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Quantification of Specific Urinary Oligosaccharide Biomarkers for Diagnosis and Treatment Monitoring of Alpha-Mannosidosis

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ABSTRACT

Alpha-mannosidosis (AM, OMIM# 248500), an ultra-rare lysosomal storage disorder (LSD), is caused by insufficient activity of alpha-mannosidase, an enzyme involved in degradation of N-glycan oligosaccharides. Hence, specific oligosaccharides accumulate in tissues, resulting in a progressive multi-organ disease. Therapeutic options exist but treatment outcome depends on early diagnosis. Thus, multiple methods have been developed for analysis of these oligosaccharides in urine that are qualitative or semi-quantitative, limiting their use for treatment monitoring. A rapid quantitative method without derivatization was developed for AM biomarkers $GlcNAc(Man)_2$, $GlcNAc(Man)_3$, and $GlcNAc(Man)_4$ in spot urine samples using ultra-performance liquid chromatography coupled to tandem-mass spectrometry. Urine samples of 208 controls, 20 AM patients, and 26 patients with other LSDs were analyzed. Method validation proved high recoveries (88%–108%) and precisions (standard deviation < 8%), low limits of quantification (0.12 μg $GlcNAc(Man)_2$/mL), and high sample stability. We observed a clear separation between controls and AM patients (0.0–9.0 vs 39.4–99.3 μmol $GlcNAc(Man)_2$/mmol creatinine, $p < 0.0001$). Furthermore, $GlcNAc(Man)_2$ concentration showed significant differences between untreated patients and those treated with enzyme replacement therapy (ERT; $n = 13$, $p = 0.0006$) or hematopoietic stem cell transplantation (HSCT; $n = 3$, $p = 0.0143$), and between both treatments ($p = 0.0143$). Thus, the developed method is well-suited for selective screening for AM and offers a simple way to monitor treatment efficacy.

1 | Introduction

Alpha-mannosidosis (AM, OMIM# 248500) is a debilitating lysosomal storage disorder (LSD) with an estimated prevalence of 1 in 500 000 to 1 000 000 [1, 2]. It follows an autosomal recessive trait and is caused by reduced alpha-mannosidase (EC 3.2.1.24) activity as a result of variants within the *MAN2B1* gene in chromosomal region 19p13.13 [3]. Alpha-mannosidase is involved in the cleavage of mannose residues from high-mannose, hybrid, and complex-glycosylated N-glycans during lysosomal glycoprotein degradation. Therefore, AM patients can be characterized by accumulation of specific non-degraded oligosaccharides [4]. As a consequence of the metabolic block, affected individuals develop symptoms such as dysmorphic features, skeletal abnormalities, organomegaly, immunodeficiency, as well as a range of neurologic symptoms including hearing impairment, ataxia, and intellectual disability [1, 3, 4]. Although the clinical presentation is highly variable, AM has often been divided into two clinical subtypes. Type I is a severe form with hepatomegaly and serious infections leading to early death, while type II is considered an attenuated but progressive form manifesting in hearing loss and mental retardation with survival into adulthood [1, 5]. In practice, there is a continuum between the two forms. Prognosis is poor, even in patients with milder presentation, which means that an independent life is generally impossible for untreated AM patients [3]. Life expectancy of patients is considerably shortened, meaning that among those who reach adulthood, the median age of death due to infectious complications or malignancies is only 45 years [5].

Treatment options such as allogeneic hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) by velmanase alfa have become available in recent years [6]. However, to achieve optimal treatment outcomes, early diagnosis is critical [5, 7]. Currently, the definitive diagnosis of alpha-mannosidosis relies on enzyme assays performed on leukocytes or dried blood spots. This approach with or without molecular genetic confirmation is technically straightforward and well-validated, although an enzyme's sensitivity to storage conditions—even for relatively short time periods at room temperature—can influence the diagnostic interpretation. Such significant effects are particularly well-documented for alpha-mannosidase and have been described, for example, in samples of cerebrospinal fluid [8]. Furthermore, this diagnostic step requires clear clinical suspicion. This is one reason why cases diagnosed primarily through untargeted genetic testing as part of a "genetics-first" approach are becoming increasingly important.

In parallel, several diagnostic methods have been developed for selective screening for LSDs via specific and unspecific urinary free oligosaccharides. These unspecific serum oligosaccharides have also been employed as one of the endpoints for the phase II and III studies for velmanase alfa treatment and they correlated to clinical endpoints like the 3-min stair climb test and the 6-min walk test [6, 9]. While unspecific testing for oligosaccharides is common, specific oligosaccharide biomarkers for AM have been identified. Three such oligosaccharide biomarkers for AM are GlcNAc$(Man)_2$, GlcNAc$(Man)_3$, and GlcNAc$(Man)_4$, which are hypothesized to accumulate specifically in AM patients [4].

Lately, the emergence of liquid chromatography (LC) in combination with tandem-mass spectrometry (MS/MS) detection has resulted in the development of various methods for the detection of free oligosaccharide biomarkers in urine and other biological materials applied for selective screening for LSDs, including AM. Piraud et al. presented an LC–MS/MS method suitable for qualitative screening for multiple oligosaccharidoses as well as some other LSDs based on a rapid and derivatization-free dilute-and-shoot method with subsequent biomarker separation on an NH_2 column [10]. Huang et al. used ultra-performance liquid chromatography (UPLC) in combination with MS/MS-detection after derivatization of free oligosaccharides in urine with butyl-4-aminobenzoate and subsequent sample purification based on solid-phase extraction [11]. After oligosaccharide separation on an amide column, the authors were able to identify patients with eight different LSDs. Additionally, semi-quantitative determination of biomarker molecules relative to the internal standard was possible. Another multiplex assay for LSDs uses high-resolution accurate mass MS combined with an iterative bioinformatics pipeline [12]. Here, an ACQUITY UPLC BEH amide column was used in a dilute-and-shoot approach. Lastly, Wongkittichote et al. have developed a UPLC–MS/MS method for the analysis of urinary oligosaccharides and glycosaminoglykans, enabling the diagnosis of LSDs including AM [13]. Following oligosaccharide derivatization with 3-methyl-1-phenyl-2-pyrazoline-5-one, the analytes were separated on an ACQUITY UPLC HSS PFP column and relative quantification was performed against one of three internal standards. When analyzing patient samples, a notable decrease in biomarker concentrations could be observed in AM patients after treatment [13]. There are more reports on qualitative and semi-quantitative methods using liquid chromatography-mass spectrometry (LC-MS) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI/TOF) for analysis of oligosaccharides [14–19]. However, the non-quantitative nature of all previously discussed methods has repeatedly been identified as a disadvantage [13, 20], especially in view of the fact that monitoring oligosaccharide concentrations in AM patients before and after therapy has been emphasized as an important measure by an international expert panel [21]. Very recently, a method for the quantitative assessment of GlcNAc$(Man)_2$ along with the semi-quantitative assessment of GlcNAc$(Man)_3$, GlcNAc$(Man)_4$, and other high-mannose oligosaccharides has been reported for plasma and serum [22]. This method includes sample preparation, a filtration step, derivatization and solid-phase extraction, and showed good separation between AM patients and normal controls, while also enabling treatment monitoring in patients after HSCT. This method demonstrates the advantages of a fully quantitative assay. However, as a result of the thorough sample preparation required for blood plasma and serum, it is time-consuming and resource-intensive. If plasma or serum could be replaced by urine, laboratories would benefit from a simpler sample clean-up and quicker turnaround, while patients would benefit from less invasive sample collection.

Our aim was therefore to develop a rapid and reliable fully-quantitative UPLC-MS/MS method for the AM oligosaccharide biomarkers GlcNAc$(Man)_2$, GlcNAc$(Man)_3$, and GlcNAc$(Man)_4$ in urine samples.

2 | Materials and Methods

2.1 | Samples

Urine samples for method development and validation were obtained from healthy volunteers and stored at 4°C up to 1 week unless declared otherwise. Urine samples of 208 controls were anonymized residuals from organic acid or amino acid analyses stored at 20°C for up to 2 months. The control cohort consisted of 87 females and 121 males of unknown ethnicity. Their median age was 3 years with an interquartile range of 8 years. According to §12 of the Hamburg Hospital Act (HmbKHG), no consent is required for anonymized samples used for the generation of reference ranges. Urine samples of AM patients and patients affected by other LSDs were stored at −80°C. An overview of the AM patients, their age, sex, and treatment status, is provided in Table S1. Additionally, we list information on enzymatic activity pre-diagnosis along with the underlying genetic *MAN2B1* variants, where available. An overview of the diagnoses, sex, and age of patients with other LSDs is compiled in Table S2. We investigated urine samples of unaffected controls ($n = 208$), untreated AM patients ($n = 13$ samples from 11 individuals), AM patients on ERT ($n = 13$ samples from 10 individuals), and AM patients who underwent HSCT at least 1 year prior to sampling ($n = 3$ samples from 3 individuals). The diagnosis of all patients was confirmed by enzyme activity and/or genetic testing (inclusion criteria for study samples; Table S1). Exclusion criteria for control samples were extreme outliers of the quantifier/qualifier-ratio of $GlcNAc(Man)_2$ (post-analysis exclusion, *see below and* Supporting Information) and the confirmed diagnosis of an LSD, resulting in 204 samples in the control cohort. Ethical approval for the study was granted by the ethics committee of the Medical Association Hamburg (Ärztekammer Hamburg, 06.02.2024, processing number 2023-101200-BO-ff), and informed consent was obtained prior to inclusion from all study participants and, if applicable, their legal guardians. All procedures were in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

2.2 | Materials

Acetonitrile (for UHPLC–MS) was purchased from Th. Geyer GmbH & Co. KG (Renningen, Germany). Water (HPLC Gradient grade) was obtained from Avantor Performance Materials LLC (Center Valley, PA, USA). Formic acid (98%–100%) was supplied by Merck KGaA (Darmstadt, Germany). $GlcNAc(Man)_2$ (95%–98%), $GlcNAc(Man)_3$ (95%–98%), $GlcNAc(Man)_4$ (95%–98%), and GlcN-[1,2-$^{13}C_2$; 2-$^{2}H_3$]$Ac(Man)_2$ (the internal standard (IS) for $GlcNAc(Man)_2$, 95%–98%) were obtained from Omicron Biochemicals Inc. (South Bend, IN, USA).

2.3 | Sample Analysis

Samples were prepared by 1/100 dilution with the extraction solvent (containing also $GlcNAc(Man)_2$-IS) and subsequently analyzed by UPLC-MS/MS. A detailed presentation of sample preparation and analysis is provided in the Supporting Information.

2.4 | Normalization

Results were normalized against the urinary creatinine concentration measured by the Jaffe method, using the commercially available kit LT-CR 0121 (Labor + Technik Eberhard Lehmann GmbH, Berlin, Germany) and a Genesys 180 spectrophotometer (Thermo Fisher Scientific GmbH, Dreieich, Germany).

2.5 | Validation

The presented method was validated for linearity, carry-over, limit of detection (LOD), limit of quantification (LOQ), lower limit of quantification (LLOQ), recovery and precision, matrix effect, stability, and analytical specificity according to the ICH guideline M10 on bioanalytical method validation and study sample analysis [23] with modifications where necessary. A comprehensive outline of the method validation is provided in Tables S3–S9, Figures S1–S2.

2.6 | Statistical Analysis and Visualization

Data acquisition was performed by MassLynx V4.2 SCN1024. Peak integration and calculation of concentrations were done by TargetLynx XS V4.2 SCN1024. The quantification was carried out by linear regression with a weighting factor of 1/×. $GlcNAc(Man)_2$ concentration was determined via external calibration based on GlcN-[1,2-$^{13}C_2$; 2-$^{2}H_3$]$Ac(Man)_2$ as internal standard, whereas $GlcNAc(Man)_3$ and $GlcNAc(Man)_4$ were quantified by external calibration without internal standards, since corresponding isotope-labeled standards are not commercially available or would have to be synthesized at substantial cost. Data visualization and statistical analyses were conducted using Python 3 in a Jupyter Notebook 7.0.8 environment, equipped with the packages pandas (version 2.2.2), matplotlib (version 3.8.4), seaborn (version 0.13.2), numpy (version 1.26.4), scipy (version 1.13.1), and statsmodels (version 0.14.2). Comparison between groups was performed using the Whitney–Mann *U*-test. To control for type I error due to multiple testing, *p*-values were adjusted using the Holm-Bonferroni method where applicable.

3 | Results

A method for the quantitative determination of the alpha-mannosidosis-specific biomarkers $GlcNAc(Man)_2$, $GlcNAc(Man)_3$, and $GlcNAc(Man)_4$ in urine was developed that showed high recovery (88%–108%) and precision (standard deviation < 8%), and a low limit of quantification. Of note is the high thermostability of these biomarkers which allows short-term storage and transport at room temperature. Details are presented in Tables S3–S9, Figures S1–S2.

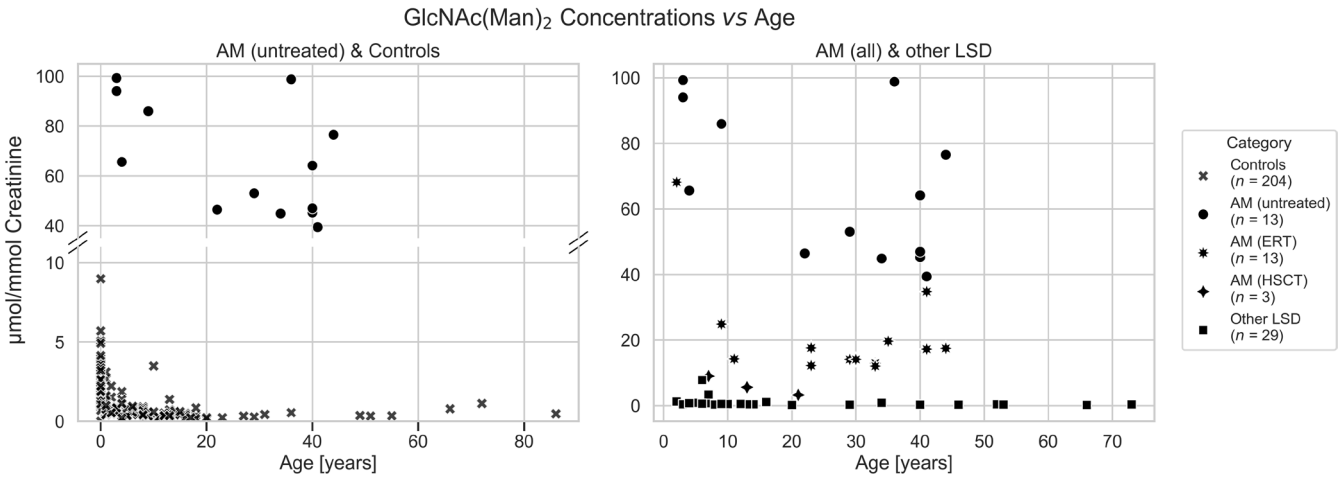

FIGURE 1 | $GlcNAc(Man)_2$ concentrations of all samples against the age at sample collection for each study cohort. The left side compares untreated AM patients and controls. The right side shows all patients with AM, including those treated with ERT or HSCT, and other LSDs. AM, alpha-mannosidosis; ERT, enzyme replacement therapy; HSCT, hematopoietic stem cell transplantation; LSD, lysosomal storage disorder.

The resulting method enabled the analysis of urine samples of AM patients and controls including patients with other lysosomal disorders. Tables S1 and S10 give an overview of all measured concentrations in samples from AM patients and patients with other LSDs, respectively. Table S11 provides the minimum and maximum concentrations measured for each analyte in each cohort. Figure 1 presents $GlcNAc(Man)_2$ concentrations plotted against the age of subjects at the time of sampling. Corresponding figures for $GlcNAc(Man)_3$ and $GlcNAc(Man)_4$ are provided in Figures S3 and S4.

We determined the 95% reference intervals from the concentrations of the control cohort by interpolating the values at the 2.5th and 97.5th percentile, which results in reference intervals for healthy individuals of 0.2–4.0 µmol $GlcNAc(Man)_2$/mmol creatinine, 0.1–18.3 µmol $GlcNAc(Man)_3$/mmol creatinine, and 0.1 to 1.5 µmol $GlcNAc(Man)_4$/mmol creatinine. We observed no differences between the analyte concentrations of the male and female sub-cohorts (*data not shown*). The biomarker concentrations were slightly higher in infants under 2 years of age. Still, the separation between this sub-cohort and untreated AM patients was very clear. Hence, we decided against the definition of age-adjusted reference intervals in order to simplify the validation of the method and the evaluation of individual samples.

The comparison of the control cohort and the untreated AM patients showed a statistically highly significant difference ($p < 0.0001$) for the concentrations of all three oligosaccharide biomarkers (Figure 2). In addition, statistically significant differences ($p < 0.05$) were observed for the concentrations of all three biomarkers between untreated AM patients and those who had received ERT or had undergone HSCT, as well as between the two forms of treatment (Figure 3). The comparison of patients with other LSDs and untreated AM patients also resulted in statistically significant differences ($p < 0.0001$) for all three biomarkers (Figure 4).

4 | Discussion

4.1 | Method Validation

The developed UPLC-MS/MS method for the analysis of $GlcNAc(Man)_2$, $GlcNAc(Man)_3$, and $GlcNAc(Man)_4$ in urine has been successfully validated for linearity, carry-over, LOD and LOQ, precision, recovery, accuracy, matrix effects, and stability, fulfilling all acceptance criteria. Notably, sample stability at room temperature was confirmed over 2 months, enabling worldwide shipping even at room temperature. The assessment of the analytical specificity showed that some other substances—most likely other N-acetylated oligosaccharides based on the transitions to m/z 222 and 204—are detected at the same retention times and with the same transitions as $GlcNAc(Man)_2$, $GlcNAc(Man)_3$, and $GlcNAc(Man)_4$. However, further investigations revealed that even the two samples we found with a high proportion of these compounds resulted in measured $GlcNAc(Man)_2$ concentrations below those of untreated AM patients. Moreover, any potential negative impact on the diagnostic sensitivity of this assay is avoided by adding the $GlcNAc(Man)_2$ quantifier/qualifier ratio as a threshold criterion (*see* Supporting Information).

4.2 | Concentration of Biomarkers in AM Patients

Evaluation of the reference ranges for the three biomarkers shows a distinct separation between the upper limit of the $GlcNAc(Man)_2$ reference range and the lower limit of the concentrations observed in samples from untreated AM patients (10-fold). Additionally, the controls and the untreated AM patients are well-separated by their $GlcNAc(Man)_4$ concentrations, with a 4-fold difference between the upper limit of the reference range and the lower limit observed in samples from untreated AM patients. The $GlcNAc(Man)_3$ concentrations, on the other hand, displayed significant overlap of the upper reference range (upper limit 18.3 µmol/mmol creatinine) and the lower concentrations in samples of untreated AM patients (lower limit

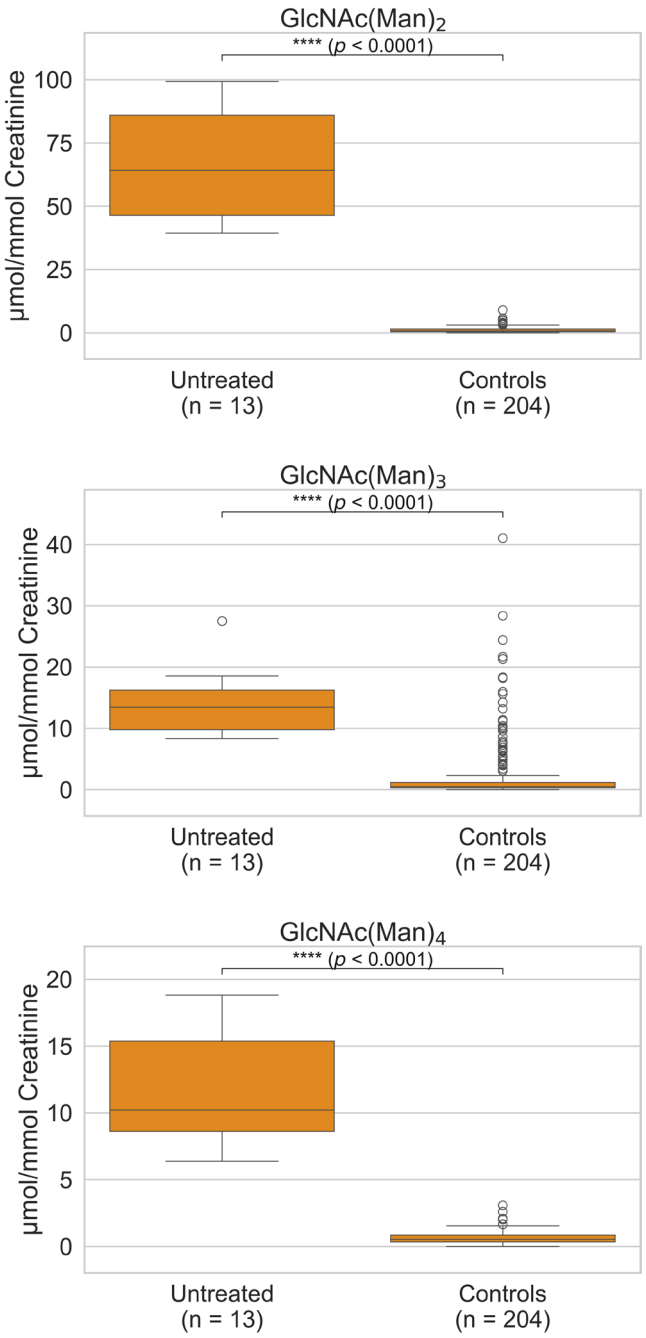

FIGURE 2 | Boxplots of the urinary biomarker concentrations measured in the control cohort and in samples of untreated AM patients. AM, alpha-mannosidosis. **** refers to statistically significance with level given in parenthesis.

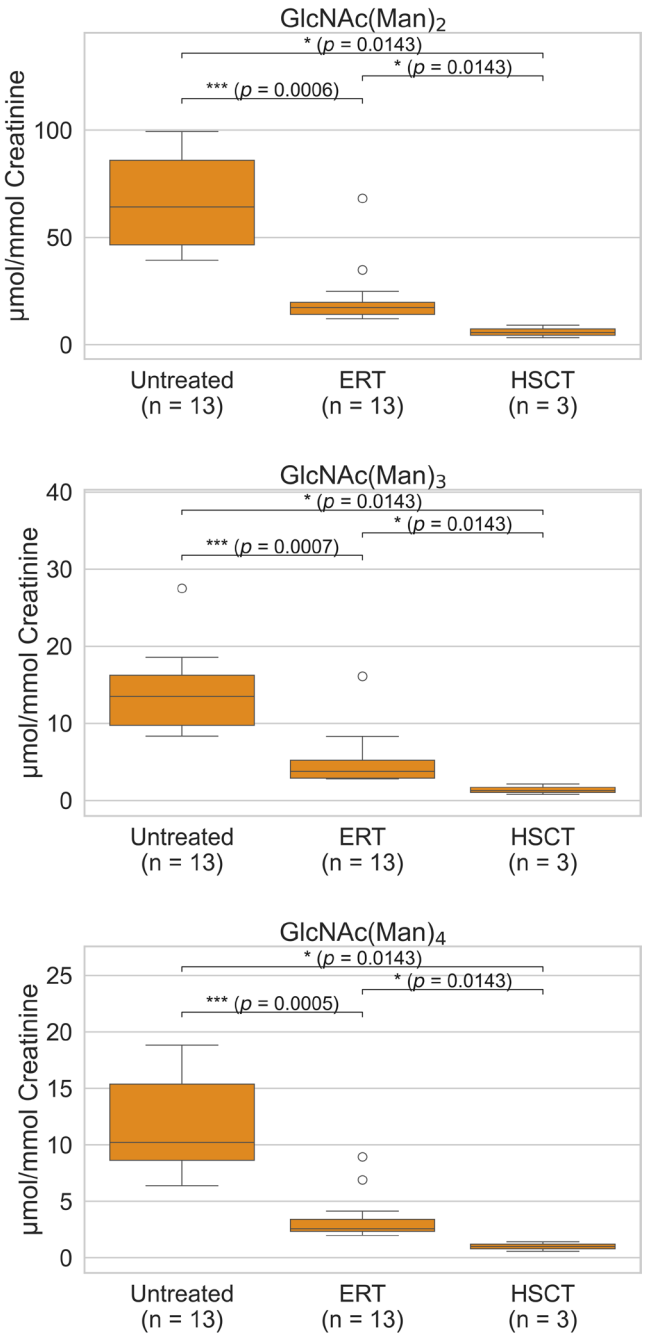

FIGURE 3 | Boxplots of the urinary biomarker concentrations measured in the samples of untreated AM patients, AM patients receiving ERT, and AM patients after HSCT. AM, alpha-mannosidosis; ERT, enzyme replacement therapy; HSCT, hematopoietic stem cell transplantation. *, *** refers to statistically significance with level given in parenthesis.

8.3 µmol/mmol creatinine). These results suggest the use of $GlcNAc(Man)_2$ as the primary biomarker, with $GlcNAc(Man)_4$ as a confirmatory marker. We chose 10 µmol/mmol creatinine as the cut-off value for $GlcNAc(Man)_2$ (range of the control cohort: 0.0–9.0 µmol/mmol creatinine) and 5 µmol/mmol creatinine as the cut-off for $GlcNAc(Man)_4$ (range of the control cohort: 0.0–3.1 µmol/mmol creatinine). In contrast, the use of the $GlcNAc(Man)_3$ concentration does not improve the diagnostic power of this method by itself. Hence, we did not determine a cut-off concentration for $GlcNAc(Man)_3$. However, the determination of $GlcNAc(Man)_3$ concentrations still holds some value in evaluating the results of this method, especially with regards to evaluating treatment efficacy, and may contribute to a reliable diagnosis of AM (*see below*).

4.3 | Diagnostic Sensitivity and Specificity and Specific Pattern of Biomarkers in AM Patients

Due to the strong separation of the control cohort and the untreated AM patients based on the $GlcNAc(Man)_2$ and

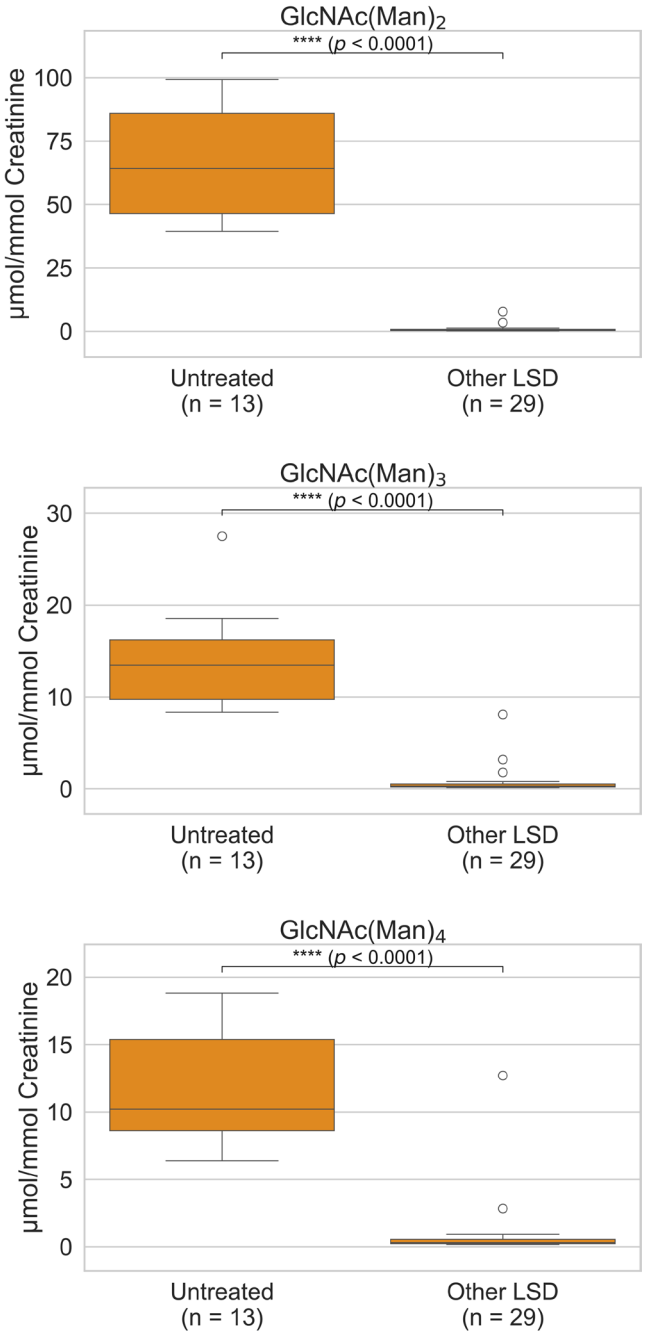

FIGURE 4 | Boxplots of the urinary biomarker concentrations measured in the samples of patients with other LSD and in the samples of untreated AM patients. AM, alpha-mannosidosis; LSD, lysosomal storage disorder. **** refers to statistically significance with level given in parenthesis.

$GlcNAc(Man)_4$ concentrations, 100% of untreated AM patients were correctly classified with the chosen cut-offs and 100% of the individuals from the control cohort would be correctly classified as not affected, resulting in an overall sensitivity of 100%. Additionally, we assessed the specificity of the method by analysis of urine samples from 26 patients with other LSDs, and none of them were classified as affected by AM. In this context, we observed that the concentrations of the three oligosaccharide biomarkers in AM patients exhibit a different pattern than in controls (Figure 5): the percentage of $GlcNAc(Man)_2$

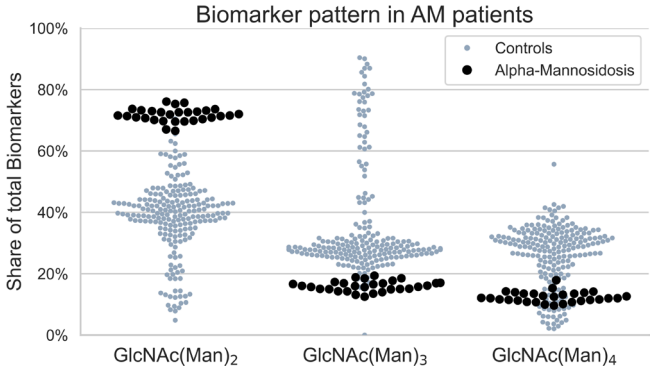

FIGURE 5 | Distribution pattern of the three biomarker oligosaccharides in patients with alpha-mannosidosis and controls. AM, alpha-mannosidosis.

in the sum of all three oligosaccharide biomarkers was in a range of 67%–76%, while the proportion of $GlcNAc(Man)_3$ and $GlcNAc(Man)_4$ was found to lie within ranges of 12%–19% and 10%–18%, respectively. The determination of this proportion, in addition to the $GlcNAc(Man)_2$ and $GlcNAc(Man)_4$ concentration, may help to prevent a misclassification of patients affected by other LSDs, in which an unspecific accumulation of various oligosaccharides may occur. Patient 0051 (patient 1 in ref. [24]) is an example for a patient who could be misclassified without assessing the biomarker pattern. He is affected by genetically proven ClC-7 hyperactivity (OMIM # 618541), a disorder causing increased acidification of lysosomes. In his sample 0124, we found a urinary $GlcNAc(Man)_2$ concentration of 7.8 µmol/mmol creatinine along with a quantifier/qualifier ratio of 1.26 and a $GlcNAc(Man)_4$ concentration of 12.7 µmol/mmol creatinine. In comparison to the reference interval for healthy individuals (0.2–4.0 µmol $GlcNAc(Man)_2$/mmol creatinine), the $GlcNAc(Man)_2$ concentration is elevated, the quantifier/qualifier ratio is within the target range (1.2% ± 20%), and the $GlcNAc(Man)_4$ concentration is well within the range for untreated AM patients (6.4–18.8 µmol/mmol creatinine). Assessing the proportion of the oligosaccharide biomarkers in this sample ($GlcNAc(Man)_2$: 27%, $GlcNAc(Man)_3$: 28%, $GlcNAc(Man)_4$: 44%), however, provides an indication that alpha-mannosidase is secondarily affected in this disorder due to a lower pH within lysosomes, but its defect is not the underlying cause. Hence, it is helpful to include $GlcNAc(Man)_3$ in the analysis and to calculate the oligosaccharide pattern, thereby improving diagnostic specificity.

To summarize, a positive diagnosis of AM with this method requires a $GlcNAc(Man)_2$ concentration of > 10 µmol/mmol creatinine along with a quantifier/qualifier ratio of 1.0–1.4, a $GlcNAc(Man)_4$ concentration of > 5 µmol/mmol creatinine. Further confirmation can be obtained by calculating the relative proportions of the three biomarkers. It is typical that the proportion of $GlcNAc(Man)_2$ in an AM patient falls in the range of 60%–80% and $GlcNAc(Man)_{3\&4}$ both contribute 10%–20% each. This protocol ensures maximum diagnostic sensitivity and specificity, and its application has successfully identified patients prior to standardized enzymatic and molecular genetic confirmation in our laboratory, which is additional evidence for the diagnostic prowess of our method.

4.4 | Treatment Monitoring

The development of this method also pursued a further objective, namely, to monitor the effectiveness of treatment of AM patients. To assess this, we compared the biomarker concentrations in samples of untreated AM patients, patients on ERT, and those who had undergone HSCT. Apart from the fact that there are statistically significant differences, the three groups (with the exception of a single relatively young patient, No. 0073, from whom only one sample could be measured so far) are also well separated from each other. Overall, we observed a clear difference of all three biomarker concentrations between samples of untreated patients and those receiving ERT or undergoing HSCT (Figure 3). Furthermore, biomarker concentrations of samples from patients receiving ERT and patients after HSCT treatment are also clearly separated, with significantly higher biomarker concentrations in patients receiving ERT than in AM patients after HSCT. Hence, this method sensitively detects differences between both treatments. One might ask—and, above all, investigate now—whether a higher dose or more continuous administration of enzyme replacement therapy produces a stronger effect, and examine the kinetics of the decline in biomarkers following a hematopoietic stem cell transplant. Of course, it is also conceivable that the smaller effect of ERT on the biomarkers reflects the inability of velmanase alfa to cross the blood–brain-barrier and the fact that the higher biomarker concentrations seen on ERT have their origin in brain tissue [25].

To further assess the suitability for treatment monitoring, we compared the biomarker concentrations in samples from individual patients before and after the start of ERT. Naturally, there were few patients who could be examined sequentially, but a very consistent picture emerged: the analysis of patient samples before and 3 and 6 months after start of ERT demonstrates that biomarker concentrations decrease within 3 months of treatment and stay rather constant thereafter (Figure 6; patients 0040 & 0041). These results are in line with the biomarker concentration change observed in the samples of patient 0028 and 0072. Here, samples were collected before starting ERT and approximately 1 year later (Figure S5). In addition, we compared the results of these four patients (No. 0028, 0040, 0041, and 0072) with the results of the samples of an untreated AM patient (No. 0039), which were obtained over a comparable time frame. The urine samples of the untreated AM patient showed elevated biomarker concentrations in all three samples, consistent with ranges of biomarker concentrations in other untreated AM patients (Figure S6). Thereby, we show that there is also good reproducibility of biomarker concentrations in a clinical setting. Lastly, we were interested in the development of biomarker concentrations in urine of patients receiving ERT for more than 6 months and analyzed two urine samples of a patient on ERT which were collected 12 months apart. The results are in line with the observations in samples taken after 3 and 6 months of ERT, as the biomarker concentrations in these two samples hardly changed (Figure S7).

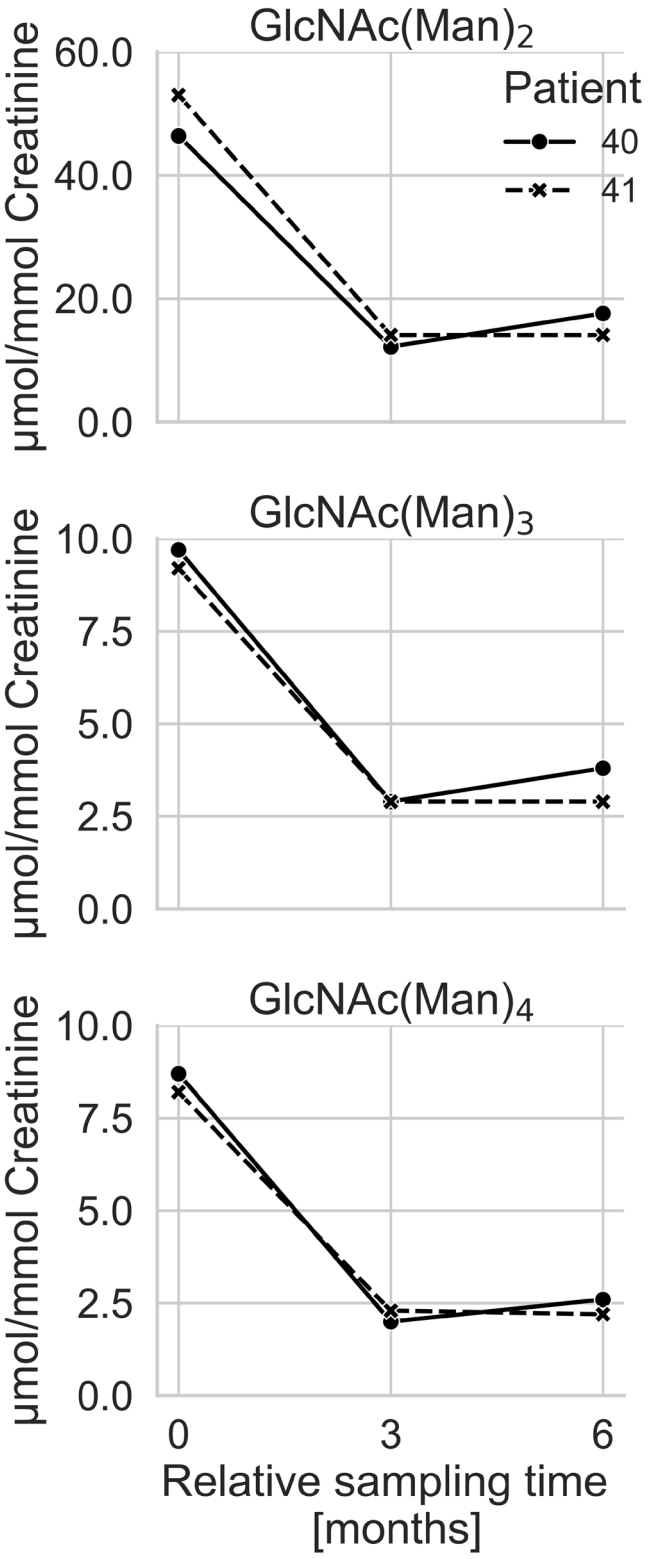

FIGURE 6 | Development of the biomarker concentrations in patients 40 and 41 before (0 months) and after ERT (3 and 6 months). ERT, enzyme replacement therapy.

In summary, we demonstrated the suitability of this method for monitoring treatment efficacy in patients undergoing ERT. Additionally, although there were no patients from whom we had both a sample before and after HSCT, the clear separation between untreated AM patients and patients after HSCT indicates that this method is also suitable for monitoring treatment efficacy after HSCT. Results from Kubaski et al. have recently demonstrated that the concentrations of the three oligosaccharide biomarkers in blood are potential predictors of engraftment status [22] and comparable results are likely to be

achieved with urine. However, further investigations are required to confirm this.

Taken together, the developed method is suitable for selective screening for AM and for monitoring treatment efficacy. A limitation of our study is the relatively low overall number of patient samples investigated and especially the low number of samples per type of treatment, which is due to the rarity of the disease. Likewise, no samples could be acquired from affected infants below the age of 2 years. Such patients might either be affected by the severe type, which often leads to early death, or by a milder form, in which diagnosis is often delayed with existing methods. As a result, no final assessment of the diagnostic power of this method for infants below the age of 2 years is possible at the moment. Nonetheless, we expect that the biomarker concentrations are elevated in AM patients already at an early age, as some of the highest biomarker concentrations were found in untreated patients at around 3 years of age (Figure 1). Finally, no clinical data is currently available for correlation with biomarker concentrations in treated patients. However, we know from Phase II and Phase III trials on enzyme replacement therapy for AM [6, 9, 25] that such a correlation exists, even when oligosaccharides were measured using less specific methods. The earlier treatment begins, the stronger the effect and the higher this correlation is likely to be.

5 | Conclusion and Outlook

We present a method which is rapid (with a preparation time of 1 h for up to 60 urine samples and an LC–MS/MS sample throughput of up to 10 samples per hour) and allows the quantitative analysis of urinary oligosaccharides that specifically accumulate in AM patients. We have demonstrated that it is well suited for diagnosis and treatment monitoring of AM. Valuable insights were gained regarding the different concentrations that can be expected in untreated AM patients, patients receiving ERT, and patients after HSCT. Additional investigations will be useful to obtain a better understanding of the oligosaccharide evolution during therapeutic measures. A denser sampling schedule would therefore allow a more detailed assessment of the effects of treatment. In addition, this approach could be transferred to other sample matrices. For example, a similar method for dried blood spots could enable the inclusion of AM into newborn screening programs (with a biomarker markedly more stable than enzyme activity) and thus allow an even earlier diagnosis of this disorder.

Author Contributions

Dominik Dörfel, **René Santer**, **Simona Murko:** conceptualization. **Dominik Dörfel:** data curation. **Dominik Dörfel:** formal analysis. **Nicole Muschol**, **René Santer**, **Simona Murko:** funding acquisition. **Dominik Dörfel**, **Benjamin Dreyer**, **René Santer**, **Simona Murko:** investigation. **Dominik Dörfel**, **Benjamin Dreyer**, **Davor Fielitz**, **Simona Murko:** methodology. **Mona Lindschau**, **Antonio Barbato**, **Nadia Altavilla**, **Giancarlo la Marca**, **Lara Maleen Marten**, **Friederike Bürger**, **Mara Botti**, **Lina Verena Sevenich**, **Ronen Spiegel**, **Anke Schumann**, **Melanie Herrmann**, **Nicole Muschol**, **René Santer**, **Simona Murko:** patient care and sample provision. **Dominik Dörfel**, **René Santer**, **Simona Murko:** project administration. **Simona Murko**, **René Santer**, **Nicole Muschol:** resources. **Dominik Dörfel:** software. **Simona Murko:** supervision. **Dominik Dörfel**, **Benjamin Dreyer:** validation. **Dominik Dörfel:** visualization. **Dominik Dörfel:** writing – original draft. **Dominik Dörfel**, **Benjamin Dreyer**, **Mona Lindschau**, **Antonio Barbato**, **Nadia Altavilla**, **Giancarlo la Marca**, **Lara Maleen Marten**, **Friederike Bürger**, **Mara Botti**, **Lina Verena Sevenich**, **Ronen Spiegel**, **Anke Schumann**, **Melanie Herrmann**, **Davor Fielitz**, **Nicole Muschol**, **René Santer**, **Simona Murko:** writing – review and editing. **Simona Murko:** correspondence. **Simona Murko:** guarantor. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work. All authors confirm the absence of previous similar or simultaneous publications.

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Ethics Statement

Ethical approval for the study was granted by the ethics committee of the Medical Association Hamburg (Ärztekammer Hamburg, 06.02.2024, processing number 2023-101200-BO-ff).

Consent

Informed consent was obtained prior to inclusion from all study participants and, if applicable, their legal guardians. According to §12 of the Hamburg Hospital Act (HmbKHG), no consent is required for anonymized samples used for the generation of reference ranges.

Conflicts of Interest

Mara Botti, Friederike Bürger, Davor Fielitz, Melanie Herrmann, Giancarlo la Marca, Mona Lindschau, Lara Maleen Marten, Anke Schumann, and Ronen Spiegel have nothing to declare. For the execution of this project, the department of Dominik Dörfel, Benjamin Dreyer, and Simona Murko received funding from Chiesi Deutschland GmbH. Antonio Barbato and Nadia Altavilla received speaker honoraria and travel reimbursement from Chiesi, Sanofi, and Takeda. Nicole Muschol received travel support, consulting fees, and/or honoraria from Amicus, Biomarin, GC Biopharma, JCR Pharmaceuticals, Sanofi Genzyme, Takeda, Ultragenyx, and Chiesi. Lina Verena Sevenich received honoraria for participation and/or presentations in advisory boards from Chiesi, Sanofi, and Takeda, and travel reimbursement from Biomarin. René Santer received speaker honoraria and travel reimbursement from Chiesi. Simona Murko received travel reimbursement from Chiesi and Orchard Therapeutics as well as speaker honoraria from Chiesi.

Data Availability Statement

The data and code underlying this article are publicly available in figshare at https://doi.org/10.6084/m9.figshare.29514059. Additional information is available from the authors on reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Overview of AM patients in this study including results of genetic analyses, residual enzymatic activity, and individual data for biomarkers $GlcNAc(Man)_2$, $GlcNAc(Man)_3$, and $GlcNAc(Man)_4$ measured in urine. **Table S2:** Overview of patients with other lysosomal storage diseases in this study. **Table S3:** MS/MS parameters for the detection of all four analytes. **Figure S1:** Linearity assessment for all three biomarkers in solvent and matrix. **Table S4:** Linear regression parameters for all three biomarkers in solvent and matrix. **Table S5:** Recoveries of $GlcNAc(Man)_3$ and $GlcNAc(Man)_4$ at each calibration level. Concentrations were calculated via the solvent calibration. **Table S6:** LOD and LOQ in urine of all three analytes. **Table S7:** Results for the accuracies and precisions of all three biomarkers. **Table S8:** Accuracies for all six individual sample matrices at low (L; 10 ng/mL) and high (H; 100 ng/mL) spiking levels ($n = 3$). **Table S9:** Assessment of matrix factor, IS-normalized matrix factor, extraction recovery, and process efficiency in low (L; 10 ng/mL) and high (H; 100 ng/mL) spiked samples ($n = 6$ matrices measured in triplicate). **Figure S2:** Long-term and freeze–thaw stability of all three biomarkers in urine. **Table S10:** Biomarker concentrations in samples of patients with other lysosomal storage diseases. **Table S11:** Overview of upper (U) and lower (L) limits of the concentration ranges of all three biomarkers for all study cohorts. **Figure S3:** Scatterplot of all samples analyzed in this study. $GlcNAc(Man)_3$ concentrations are plotted against the age of the subject at the time of sampling. AM, alpha-mannosidosis; ERT, enzyme replacement therapy; HSCT, hematopoietic stem cell transplantation. **Figure S4:** Scatterplot of all samples analyzed in this study. $GlcNAc(Man)_4$ concentrations are plotted against the age of the subject at the time of sampling. AM, alpha-mannosidosis; ERT, enzyme replacement therapy; HSCT, hematopoietic stem cell transplantation. **Figure S5:** Course of biomarker concentration in patients 28 and 72 before (0 months) and approximately 1 year after starting ERT treatment (13 and 14 months, respectively). ERT, enzyme replacement therapy **Figure S6:** Course of biomarker concentration in patient 39 (untreated) over time. **Figure S7:** Course of biomarker concentration in patient 35 (ERT) over time.