesso et al.[9]
	3.36	1315_GM2	1315.4793	[M-H]-	C50H84N4O36	GlcNAc-Man(-Man-GlcNAc)-Man-(GlcNAc)-GlcNAc	3	Bonesso et al.[9]
	3.36	1351_GM2	1351.4559	[M + Cl]-	C50H84N4O36	GlcNAc-Man(-Man-GlcNAc)-Man-(GlcNAc)-GlcNAc	3	Bonesso et al.[9]
NAGA deficiency ($n = 3$)	3.25	307_alfa-NAG	307.1147	[M-H]-	C11H20N2O8	Ser-GalNAc	3	Piraud et al.[10]
	3.42	321_alfa-NAG	321.1303	[M-H]-	C12H22N2O8	Thr-GalNAc	3	Piraud et al.[10]
Aspartylglucosaminuria ($n = 4$) NGLY1 deficiency ($n = 2$)	3.38	334_aspartylglucos	334.1256	[M-H]-	C12H21N3O8	Asn-GlcNAc	3	Bonesso et al.[9]
	3.47	496_aspartylglucos	496.1784	[M-H]-	C18H31N3O13	Asn-GlcNAc-Gal	3	Bonesso et al.[9]
	3.18	787_aspartylglucos	787.2738	[M-H]-	C29H48N4O21	Asn-GlcNAc-Gal-NANA	3	Bonesso et al.[9]
α-Mannosidosis ($n = 8$)	3.11	544_α-mann	544.1883	[M-H]-	C20H35NO16	GlcNAc-Man-Man	3	Bonesso et al.[9]
	3.11	580_α-mann	580.1650	[M + Cl]-	C20H35NO16	GlcNAc-Man-Man	3	Bonesso et al.[9]
	3.35	868_α-mann	868.2940	[M-H]-	C32H55NO26	GlcNAc-Man-Man-Man-Man	3	Piraud et al.[10]
	3.34	904_α-mann	904.2706	[M + Cl]-	C32H55NO26	GlcNAc-Man-Man-Man-Man	3	Piraud et al.[10]
	3.44	1030_α-mann	1030.3462	[M-H]-	C38H65NO31	GlcNAc-Man-Man-Man-Man-Man	3	Bonesso et al.[9]

(Continues)

TABLE 2 (Continued)

IEM	Rt (min)	Component	m/z	Adduct	Chemical formula	Oligosaccharides	ID	Reference
β-Mannosidosis ($n = 4$)	2.85	382_β-mann	382.1355	[M-H]-	C14H25NO11	GlcNAc-Man	3	Piraud et al.[10]
	2.83	418_β-mann	418.1122	[M + Cl]-				
Sialidosis ($n = 6$), Galactosialidosis ($n = 3$), ML II/ML III ($n = 10$)	3.16	1008_(galacto)sia	1008.8445	[M-2H]2-	C76H125N5O57	GlcNAc-Man-(Man-GlcNAc-Gal-NANA)-Man-GlcNAc-Gal-NANA	3	Xia et al.[13]
	3.11	1200_(galacto)sia	1200.4159	[M-H]-	C45H75N3O34	GlcNAc-Man-Man-GlcNAc-Gal-NANA	3	Bonesso et al.[9]
Sialic acid storage disease ($n = 11$)	2.80	*N*-acetylneuraminic acid	308.0987	[M-H]-	C11H19NO9	NANA	1	van den Bosch et al.[17]
GSD type 2 (M. Pompe) ($n = 18$)	3.31	665_Glc4	665.2146	[M-H]-	C24H42O21	Tetraglucoside	3	Sluiter et al.[18]
	3.44	989_Hex6 (sec)	989.3202	[M-H]-	C36H62O31	Hex6	4	
	3.50	1151_Hex7	1151.3731	[M-H]-	C42H72O36	Hex7	4	This study
Sialyllactose	3.52	sialyllactose	632.2044	[M-H]-	C23H39NO19	Gal-Glc-NANA	4	This study

Abbreviations: Asn, asparagine; Fuc, fucosidose; Gal, galactose; GM1, monosialotetrahexosylganglioside; GM2, disialotetrahexosylganglioside; GSD, glycogen storage disorder; ML; mucolipidosis; GlcNAc, *N*-Acetylglucosamine; Hex, hexose; ID, confidence level of metabolite annotation according to MSI initiative reporting standard[19]; Man, mannose; ML, mucolipidosis; MPS, mucopolysaccharidose; NAGA, α-*N*-Acetylgalactosaminidase; NGLY1, *N*-glycanase 1; sec, secondary marker; Ser, serine; Thr, threonine.

($n = 11$), together with normal control urine samples ($n = 121$) of patients who were not diagnosed with an IMD. An overview of the results is given in Figure 2 and Figure S3.

Aspartylglycosaminuria, α-mannosidosis, and β-mannosidosis demonstrated oligosaccharide-specific metabolic profiles with clearly elevated Z-scores without substantial elevation of any of the other investigated biomarkers. Although a specific expression pattern was also observed for fucosidosis, GlcNAc-Fuc (366_fuco) was not consistently elevated in all four patient samples and is considered to be a secondary marker. As Z-scores of both NAGA deficiency biomarkers (Z-scores ≥10) were increased, with only a subset of the aspartylglucosaminuria and α-mannosidosis markers being slightly elevated in two out of three samples, NAGA deficiency could successfully be detected. The two (galacto)sialidosis markers were increased in both sialidosis (Z-scores >100) and galactosialidosis with average Z-scores being 3- and 5-fold higher in sialidosis samples than in galactosialidosis samples.

Distinct biomarker clusters were present in the Z-score plots of the analyzed sphingolipidoses samples. Two out of six GM2 gangliosidosis (M. Sandhoff) markers (GlcNAc-Man2-GlcNAc, *m/z* 747 and *m/z* 783) were increased to a lesser extent compared to the other markers and were designated as secondary markers. Interestingly, both sialidosis/galactosialidosis markers were elevated in the GM1 gangliosidosis samples as well. GM1 gangliosidosis and GM2 gangliosidosis (M. Sandhoff) can be detected using our screening platform provided that all primary biomarkers for each sphingolipidosis are elevated.

As expected, the ML II/ML III Z-score plot revealed a clear increase of both (galacto)sialidosis biomarkers. Importantly, in contrast to the biomarker profile of sialidosis (ML I) patients in which only the sialidosis/galactosialidosis markers were increased, additional moderate-to-high levels of various oligosaccharide species were detected in the majority of the samples (particularly several GM1 gangliosidosis and α-mannosidosis markers). As such, the appearance (or lack) of a combination of these biomarker clusters might facilitate discrimination of ML II/ML III from sialidosis.

The only component that is measured quantitatively in our screening platform is NANA to diagnose sialic acid storage disorders. NANA responses were linear up to 1300 μmol/L with linear regression values (R^2) ≥ 0.999 in three urine samples. The limit of detection (LOD) was 4 μmol/L and the limit of quantitation (LOQ) was 13 μmol/L. The precision of NANA quantification was determined with inter-assay coefficients of variation (CV) of 9.5% ($n = 121$, SD = 7) and 8.1% ($n = 45$,

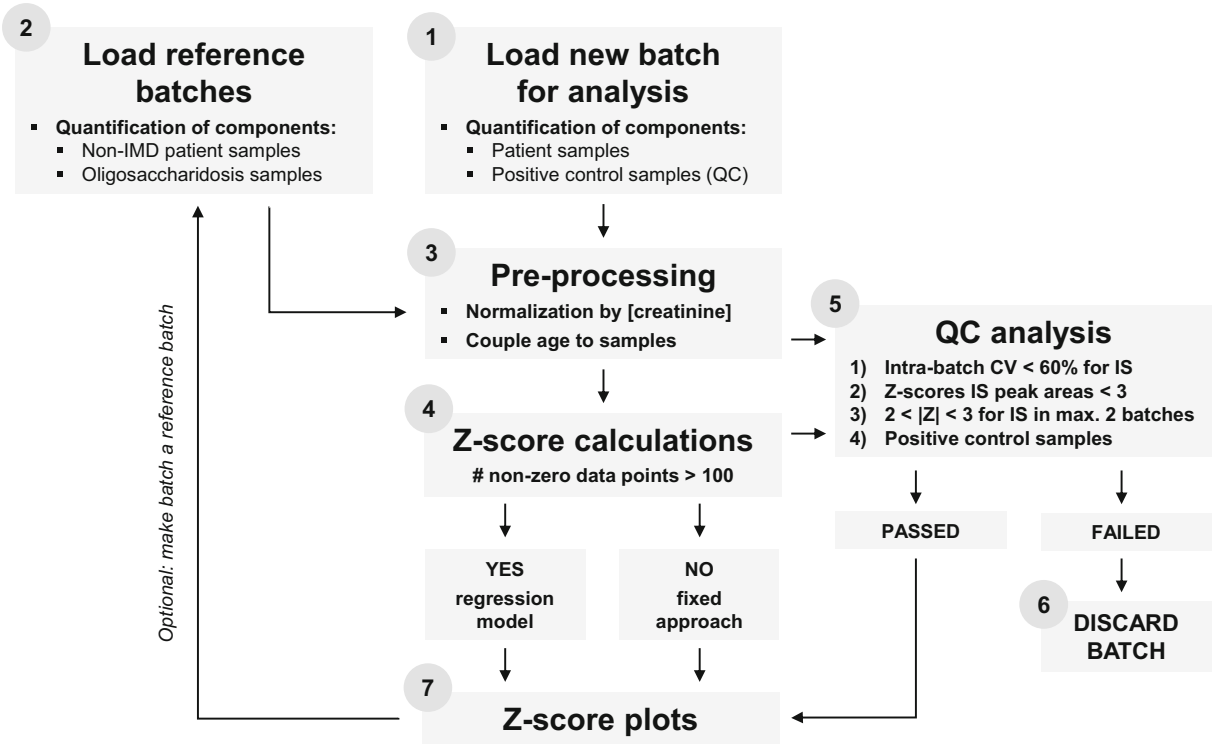


FIGURE 1 Overview of the bioinformatics pipeline. The batch file obtained from the mass spectrometer raw data processing software (Tracefinder) is loaded into the pipeline for analysis (Step 1). In parallel, (previously marked) reference batches with (semi)-quantified components of (non)-IMD patient samples and QC samples are loaded into the pipeline as well (Step 2). Subsequently, these batch files are preprocessed (Step 3), followed by calculation of the Z-scores for each individual component (either using a regression model or a "fixed" approach; Step 4). Simultaneously, QC is performed using internal standard (IS) and positive control samples (Step 5). If the QC fails, the batch will be discarded and the experiment has to be repeated (Step 6) but if it passes the QC requirements Z-score plots will be generated (Step 7). The analyzed batch file can subsequently be marked as a *reference batch* for future analyses.

SD = 17) for QC samples containing 79 μmoL/L or 206 μmoL/L NANA, respectively.

We investigated the excretion of NANA in patient samples with Salla disease and infantile sialic acid storage disease (ISSD). In the 2021 and 2022 ERNDIM "Special Assays in Urine" surveys all our sialic acid results had Z-scores <2 (interlaboratory variation). Age-matched Z-scores were ranging from 4 to 24 in these samples with urinary excretions ranging from 62 to 412 μmol/mol creatinine. In several of these samples, GlcNAc-Man-Man-GlcNAc-Gal-NANA (1200_(galacto)sia) excretion was also detected with similar Z-scores as NANA. Although, in two of the patient samples, various nonclustered oligosaccharide species were detected these did not interfere with the analysis.

Taken together, disease-specific urinary biomarker profiles were identified by our screening approach, which function as a visual reference set and provide assistance for the interpretation of the Z-score plots generated by the analysis pipeline (an example is given in Figure 3).

3.4 | Detection of MPS type IV B and NGLY-1 deficiency

Increased excretion of GM1 gangliosidosis markers was detected in three urine samples from mild MPS IV B patients but not in any of the other MPS samples (see Figure S4). This makes sense as both GM1 gangliosidosis and MPS IV B are caused by a deficiency in β-galactosidase (GLB1). Component GlcNAc-Man2-GlcNAc-Gal (909_GM1) was the only biomarker elevated in all MPS IV B samples with variable expression of the other GM1 gangliosidosis markers. GlcNAc-Man3-GlcNAc-Man2 (1274_GM1) and GlcNAc-Man(-Man-GlcNAc-Gal)-Man-GlcNAc-Gal (1472_GM1) were absent in the three MPS IV B samples. The differences between GM1 gangliosidosis and MPS IV B may be due to high residual β-galactosidase activity in MPS IV B compared to low activity in GM1 gangliosidosis or changed specificity of mutated GLB1 for protein- or glycolipid-derived oligosaccharides.

Additionally, we investigated oligosaccharide patterns in other MPS samples (n = 24). We observed increased excretion of sialyl-oligosaccharide markers in MPS I,

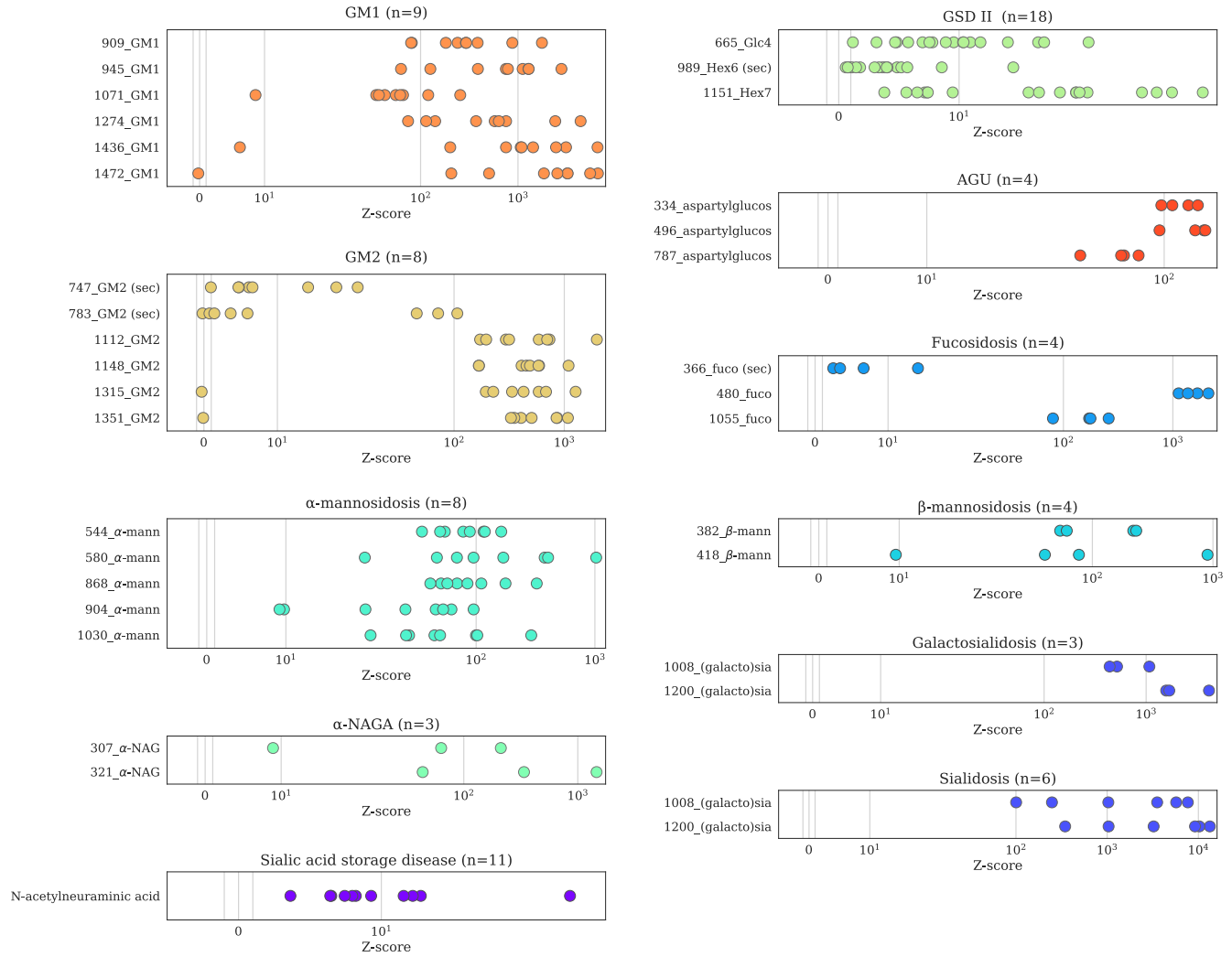


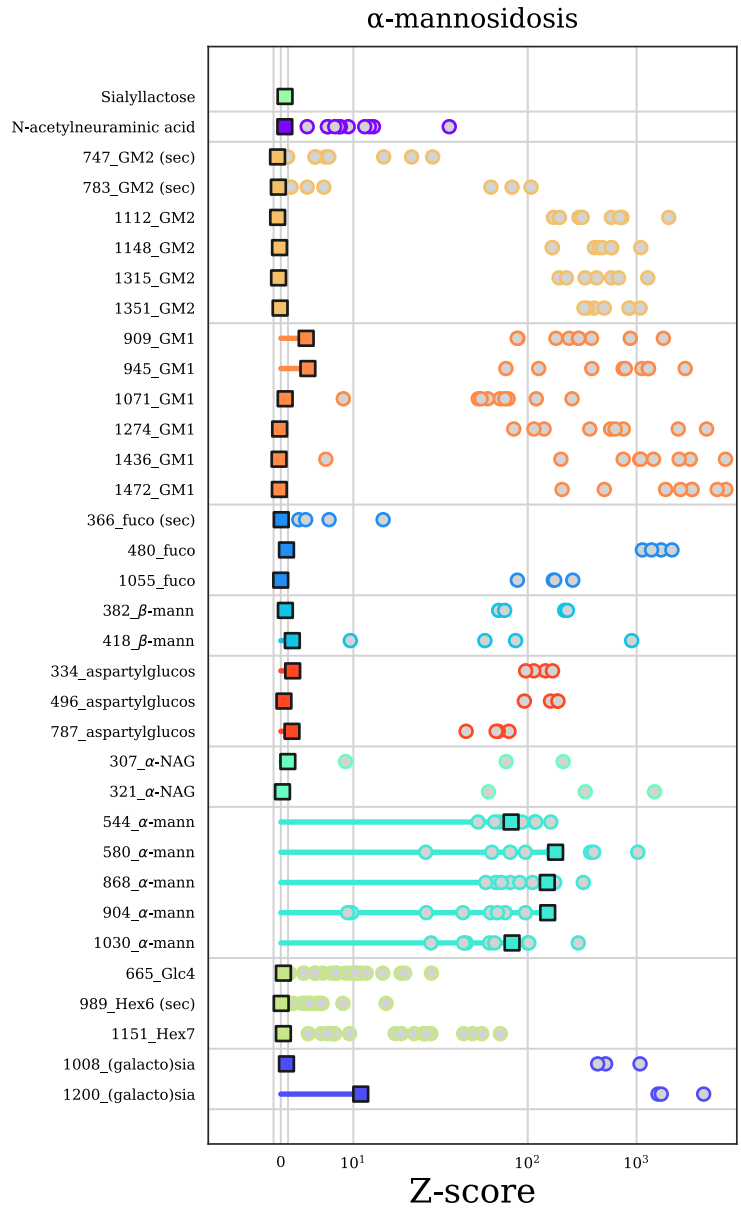
FIGURE 2 Z-score plots of all diseases included in this study. Oligosaccharide-specific metabolic profiles in samples of known patients diagnosed with the disorders as indicated in the figure are depicted. Dots represent Z-scores of disease-specific biomarkers of individual patients. Secondary markers are indicated by the affix *sec*. *AGU*, apartylglucosaminuria; *GM1*, GM1 gangliosidosis; *GM2*, GM2 gangliosidosis; *α-NAGA*, α-*N*-acetylgalactosaminidase deficiency; *GSD II*, glycogen storage disease 2 (Pompe disease).

MPS II, MPS III A, MPS IV A, MPS VI, and MPS VII samples (see Figure S4). Detection of these markers alone does not allow for MPS identification as they were also present at high levels in other diseases. No other abnormal oligosaccharide excretions were detected for the MPS subtypes investigated in this study.

Urine samples of patients suffering from the congenital disorder of deglycosylation (CDDG) *N*-glycanase 1 deficiency (NGLY-1)[20] were investigated as well. As can be seen in the representative Z-score plot depicted in Figure S5, analysis of NGLY-1 samples ($n = 2$) revealed moderately elevated Z-scores of all aspartylglucosaminuria biomarkers. As these markers as a group were not elevated to the same extent as observed in aspartylglucosaminuria samples, our screening platform allows for the detection of NGLY-1 deficiency.

3.5 | GSD2 patients excrete elevated levels of urinary Hex7

Individuals affected with GSD type II (M. Pompe) excrete, among other oligosaccharides, elevated levels of tetrasaccharide 6-α-D-glucopyranosyl-maltotriose (Glc4) in urine, which serves as a biomarker for disease and therapy monitoring despite not being specific for this disease.[21–24] We investigated whether urinary Glc4 and other previously published aberrant glycogen breakdown products,[23,25–27] were detectable in Pompe disease samples ($n = 18$). As can be seen in Figure S6a, elevated urinary excretion of Glc4 (range 7–136 mmol/mol creatinine) and Hex7 (range 5–61 mmol/mol creatinine) was detected in all samples, except for one (from a 13-year-old patient) that demonstrated elevated Hex7 but



Patient	Mean	Std	Model
4.25	1.33	5.01	Regression
4.99	0	9.02	Regression
0.0	0.15	0.34	Regression
0.0	0.05	0.16	Regression
0.0	0.02	0.03	Regression
0.0	0.0	0.01	Fixed
0.0	0.0	0.02	Fixed
0.0	0.0	0.0	Fixed
2.26	0.18	0.6	Regression
0.12	0.01	0.03	Regression
0.12	0.01	0.18	Regression
0.0	0.0	0.02	Regression
0.0	0.01	0.04	Fixed
0.0	0.0	0.01	Fixed
0.33	0.29	0.78	Regression
0.41	0.16	0.32	Regression
0.0	0.0	0.15	Regression
0.2	0.06	0.22	Regression
1.69	0.39	0.82	Regression
3.2	0.7	1.53	Regression
0.18	0.11	0.16	Regression
1.8	0.55	0.81	Regression
1.05	0.37	0.71	Regression
0.26	0.14	0.51	Regression
15.52	0	0.22	Regression
12.95	0.01	0.07	Regression
132.92	0.19	0.88	Regression
16.24	0.03	0.11	Regression
22.36	0.11	0.31	Regression
2.1	0.6	4.19	Regression
0.61	0.45	2.81	Regression
0.82	0.46	1.03	Regression
0.94	0.25	0.88	Regression
1.24	0.04	0.11	Regression

FIGURE 3 Representative Z-score plot of an α-mannosidosis patient. Z-scores of the disease-specific biomarkers in the patient sample under investigation were calculated using the analysis pipeline and depicted as squares. Pathological disease-specific reference values of multiple known patients suffering from the indicated diseases are depicted as dots. All of the α-mannosidosis biomarkers in the urine sample of the patient under investigation are elevated and in the disease range. Semi-quantitative concentrations of each component are listed next to the graph, together with the model utilized to calculate the Z-scores.

normal Glc4 excretion. Hex6 urinary excretion (range 3–49 mmol/mol creatinine) was elevated in 14 out of 18 patient samples (Z-score >1). The absolute concentration of Glc4 was the highest, when compared to Hex6 and Hex7, with corresponding median normal control sample concentrations being lower (Figure S6b). Although absolute Glc4 concentrations were higher than Hex7, the corresponding median Z-score values were 18, 9, and 4 for Hex7, Glc4, and Hex6, respectively. During routine use of the test, median values obtained with undiagnosed patient samples ($n = 1916$, >31 days of age) were similar to the reference control data set but, as expected, more outliers were observed with the larger data set (Figure S6b).

Next, we generated receiver operating characteristic (ROC) curves to examine the predictive potential of these biomarkers for Pompe disease (see Figure S6c). Calculations of the area under the curve (AUC) of the ROC curves of Hex7 and Glc4 compared to the control samples, revealed both markers excellent accuracy with AUCs of 0.995 and 0.987, respectively. A moderate increase in specificity and sensitivity for the Hex7 marker compared to Glc4 was observed. Hex6 accuracy was the lowest when compared to Hex7 and Glc4 and as such

designated as a secondary marker. Similar results were obtained for Pompe samples versus undiagnosed patient samples with AUCs of 0.981, 0.973, and 0.921 for Hex7, Glc4, and Hex6, respectively. The optimal Z-score cut-off values (the point on the ROC curve closest to the (0,1) point) for Glc4 and Hex7 were 3.10 and 3.59 when using undiagnosed patients as a reference, respectively (see Figure S6c). Taken together, these results suggested that Hex7 might be a potential novel biomarker, slightly outperforming Glc4, for the diagnosis and monitoring of Pompe disease.

3.6 | Routine diagnostics; sensitivity and specificity of the screening platform

Since the implementation of the oligosaccharidoses screening platform, a total of 2277 analyses have been performed. In line with expectations, the majority (94.2%) of these analyses demonstrated normal oligosaccharide excretion patterns. This allowed for (rapid) expansion of the reference batch datasets and improvement of the sensitivity of the method. In 3.5% of the cases, results were not conclusive calling for additional testing or a repeat sample for analysis. This results in a specificity of 96.5% on a group level for our screening method. Reasons for re-analysis were due to elevated sialic acid, elevated Glc4-Glc7 markers or patterns with multiple elevated markers of one or more oligosaccharidoses. The latter typically consisted of nonspecific oligosaccharide profiles in neonates in which sialyllactose (a "breastfeeding marker") was generally also elevated. A representative Z-score plot of such a "neonate" profile is presented in Figure S7. The remaining abnormal profiles (2.2%) included newly diagnosed oligosaccharidosis patients, samples from previously diagnosed patients investigated during regular follow-up or samples related to external quality assurance programs (ERNDIM diagnostic proficiency testing in urine [DPT] schemes). In 24 patients diagnosed with IEM other than oligosaccharidoses or MPS (including aminoacidopathies, organic acidurias, and purine-pyrimidine defects) oligosaccharide patterns were normal. Until now, a total of 19 oligosaccharidoses have been detected and/or confirmed in urine samples of both newly identified ($n = 5$) and previously known patients ($n = 14$). Importantly, to date, no false-negative results have become apparent during the application of our screening platform (that is, sensitivity of 100%).

4 | DISCUSSION

We describe a unique UHPLC/HRAM mass spectrometry screening platform, which includes a novel intuitive bioinformatics analysis pipeline, for screening of pathological urinary oligosaccharides. This screening approach was able to identify oligosaccharidoses, sphingolipidoses, mucolipidoses, other LSDs, and NGLY1 deficiency. Investigation of samples from patients with Pompe disease resulted in the identification of a potential biomarker, a heptaglucoside (Glc7), for diagnosis and monitoring of this disease.

Despite several disadvantages, the most commonly used method to detect (pathological) urinary glycan excretions for screening of oligosaccharidoses is TLC.[3–5] To overcome these difficulties MS-based approaches have recently been developed (see Table 1). A few of these methods did not require oligosaccharide derivatization prior to analysis, initially reported in the pivotal papers of Bonesso et al.[9] and Piraud et al.,[10] followed by two other recent publications.[11,12] All these methods were able to successfully identify oligosaccharidoses (and other LSDs) in patient material and are, regardless of the utilized approach, adequate screening tools. Several differences are worth mentioning when compared to our approach.

We developed a negative-ESI MS screening method, whereas other methodologies applied polarity switching during data acquisition. Transitioning between positive and negative ionization modes might result in less data points per peak potentially leading to decreased sensitivity. Additionally, we utilized three internal standards in each run while others only used one (Glc7; maltoheptaose)[10,11] or none.[12] Usage of multiple IS generally increases accuracy and, importantly, allowed for quantitative analysis of NANA (others reported qualitative values). Also, all three IS in our method are monitored over time for QC purposes. The lack of appropriate (isotope labeled) internal standards for each analyte is a limitation and results in the inability to quantify the oligosaccharide biomarkers. The use of compounds that are normally not present in urine (e.g., acarbose) as internal standards allow for semi-quantification, that is, estimation of the approximate concentration of the analytes. Instead of utilizing tandem-MS,[10–12] we developed our screening platform around HRAM-MS technology. A major advantage of this technique is that it has a higher mass range of m/z 2000 and enables the analysis of large *complete* molecules. When compared with tandem-MS (using multiple reaction monitoring; MRM), HRAM-MS acquires data in full-scan mode, which in addition to the targeted analysis allows for retrospective analysis of new targets or unknown compounds. Finally, not all of the disease-specific biomarkers adapted from the literature were consistently elevated in all corresponding patient samples using our HRAM-MS screening approach, that is, components 366_fuco, 747_GM2, 783_GM2 and 989_Hex6 (see Figure 2 and Figure S6). As such, these markers were designated as *secondary markers*

of which elevated levels are not required but can assist in diagnosing a potential LSD. The remaining disease-specific markers were designated as primary biomarkers as these were consistently elevated in all matching patient samples and are required to be elevated as a group for diagnosis of the specific disease.

Occasionally, inconclusive biomarker profiles were obtained in samples from (very) young patients. In such profiles, variable subsets of biomarkers were elevated, sometimes even to the same extent as observed in known patient samples. In particular, the (galacto)sialidosis markers were often elevated in neonate profiles and required further investigation to exclude (galacto)sialidosis. We hypothesize that this interference is related to the young age and nutritional status of the patient. As such, sialyllactose excretion (a nutritional marker for breastfeeding[28]) could aid in the interpretation of such inconclusive biomarker profiles.

Piraud et al.[10] noticed a moderate unspecific increase of galactosyl oligosaccharides in sialidosis samples, which we did not detect with our assay possibly due to the high specificity of our HRAM system. The nonspecific mixed mannosyl- and galactosyl-oligosaccharide patterns in ML II and ML III urine samples have been previously reported.[11] We were able to detect ML II/III by concurrent increased levels of sialyl-oligosaccharide biomarkers but were unable to discriminate between ML II or ML III. Mak and Cowan,[12] however, recently published markers, which allows for the identification of each type. Others were able to discriminate between infantile and late-onset forms of sialidosis, GM1 gangliosidosis, and Sandhoff disease. Unfortunately, the disease severity of the patients we investigated was unknown, but we believe that our screening platform can distinguish between these forms as well.

We found similarly to Piraud et al.[10] that the sialyl-oligosaccharide biomarkers were not disease-specific (see Figure S3). Although the exact reason for this observation is unknown, we speculate that these markers were elevated because the lysosomal multienzyme complex, consisting of neuraminidase, β-galactosidase, and protective protein/cathepsin, might be sensitive to disturbances of lysosomal functioning and may lead to secondary storage of sialyl-oligosaccharides. Hence, when exclusively elevated Z-scores of sialyl-oligosaccharide species are observed for these LSDs additional metabolic, enzymatic, or genetic investigations should be considered.

A unique element in our method is the bioinformatics data processing pipeline to assist in data interpretation and extensive QC monitoring (see Figure 1). It can easily be modified to specific user needs, for example, adding newly discovered biomarkers or adjusting QC-specific parameters. We designed our pipeline to operate in an iterative way that refines and optimizes (age dependent) reference range and pathological values for each biomarker over time. This hardly requires user training and can be achieved by simply designating batch runs with normal oligosaccharide excretion profiles as *reference batches* and aberrant profiles as being *disease-specific*. As it is known that glycan excretions decrease with age and concentrations can be high in infants and as such the analysis will become more accurate over time, which is a major advantage of our screening pipeline.

The final output file generated by our analysis pipeline is a graphical overview of disease-specific Z-scores grouped accordingly, color-coded, and plotted together with disease-specific pathological values for individual patient samples. This allows for fast and intuitive interpretation of comprehensive biomarker profiles, which is in contrast to previously published complex large data tables, profiles, and heatmaps.[10–12] As not all biomarkers are exclusively elevated in a particular storage disorder, interpretation of *patterns* of aberrant oligosaccharide markers is essential and greatly benefits from our data visualization approach. Whereas others reported their results as multiple of the median (MoM)[10,11] we favor usage of Z-scores as this takes the population spread into consideration, which is not covered when using MoM, and in our opinion produces more reliable results.

Importantly, the data processing and visualization pipeline are not exclusively confined to data generated by a UHPLC-HRAM MS but can also be utilized for data obtained by other MS systems as long as the data output file has the same format as the accompanying online demo output file (see our GitHub repository for more details).

Investigation of urinary excretions in samples of patients with Pompe disease demonstrated, similar to previous publications,[23,25–27] elevated Glc4 excretions but also of Hex7 and to a lesser extent of Hex6 (see Figure S6). Only the Glc4 biomarker was investigated in the previously discussed screening methods for the detection of GSD II. Although Piraud et al.[10] mentioned that Glc7 urinary excretion in the absence of the IS maltoheptaose was increased in GSDII (and GSD III) samples, no data were presented. Others reported that these larger urinary oligosaccharides were considerably less excreted than Glc4 in patient samples but substantially increased when compared to normal control samples,[25,26] which is in agreement with our observations. Until now, the definitive structure of Hex7 has not been elucidated, but it seems likely that it will be a branched glucoside similar to Glc4.

Interestingly, Z-scores of Hex7 in our study were clearly higher than Glc4 and Hex6, suggesting that Hex7

might be a potential novel biomarker for the diagnosis and monitoring of Pompe disease (underscored by our ROC curve analyses). Hex7 could serve as a secondary biomarker, in addition to Glc4, for the potential identification of patients that otherwise might have been missed by solely investigating Glc4 excretions. However, the majority of Pompe samples investigated in our study were young-onset cases with only two patients with later-onset (>10 years) forms of the disease. As such, we cannot exclude that adult-onset Pompe patients may be missed by our screening method, which also occurs frequently in TLC investigations. Nonetheless, several other diseases exist in which Glc4 is elevated, for example, several GSDs[18] and autophagy-related disorders,[11] where Hex7 excretion in conjunction with Glc4 might assist in the correct diagnosis of these disorders as exemplified by the GSD VI patients detected by our screening method. Additional research is required to explore the potential use of Hex7 as a novel biomarker for Pompe disease and various GSDs.

In conclusion, we developed a UHPLC/HRAM-MS screening platform with a bioinformatics analysis pipeline that allows for straightforward screening of oligosaccharidoses, as exemplified by five newly diagnosed patients. Our screening approach may also serve as a valuable tool, in addition to specific enzyme testing, for elucidating the clinical importance of variants of unknown significance (VUS) in genes encoding for proteins involved in oligosaccharide degradation.

AUTHOR CONTRIBUTIONS

Conception and design of the article: Marne C. Hagemeijer and George J. G. Ruijter. *Analysis and interpretation*: Marne C. Hagemeijer, Jeroen C. van den Bosch, Michiel Bongaerts, Edwin H. Jacobs, and George J. G. Ruijter. *Drafting the article*: Marne C. Hagemeijer and George J. G. Ruijter. *Revising the article for intellectual content*: Jeroen C. van den Bosch, Michiel Bongaerts, Edwin H. Jacobs, Johanna M. P. van den Hout, and Esmee Oussoren. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

Marne Hagemeijer, Jeroen van den Bosch, Michiel Bongaerts, Edwin Jacobs, Johanna M. P. van den Hout, Esmee Oussoren and George Ruijter declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). All patient urine samples were pseudonymized and were obtained in accordance with national legislation and institutional guidelines of the Erasmus MC.

ANIMAL RIGHTS

This article does not contain any studies with animal subjects performed by the any of the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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