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ORIGINAL ARTICLE



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MOGS-CDG: Quantitative analysis of the diagnostic Glc₃Man tetrasaccharide and clinical spectrum of six new cases

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Abstract

Congenital disorders of glycosylation (CDG) are a clinically and biochemically heterogeneous subgroup of inherited metabolic disorders. Most CDG with abnormal N-glycosylation can be detected by transferrin screening, however, MOGS-CDG escapes this routine screening. Combined with the clinical heterogeneity of reported cases, diagnosing MOGS-CDG can be challenging. Here, we clinically characterize ten MOGS-CDG cases including six previously unreported individuals, showing a phenotype characterized by dysmorphic features, global developmental delay, muscular hypotonia, and seizures in all patients and in a minority vision problems and hypogammaglobulinemia. Glycomics confirmed accumulation of a $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ glycan in plasma. For quantification of the diagnostic $\text{Glc}\alpha 1\text{-3Glc}\alpha 1\text{-3Glc}\alpha 1\text{-2Man}$ tetrasaccharide in urine, we developed and validated a liquid chromatography-mass spectrometry method of 2-aminobenzoic acid (2AA) labeled urinary glycans. As an internal standard, isotopically labeled $^{13}\text{C}_6\text{-2AA}$ Glc_3Man was used, while labeling efficiency was controlled by use of $^{12}\text{C}_6\text{-2AA}$ and $^{13}\text{C}_6\text{-2AA}$ labeled laminaritetraose. Recovery, linearity, intra- and interassay coefficients of variability of these labeled compounds were determined. Furthermore, Glc_3Man was specifically identified by retention time matching against authentic MOGS-CDG urine and compared with Pompe urine. Glc_3Man was increased in all six analyzed cases, ranging from 34.1 to 618.0 $\mu\text{mol}/\text{mmol}$ creatinine (reference <5 μmol). In short, MOGS-CDG has a broad manifestation of symptoms but can be diagnosed with the use of a quantitative method for analysis of urinary Glc_3Man excretion.

KEYWORDS

biomarker, MOGS-CDG, multisystem, tetraglucoside, urine oligosaccharide

1 | INTRODUCTION

Congenital disorders of glycosylation (CDG) are a group of inherited metabolic disorders that affect the glycosylation of proteins and lipids needed for intercellular and intracellular processes. Defects in multiple glycosylation pathways have been associated with human disease, summing up to more than 160 types of CDG.¹ Consequently, the presenting symptoms are heterogeneous. Most CDG with defective protein N-glycosylation can be diagnosed by screening for transferrin N-glycosylation in blood. However, some N-glycosylation defects such as MOGS-CDG (OMIM 606056) escape diagnosis using this transferrin assay.² In MOGS-CDG, biallelic pathogenic variants in *MOGS* result in loss-of-function of the enzyme mannosyl-oligosaccharide glucosidase (glucosidase I). Glucosidase I is the first enzyme involved in the processing of N-linked glycans after their transfer from the dolichol donor to proteins in the endoplasmic reticulum.

Glucosidase I removes the distal $\alpha 1,2$ -linked glucose residue from $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ glycans. When glucosidase I is inactive, subsequent mannosidases produce $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ glycans.³ Alternatively, endomannosidase may remove a $\text{Glc}\alpha 1\text{-2Glc}\alpha 1\text{-3Glc}\alpha 1\text{-3Man}$ tetrasaccharide (Glc_3Man), which is excreted in urine, while the resulting $\text{Man}_8\text{GlcNAc}_2$ glycans are further processed in the Golgi resulting in normal transferrin N-glycosylation.⁴

Thus far, 24 cases of MOGS-CDG have been described.^{2,5-13} The reported clinical features include facial dysmorphisms, neurologic symptoms (global developmental delay, muscular hypotonia, seizures), immune dysfunction related to hypogammaglobulinemia, hepatomegaly, and other less common features. Nevertheless, the phenotypic spectrum and natural history remain to be delineated, especially given the limited number of cases.

Biochemical diagnosis of MOGS-CDG can be challenging. In traditional assays for N-glycosylation disorders such

as transferrin isoelectric focusing and quadrupole time-of-flight mass spectrometry (QTOF), there is no consistent alteration compared to the glycosylation pattern in unaffected individuals.¹⁴ However, diagnosis can be established through biomarker screening which is not based on transferrin. In literature, two biomarkers have been reported for MOGS-CDG; accumulation of the $\text{Glc}_3\text{Man}_{7-9}\text{GlcNAc}_2$ N-glycan in patient serum and on immunoglobulin (Ig)G and the urinary excretion of Glc_3Man .^{5,15} The urinary marker is pathognomonic for MOGS-CDG and has previously been analyzed by thin layer chromatography and mass spectrometry (MS), such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF).^{2,5,11} Since Glc_3Man has an identical mass as several other tetra-glucosides that are present in normal urine and specifically as $\text{Glc}\alpha 1-6\text{Glc}\alpha 1-4\text{Glc}\alpha 1-4\text{Glc}$ in individuals with Pompe disease and several glycogenoses, chromatographic separation is preferred for quantitative analysis.

In this report, we aimed to improve the biochemical identification of MOGS-CDG by developing a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to identify and quantify the presence of Glc_3Man in the urine. The current report furthermore aims to delineate the phenotypic spectrum by providing data on six novel cases in which we found variants in *MOGS*, as well as a follow-up of three reported patients.

2 | METHODS

2.1 | Clinical data collection

Subjects were identified at MetabERN recognized CDG expertise center in Radboudumc, The Netherlands as well as via international collaborations, revealing six novel cases of MOGS-CDG. All patients in whom biallelic variants in *MOGS* were identified were included. Variants were classified as class 4 or 5 in all but three patients (following ACMG criteria¹⁶). The homozygous variant found in patients 4, 5, and 6 was initially classified as 3B, a variant of uncertain significance. Informed consent was obtained. Ultimately, 10 patients were included in this study.

2.1.1 | Data collection

A questionnaire for clinicians was created to obtain the clinical patient information, which subsequently was extracted from medical records. This questionnaire was based on features that were found in previous reports and include general characteristics, details on pregnancy and neonatal course, and phenotypic features and overall history (dysmorphisms, neurological, and systemic symptoms). In

addition, we obtained brain imaging reports and whenever possible MRI brain scans were reevaluated.

2.2 | Plasma N-glycan profiling

2.2.1 | Samples

Plasma was available for two patients (1 and 6). Six sex-matched control samples were added which were received from our local blood bank.

2.2.2 | Sample preparation

Then, 10 μl plasma from controls and patients was mixed with 10 μl 8 M urea/10 mM Tris-HCl pH 8.0. Samples were reduced with 15 μl dithiothreitol (10 mM) and incubated for 30 min at room temperature. Samples were then alkylated using 15 μl 2-chloroacetamide (50 mM) and incubated for 20 min in the dark. Of this mix, 15 μl was used for PNGase F digestion. Samples were diluted using 80 μl 50 mM ammonium bicarbonate buffer before PNGase F (Biolabs) treatment and left to incubate overnight at 37°C to release N-glycans. Samples were stored at 4°C for 2 days to complete the conversion of ammonium groups to hydroxyl groups. Proteins were precipitated using 96% ethanol with the ratio of 1:3 and stored at -80°C for 3 h. Samples were centrifuged at 4°C for 5 min before transferring the supernatant. Samples were dried by centrifugation under reduced pressure.

Glycans were enriched using Hypersep Tip Microscale SPE Extraction tips (Thermo Fisher Scientific). Samples were dissolved in 50 μl water with 0.01% formic acid (FA). Tips were washed using acetonitrile (AcN) +0.01% FA and equilibrated with water +0.01% FA. The sample was loaded onto the tip and washed with water +0.01% FA and eluted in 40:60 water/AcN with 0.01% FA. Finally, the solution was neutralized using aqueous 0.4% NH_3 for storage. Samples were centrifuged under reduced pressure for 10 min to remove AcN before injection in the mass spectrometer.

2.2.3 | LC-MS/MS analysis of N-glycans

LC-MS/MS was performed using a Waters Acquity UPLC (Waters) coupled to a TimsTOF pro 2 mass spectrometer (Bruker). The instrument is equipped with an electrospray ionization source. Samples were separated by liquid chromatography using porous graphitized carbon column (10 cm length, 1 mm I.D., and 3 μm particle size, Thermo Fisher Scientific) which was heated at 60°C. Separation was achieved at 0.050 ml/min using mobile phase A (water + 0.1% FA and 0.04% trifluoroacetic acid (TFA)) and B

(AcN + 0.1% FA and 0.04% TFA) using partial loop injection. The following gradient was used with buffer A; 0–14 min 90–35%, 14–17 min 35–10%, and 18–22 min 10–90%. The TimsTOF pro 2 instrument was operated in positive ionization QTOF mode. The MS1 scan event was achieved with MS scan range of 100–3500 m/z , 1 Hz spectrum acquisition rate, 100 μ s transfer time, 12 μ s pre-pulse storage time, and 1500 Vpp collision cell RF.

2.2.4 | Data analysis

Data analysis was performed using targeted extraction using Skyline (version 20.2.0.343) with a target list consisting of 35 precursors (both M + 2H and M + 3H). Manual verification of correct peak integration was performed for all targets. Absolute intensities were exported after which relative normalization within samples was performed by dividing the individual N-glycan peak area by the total N-glycan peak area per sample.

2.3 | Quantitative analysis of urinary tetrasaccharides

2.3.1 | Synthesis of the standards $^{13}\text{C}_6$ -2AA-T4 and $^{13}\text{C}_6$ -2AA-L4

As internal standard (IS), we prepared $^{13}\text{C}_6$ -labeled T4 (Glc α 1-6Glc α 1-4Glc α 1-4Glc, Merck), and as labeling control we used $^{13}\text{C}_6$ -labeled laminaritetraose (L4, Glc β 1-3Glc β 1-3Glc β 1-3Glc, Carbosynth). To an Eppendorf tube containing 100 μ l 1 mM unlabeled T4 or L4, 100 μ l 30 mg/mL $^{13}\text{C}_6$ -aminobenzoic acid (2AA) (Merck) was added which was dissolved in 95% methanol (Merck) buffered with acetate: borate (2:1, v/v Merck). Additionally, 50 μ l 1 M 2-picoline borane (Merck) dissolved in 95% methanol was added. After incubation for 1 h at 80°C, the standards were purified using a DPA-6 S (Merck) column made in-house to remove access label. The column was equilibrated with AcN. Samples were dissolved in 96% AcN and loaded on the column. After washing, the samples were eluted with 20% AcN and dried completely under reduced pressure. After reaching dryness, the standards were dissolved in 1 ml water to a concentration of 100 μ M. Collision energies were optimized and using daughter scans transitions were determined (Supplementary Figure 1A).

2.3.2 | 2-AA labeling of urinary oligosaccharides

Single urine samples (as portion) were received in vials (patients 1, 2, and 4) or on filter paper (patients 5, 6, and

7). Control samples were obtained via routine diagnostics of patients with a suspicion of an inborn metabolic disorder and without confirmed diagnosis. Controls included 75 male and 46 female samples and mean age per age group in years was; 0–1: age of 0.3 ± 0.5 , 1–10: age of 4.9 ± 2.5 and 10–100: age of 37.1 ± 21.2 . For elution of oligosaccharides from filter paper, the sample area was cut in a V shape at the end for easier extraction of the oligosaccharides. The sample was then eluted with Millipore water from the top which flowed through the filter paper and dripped from the V shape into a tube. This continued until \sim 1 ml of diluted urine was collected. Creatinine levels were measured using common assays and results expressed relative to creatinine. A urine sample of the first described case² was used as a positive control. For validation of our method, we used three Pompe patient samples with low, medium, and high levels of T4 accumulation. Urine samples were diluted to a concentration of 0.2 mmol creatinine/L. To 10 μ l of the diluted urine samples, 10 μ l of a solution of freshly made 30 mg/ml $^{12}\text{C}_6$ -2AA and 107 mg/ml 2-picoline borane in methanol and 10 μ l of L4 as $^{12}\text{C}_6$ -2AA labeling control were added. The reaction mixtures were incubated at 60°C and left for 90 min until complete dryness.

2.3.3 | Measurement of tetrasaccharide using LC-MS/MS

Dried samples were reconstituted in 100 μ l MiliQ in which 75 nM $^{13}\text{C}_6$ -labeled L4 and 150 nM $^{13}\text{C}_6$ -labeled T4 were dissolved. To 6 μ l of this mixture, 50 μ l of 0.5% acetic acid in AcN was added. Of this mixture 5 μ l was injected. Separation of the oligosaccharides was achieved using a 3.5 μ m 200 Å zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) column (PEEK coated, 50 mm length, 2.1 mm I.D., 3.5 μ m particle size., 200 Å pore size, Thermo Fischer Scientific) coupled to a Xevo TQ-S micro mass spectrometer (Waters) in positive ionization mode with a flow rate of 0.25 ml/min. The LC solvents were mobile phase A 0.5% acetic acid in water and B 100% AcN. An isocratic elution gradient of 90% mobile phase B was used between 0.1 and 4.1 min and then a linear gradient followed with 30–60% A until 6.1 min. The column was then stripped with 99.5% mobile phase A until 8.1 min. Finally, it was reequilibrated from 8.6 to 12 min.

The single reaction monitoring (SRM) transition was set from 788.2 > 302.2 to detect $^{12}\text{C}_6$ 2AA-labeled tetraglucosides (Supplementary Figure 1B). For the $^{13}\text{C}_6$ 2AA-labeled standards, the SRM transition was set from 794.2 > 308.2 (see Supplementary Figure 1A). Both transitions were measured with 28 V collision energy and 33 V cone voltage. The MOGS-CDG specific Glc₃Man

TABLE 1 Most important clinical features in MOGS-CDG patients. Patient 7 is excluded from this table.

| | Present in current novel cases | Present in literature cases ^{2,6,10–13} | Total |
|------------------------|--------------------------------|--|------------------|
| Dysmorphic features | 6 of 6 (100%) | 23 of 23 (100%) | 29 of 29 (100%) |
| Developmental delay | 6 of 6 (100%) | 24 of 24 (100%) | 30 of 30 (100%) |
| Hypotonia | 6 of 6 (100%) | 22 of 24 (84.6%) | 28 of 30 (93.3%) |
| Seizures | 6 of 6 (100%) | 16 of 23 (75%) | 22 of 29 (75.9%) |
| Hypogammaglobulinemia | 2 of 4 (50%) | 18 of 20 (84.6%) | 20 of 24 (83.3%) |
| Recurrent infections | 3 of 6 (50%) | 18 of 23 (84.6%) | 21 of 29 (72.4%) |
| Vision problems | 2 of 6 (33.3%) | 17 of 18 (94.4%) | 19 of 24 (79.2%) |
| Respiratory infections | 4 of 6 (66.7%) | 11 of 21 (66.7%) | 15 of 27 (55.5%) |
| Feeding problems | 5 of 6 (83.3%) | 6 of 10 (55.6%) | 11 of 16 (68.8%) |
| Hepatomegaly | 1 of 6 (16.7%) | 13 of 22 (72.7%) | 14 of 28 (50%) |

Abbreviation: CDG, congenital disorders of glycosylation.

tetrasaccharide eluted at 3.76 min, close to the retention time (3.72 min) of the T4 tetraglucoside, diagnostic for Pompe disease. Peak integration and quantification were performed by Quanlynx and MS Excel. Quantification was performed using peak areas and concentration of the ISs added. A dilution curve of ¹²C₆ 2AA-labeled T4 standard was used to determine the linearity of the labeling, where the slope was 1.037 with an *R*² of 0.9987. A one-point calibration coefficient was based on this curve. Using the following formula, the concentration of T4 in patient samples was determined: Concentration ¹²C₆ 2AA-labeled T4 (μmol/mmol creatinine) = area ¹²C₆ 2AA-labeled T4/area ¹³C₆ 2AA-labeled IS T4 (intensity)/calibration coefficient × IS concentration ¹³C₆ 2AA-labeled T4 (μmol/mmol creatinine). This formula is also applied to labeling control L4. After these calculations, the concentration of ¹²C₆ 2AA-labeled T4 was corrected with the use of L4 via formula: Corrected concentration ¹²C₆ 2AA-labeled T4 (μmol/mmol creatinine) = calculated concentration ¹²C₆ 2AA-labeled T4 (μmol/mmol creatinine)/calculated concentration ¹²C₆ 2AA-labeled L4 (μmol/mmol creatinine) × added ¹³C₆ 2AA-labeled IS L4 (μmol/mmol creatinine).

3 | RESULTS

3.1 | Clinical characterization of novel and previously reported MOGS-CDG patients

Seven novel patients were identified with biallelic *MOGS* variants, and clinical data of three previously reported patients were obtained. The total of 10 patients included 5 male and 5 female patients with a mean age of 2 years and 2 months and ranged from 87 days to 6 years and 11 months. Patient 7 was initially identified with biallelic

MOGS variants. Reexamination of the exome sequencing data after obtaining negative biochemical results revealed that this patient had a heterozygous variant and was therefore excluded from the results of this study henceforth.

Overall, a rather heterogeneous phenotypic presentation was observed within this patient group (Table 1) without clearly distinguishing diagnostic features. Dysmorphic features included narrow forehead, narrow palpebral fissures with long eyelashes, broad nose, and retrognathia (Figure 1). A summary of phenotypic features of these novel patients together with literature cases includes moderate to severe global developmental delay, facial dysmorphism (Table 2), hypotonia, and seizures. Immune dysfunction was reported in the majority of the patients. Hepatomegaly (without functional abnormalities) was also reported in these novel patients. Furthermore, recurrent respiratory infections and feeding problems often requiring G-tube feeds were common. Surprisingly, these novel patients did not have abnormal MRI images, which is something that is frequently reported in literature. Also less frequently reported were vision problems, hearing problems, hypogammaglobulinemia, recurrent infections, and hepatomegaly. The clinical details of seven individual cases and an update of three reported cases in literature^{7,8} can be found in supplementary case reports, and Tables S1, S2, S3, and S4.

3.2 | Plasma N-glycan analysis reveals the characteristic oligomannose N-glycan in MOGS-CDG patients

Plasma from MOGS-CDG patients 1 and 6 was available to determine the N-glycan profile. Evaluating the glycan class distribution (Figure 2A) revealed a slight elevation



FIGURE 1 Dysmorphic features in novel MOGS-congenital disorders of glycosylation (CDG) patients. (A) Patient 4 at 2 years of age. (B) Patient 5 at 3 months of age. (C) Patient 2 at 11 months of age. (D) Summary of dysmorphic features of the six novel cases described in this study

in relative abundance of hybrid and high-mannose glycan structures compared to controls ($p < 0.01$), and a consequently slight decrease in abundance of complex glycans. Known biomarker with composition $\text{Hex}_{10}\text{HexNAc}_2$ was distinctly increased in both patients (Figure 2B). Additionally, the hybrid $\text{Hex}_6\text{HexNAc}_3\text{NeuAc}_1$ glycan was significantly increased in MOGS patients ($p < 0.01$).

3.3 | Development of a quantitative assay for analysis of the urinary Glc3Man tetrasaccharide

Multiple tetrasaccharides with isobaric molecular mass can be present in urine. The specific tetraglucoside consisting of four glucose residues in $\text{Glc}\alpha 1-6\text{Glc}\alpha 1-4\text{Glc}\alpha 1-4\text{Glc}$ linkage (T4) is increased in the urine of individuals with

TABLE 2 Most important dysmorphic features in MOGS-CDG patients. Patient 7 is excluded from this table. Patients from literature are shown in bold corresponding to reference number

| Patient number | 1 | 2 | 3 | 4 | 5 | 6 | 8 | 9 | 10 | 2 | 6 | 10 | 11 | 12 | Frequency |
|------------------------------|---|---|---|---|---|---|---|---|----|---|---|----|----|----|------------------|
| Narrow forehead | – | + | – | + | + | + | + | + | + | – | + | + | ND | ND | 9 of 12 (75%) |
| Long eyelashes | + | + | – | + | + | + | + | + | + | + | – | + | ND | + | 17 of 22 (77.2%) |
| Short palpebral fissure | + | + | + | – | – | – | + | + | + | + | + | – | + | + | 15 of 26 (57.7%) |
| Broad/prominent nose | – | – | – | + | + | + | + | + | + | + | – | – | + | + | 18 of 26 (69.2%) |
| High/cleft palate | + | – | + | – | – | – | + | + | + | + | – | – | + | + | 15 of 21 (71.4%) |
| Retrognathia/micrognathia | + | – | + | + | + | + | + | + | + | + | – | + | + | + | 21 of 25 (84%) |
| Clenched/overlapping fingers | – | – | + | – | – | – | – | + | + | + | – | + | + | + | 15 of 25 (60%) |

Note: ND: not described.

Abbreviation: CDG, congenital disorders of glycosylation.

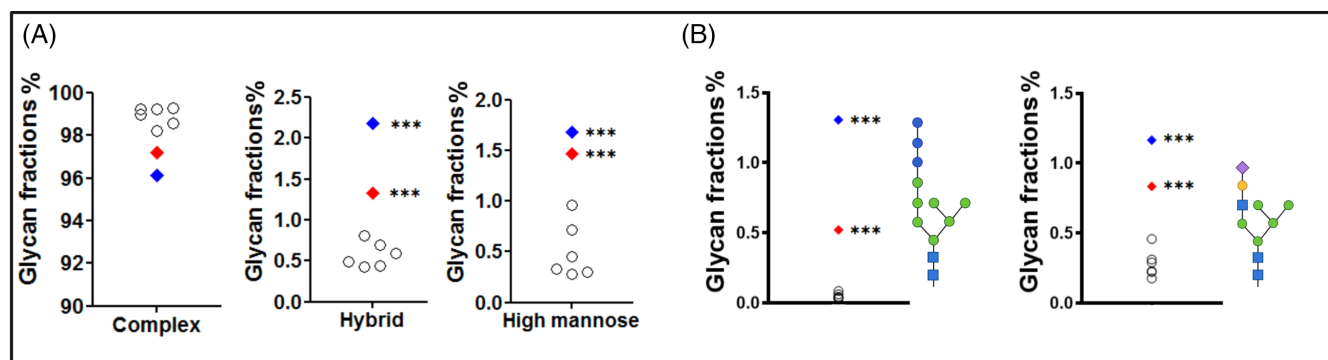


FIGURE 2 Summary of the N-glycan profiles of patients 1 and 6. (A) Glycan class distribution of complex, hybrid, and high mannose glycans in patient 1 (in red), patient 6 (in blue), and controls (white open circles). (B) Hex₁₀HexNAc₂ and Hex₆HexNAc₃NeuAc₁ glycans of control and patients. Patient 1 is in red, patient 6 in blue. Significance *** indicates $p < 0.01$. All annotated glycans with their relative abundance are reported in Supplementary Table S5

Pompe disease, several glycogen storage disorders and disorders of autophagy.¹⁷ For MOGS-CDG, the Glc₃Man tetrasaccharide with isobaric molecular mass has been identified before as diagnostic marker by thin layer chromatography and MALDI MS.^{2,11} In view of the different (diagnostic) tetrasaccharide isomers, we aimed to develop a quantitative assay by liquid chromatography-MS for analysis of the T4 and Glc₃Man diagnostic biomarkers. Since no commercial standards are available, a labeling strategy with light and heavy labels was exploited for quantification. Reductive amination with 2AA was selected as this is compatible with aqueous solutions. Commercial T4 was labeled with ¹³C₆-2AA for use as an IS and laminaritetraose (Glcβ1-3Glcβ1-3Glcβ1-3Glc) as non-mammalian tetraglucoside was used as labeling control to correct for labeling efficiency. Different standard tetraglucoside isomers were separated by ZIC-HILIC chromatography (Figure 3B). In infants (<1 year), we see an overall higher level and high fluctuation of different tetraglucoside isomers in urine, possibly due to human milk oligosaccharides. With our method, we could also achieve separation of the Glc₃Man peak in these samples

(Figure 3C). We then analyzed urine from one of our six suspected MOGS-CDG patients and identified a strong accumulation of tetrasaccharide Glc₃Man at RT 3.76 min (Figure 3E). Here, the first peak at 3.35 min corresponds to the ¹²C₆ 2AA-labeled L4 and ¹³C₆ 2AA-labeled L4 standards, while both the ¹³C₆ 2AA-labeled T4 standard and elute at 3.73-3.76 minutes respectively. Inspection of urine samples of 150 disease controls did not show a peak at 3.76 minutes (data not shown). A clear distinction is visible from patients with Pompe disease as both the T4 peaks coelute (Figure 3D).

3.4 | Analysis of urinary tetraglucosides

The ¹³C₆ 2AA-labeled tetraglucoside standards were used to establish a workflow for quantitative analysis of urinary tetraglucosides (Figure 3A). L4 as labeling control and labeling reagents were directly added to patient and control urine samples. After reductive amination with ¹²C₆-2AA, ¹³C₆-2AA labeled standards were added. Analysis was performed by LC-MS/MS, using ZIC-HILIC

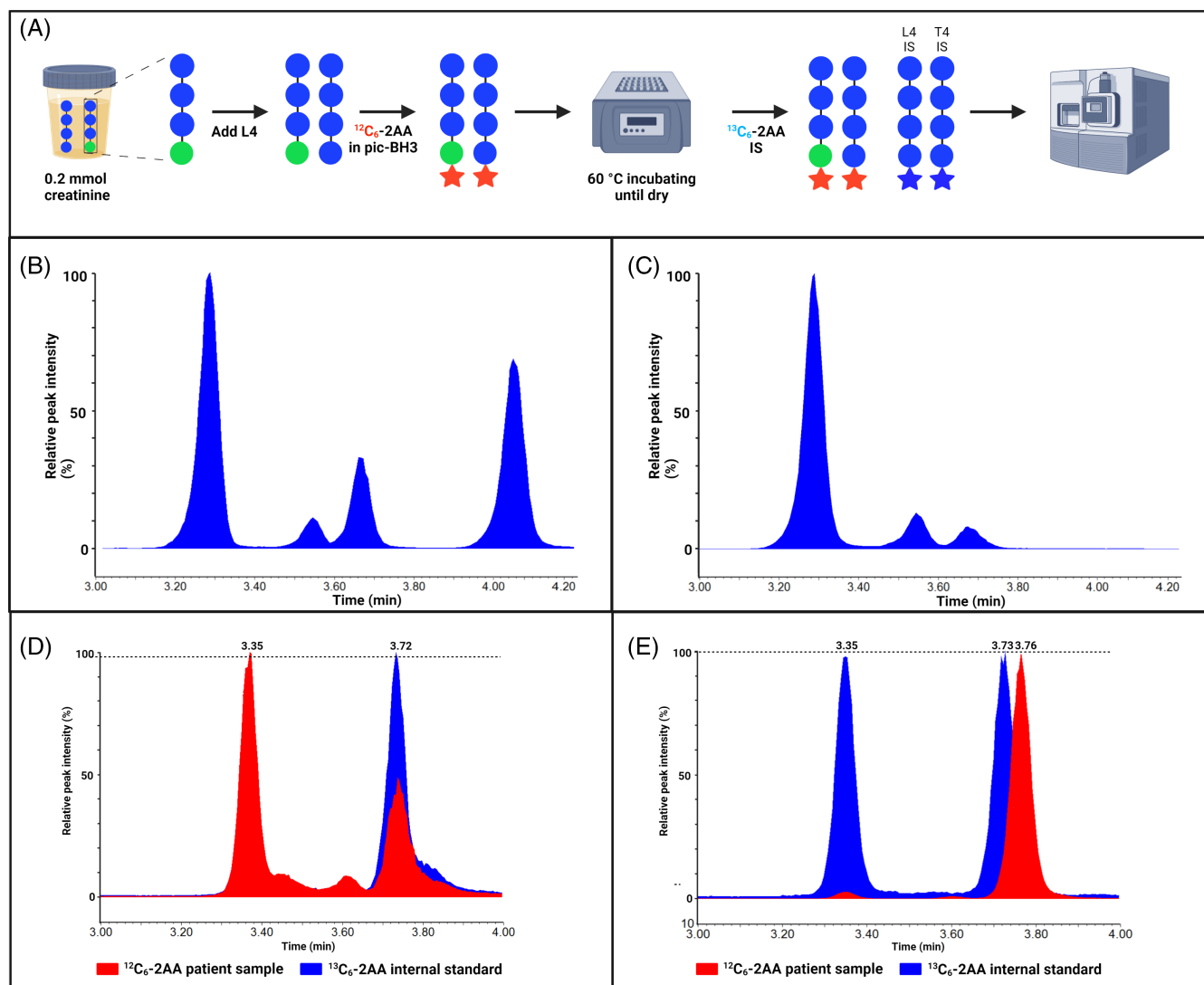


FIGURE 3 Workflow and chromatographic separation of tetraglucoside standards and patient-derived tetrasaccharides. (A) Schematic representation of sample preparation workflow. First, a Glc4 labeling control (L4) is added to the urine sample containing T4. Both are labeled with $^{12}\text{C}_6$ 2-aminobenzoic acid (2AA) labeling, after which $^{13}\text{C}_6$ 2AA-labeled internal standards (IS) are mixed in the samples. This is measured on a QqQ mass spectrometer. (B) Separation of different $^{13}\text{C}_6$ 2AA-labeled tetraglucoside standards in a single measurement. From left to right; laminaritetraose, maltotetraose, tetraglucoside, and isomaltotetraose. (C) Separation of different tetraglucosides in urine of an infant of <1 year. (D) Chromatogram for a Pompe patient. Peak with maximum peak height at 3.35 min is $^{12}\text{C}_6$ -2AA-L4 and $^{13}\text{C}_6$ -2AA-L4, whereas peak with maximum peak height at 3.72 min is $^{12}\text{C}_6$ -2AA-T4 and $^{13}\text{C}_6$ -2AA-T4. Red is $^{12}\text{C}_6$ trace, blue is $^{13}\text{C}_6$ trace. Both traces for L4 and T4 IS and patient T4 are overlapping. The dotted line represents the level of L4, which is the same in $^{12}\text{C}_6$ and $^{13}\text{C}_6$ traces. Peaks are baseline normalized, and the highest peak in each trace is set to 100%. (E) Chromatogram for a patient 6. Peak with maximum peak height at 3.35 min is $^{12}\text{C}_6$ -2AA-L4 and $^{13}\text{C}_6$ -2AA-L4, whereas peak with maximum peak height at 3.73 min is $^{13}\text{C}_6$ -2AA-T4 and at 3.76 min $^{12}\text{C}_6$ -2AA-Glc₃Man. Red is $^{12}\text{C}_6$ trace, blue is $^{13}\text{C}_6$ trace. Traces for L4 are overlapping, whereas T4 IS and patient Glc₃Man have a retention time difference (0.03 min). The dotted line represents the level of L4, which is the same in $^{12}\text{C}_6$ and $^{13}\text{C}_6$ traces. Peaks are baseline normalized, highest peak in each trace is set to 100%

chromatography. After optimization of collision energies, MRM transitions were selected for analysis of $^{12}\text{C}_6$ 2AA-labeled ($788.2 > 302.2$) and $^{13}\text{C}_6$ 2AA-labeled ($794.3 > 308.14$) tetraglucosides.

After correction for labeling efficiency, quantification of the diagnostic T4 tetraglucoside in urine samples was achieved by using the $^{13}\text{C}_6$ 2AA-labeled T4 IS. Coefficient

of variability (CV) was determined in urine samples with low, medium, and high secretion of T4 within 1 batch and several batches and showed to be <10% (Supplementary Table 6). Accuracy and precision was determined in urine samples with low, medium, and high secretion of T4 where the recovery was >90%. The assay was linear from 0.5 $\mu\text{mol}/\text{mmol}$ creatinine (lower limit of detection,

S/N ratio >10) to 200 $\mu\text{mol}/\text{mmol}$ creatinine for T4, and carry over was 0.15%.

TABLE 3 Validation of stability of Glc4 labeling of $^{12}\text{C}_6$ 2AA-labeled patient T4 peak area *with* correction using the IS. Samples of seven Pompe patients were quantified and stored up to 2 months at -18°C . Quantification of Glc4 accumulation was on day 1, day 6, 3 weeks and 2 months. Levels are shown in $\mu\text{mol}/\text{mmol}$ creatinine

| Sample nr | Day 1 | Day 6 | 3 weeks | 2 months |
|-----------|-------|-------|---------|----------|
| 1 | 2.5 | 2.9 | 2.2 | 2.3 |
| 2 | 169.1 | 162.5 | 175.2 | 191.7 |
| 3 | 1.3 | 1.5 | 1.5 | 1.5 |
| 4 | 1 | 1.0 | 1.2 | 1.4 |
| 5 | 3.5 | 3.5 | 3.2 | 3.9 |
| 6 | 7.2 | 7.4 | 7.9 | 7.7 |
| 7 | 1.9 | 2.0 | 2.1 | 2.2 |

Abbreviations: 2AA, 2-aminobenzoic acid; IS, internal standard.

TABLE 4 Validation of stability of Glc4 labeling of $^{12}\text{C}_6$ 2AA-labeled L4 peak area and patient T4 peak area *without* correction using the IS. Samples of seven Pompe patients were prepared using fresh and stored labeling mix. Quantification of L4 label and the non-corrected T4 accumulation was on day 1, 1–2 weeks and 3 months. Levels of day 1 and 3 months were determined in duplicates. Levels are shown in $\mu\text{mol}/\text{mmol}$ creatinine

| Sample nr | $^{12}\text{C}_6$ -2AA-L4 | | | $^{12}\text{C}_6$ -2AA-T4 | | |
|-----------|---------------------------|-----------|----------|---------------------------|-----------|----------|
| | Day 1 | 1–2 weeks | 3 months | Day 1 | 1–2 weeks | 3 months |
| 1 | 2.8–3.0 | 2.9 | 0.1 | 11.9–12.8 | 13.0 | 0.5 |
| 2 | 2.9–3.0 | 2.9 | 0.0 | 34.4–34.6 | 34.1 | 0.3–0.4 |
| 3 | 2.7–2.9 | 3.0 | 0.1 | 39.8–40.9 | 40.8 | 0.7–0.8 |
| 4 | 3.0–3.1 | 3.1 | 0.0 | 16.9–18.1 | 19.0 | 0.1 |
| 5 | 3.8–3.9 | 3.9 | 0.0 | 58.2–59.8 | 59.8 | 0.1 |
| 6 | 2.8–3.0 | 3.1 | 0.0–0.1 | 84.3–85.9 | 90.5 | 1.5 |
| 7 | 2.8 | 3.2 | 0.1 | 8.6–8.7 | 9.0 | 0.3 |

Abbreviations: 2AA, 2-aminobenzoic acid; IS, internal standard.

TABLE 5 Urinary analysis of the Glc₃Man tetrasaccharide for six suspected MOGS-CDG patients. Top: Control ranges of the T4 tetraglucoside in urine per age category. Bottom: Quantitative levels of Glc₃Man tetrasaccharide for six suspected MOGS-CDG patients and three individual controls. Patient from De Praeter et al. was used as positive control²

| | Age range | Concentration Glc ₃ Man ($\mu\text{mol}/\text{mmol}$ creatinine) | Diagnosis confirmed? |
|-------------------------------|--------------|--|----------------------|
| Controls ($n = 18$) | 0–1 years | 0–10 | |
| Controls ($n = 55$) | 1–10 years | 0–5 | |
| Controls ($n = 48$) | 10–100 years | 0–2 | |
| Control 1 | 1–2 years | 1,6 | No |
| Control 2 | 3–4 years | 2,0 | No |
| Control 3 | 4–5 m | 3,0 | No |
| Patient 1 | 7–8 years | 34.1 | Yes |
| Patient 2 | 1–2 years | 618.0 | Yes |
| Patient 4 | 1–2 years | 294.1 | Yes |
| Patient 5 | 3–4 years | 542.8 | Yes |
| Patient 6 | 4–5 months | 521.4 | Yes |
| Patient 7 | 6–7 years | 1.6 | No |
| Positive control ² | 0–7 days | 347.0 | Yes |

Abbreviation: CDG, congenital disorders of glycosylation.

Next, we evaluated the robustness of the sample preparation, including labeling. Labeled urine extracts of seven different Pompe patients were stored for 24 h at room temperature or a maximum of 2 months at -18°C , and were shown to be stable for at least 2 months (Table 3). Additionally, the labeling mix itself was stored for up to 3 months and shown to be stable for 1 week (Table 4).

3.5 | Clinical validation and application to MOGS-CDG

Reference ranges were established by analysis of 121 samples and shown to be age dependent (Table 5). Mean tetrasaccharide per age group in $\mu\text{mol}/\text{mmol}$ creatinine: 0–1: 5.27 ± 1.96 , 1–10: 1.82 ± 0.85 , and 10–100: 0.87 ± 0.29 (Table 5). Patients with known diagnosis of Pompe

disease showed elevated excretion of T4 tetraglucoside (data not shown).

We then analyzed the available urine samples in our cohort of suspected MOGS-CDG patients. In all cases, a clear accumulation of the Glc₃Man tetrasaccharide was visible, with a clearly distinct retention time as compared to classical Pompe disease patients (Figure 3D,E and Supplementary Figure 2). Quantitative analysis (Table 5) showed strong but variable elevations, not directly correlating with clinical severity or age.

Patient 7 had been diagnosed with MOGS-CDG based on the identification of a compound heterozygous variant (c.1079C > T, p.(Ala360Val); c.1851 T > A, p.(His617Gln)) in the *MOGS* gene. Urinary excretion of Glc₃Man was normal. Reanalysis of the variants revealed that the c.1851 T > A variant was found in homozygous state in GnomAD (which was not available upon first analysis/variant interpretation several years before).

In conclusion, our novel quantitative method allowed to functionally confirm or exclude a diagnosis of MOGS-CDG in six patients.

4 | DISCUSSION

MOGS-CDG is a rare disease and therefore little is known about the consistency of the clinical phenotype. Despite being a N-glycosylation disorder, classical CDG diagnostic assays using transferrin fail to diagnose MOGS-CDG as it shows a normal pattern. Therefore, in this study we have developed a novel quantitative diagnostic assay based on the tetrasaccharide accumulation in urine.

Furthermore, this study contributes to the existing knowledge of MOGS-CDG by providing a description of six new patients and a follow-up of three already reported patients. Recently, Shimada et al. reported an extensive clinical review of MOGS-CDG patients.¹² Most clinical features found in our novel cases are in line with those reported in that study and other previous studies. The core phenotype includes dysmorphic features, global developmental delay, hypotonia, and seizures. In addition, vision problems and hypogammaglobulinemia are notable hallmarks in some but not all patients. This constellation of features should prompt a differential diagnosis of MOGS-CDG. Quite surprisingly, brain imaging abnormalities were only present in one patient. It is possible that subtle abnormalities were missed due to the (very) young age of the patients resulting in fast low resolution sequences and motion artifacts. Furthermore, the clinical spectrum of MOGS-CDG does include patients without congenital brain abnormalities. In the literature cases analyzed in this study, abnormalities were found in

79.2% of reported patients.^{2,5-13} Analysis of urinary excretion of the Glc₃Man biomarker can be initiated to screen for MOGS-CDG, followed by genetic confirmation via targeted exome or single analysis. Or vice versa, variants (of unknown significance) in *MOGS* can be validated via biochemical testing in urine.

Progressive intellectual neurologic deterioration (PIND; Warmerdam et al.) with loss of speech in patient 1, hypocortisolism (patient 3), and microcolon (patient 3) could represent novel features.²⁰ Hyporeflexia suggesting polyneuropathy as seen in patient 6 is uncommon albeit reported before in one of the patients by Shimada et al., 2022.¹²

In patient 4, a dual diagnosis was made. The cataract (OMIM 604209) was caused by a heterozygous known pathogenic variant in *CRYAA* (c.346C > T; p(Arg116Cys)) rather than *MOGS*, which prevented an unjust attribution of this feature to the spectrum of MOGS-CDG. The importance of a full analysis of the exome to identify all possible genetic conditions in patients is essential to avoid erroneous clinical spectrum descriptions. Indeed, dual diagnoses occur, as illustrated by the study of Posey et al. and Tarailo-Graovac et al. reporting dual diagnoses causing blended phenotypes in up to 1 out of 20 rare disease cases in which whole-exome sequencing was informative.^{18,19} Patient 7 was also excluded in this study due to the identification of a single heterozygous variant in *MOGS*. Still, this case report illustrates the importance of biochemical testing to determine the pathogenicity of molecular variants.

One unanticipated finding was regression with loss of speech present in patient 1. As far as we are aware, this is the first time PIND was reported in MOGS-CDG.²⁰ The only other CDG in the list of IMDs presenting with PIND is PMM2-CDG, which is also a defect in the N-glycosylation.²¹

The apparent paradox of hypogammaglobulinemia without a corresponding infection rate as previously described by Sadat et al. was also found in our patient 2.⁵ The patient was exposed to the Influenza and Varicella Zoster viruses but was not infected. This supports the finding of their study that MOGS-CDG patients seem to have a markedly reduced susceptibility to glycosylation-dependent enveloped viruses.⁵ This might be because these viruses depend on glycoproteins synthesized by infected host cells, which are probably absent in MOGS-CDG patients.²² On the contrary, some patients of our study (patients 3 and 10) were infected with enveloped viruses, which would reject this hypothesis. Similarly, studies of the pathophysiology of COVID-19 using *MOGS* deficient cells did not show changes in cytokine production.²³ Further study is therefore needed to understand the pathophysiology of this reduced susceptibility

for infection and at the same time more patients should be followed clinically.

Evaluating N-glycan profiles of patients 1 and 6 showed an accumulation of the biomarker Hex₁₀HexNAc₂. This biomarker has been described before in multiple studies.^{5,15} This glycan is disease-specific for MOGS-CDG and shown to present on IgG. IgG glycans can be measured with multiple techniques including glycomics which has been done in this study. This glycan can also be used for a diagnostic assay; however, we did not have plasma samples available from all the patients. The other glycan that was significantly increased was the hybrid glycan Hex₆HexNAc₃NeuAc₁. This glycan is also seen in the study of Messina et al.¹⁵ It is not disease-specific, however, having an overview of all glycans present on IgG or serum could be a first step to understand disease mechanisms as well as a start to combine biomarkers. In our glycomics data, we have observed some different isoforms specific for patient samples (data not shown). Even so, due to our small sample size ($n = 2$), further testing is needed before we can determine if it is pathognomonic. A future step could be to perform glycoproteomics, to find protein specific changes to get a deeper knowledge of glycosylation changes.

Our tetrasaccharide method is a specific method to diagnose MOGS-CDG. Specificity of this tetrasaccharide for MOGS-CDG is due to the mannose present in tetrasaccharide resulting from the activity of the endomannosidase present in liver cells.²⁴ Our new biomarker screening technique is very reliable: in all the five measured novel cases, the clear peak for the tetrasaccharide was found. The reevaluation of the diagnosis of patient 7 also highlights the importance of biochemically confirming the disease. In this case, variants of unknown significance (VUS) were found for this patient and the mutations were not pathognomonic. This clearly shows the importance of the biomarker. From this, we can infer that Glc₃Man measured by LC-MS/MS is a very specific biomarker for MOGS-CDG and should be performed when this diagnosis is suspected. MALDI TOF systems have been used to determine excretion of the Glc₃Man tetrasaccharide, but these methods are not quantitative.^{2,11} Apart from its reliability, the straightforward sample preparation method should be noted. Once the sample is received it takes just 2 hours to be ready for injection, making this diagnostic assay a fast procedure.

In addition, it is in current diagnostic use for analysis of the diagnostic tetraglucoside for Pompe disease and glycogenosis. Additionally, this method can be extended to other oligosaccharide accumulation disorders such as mucopolysaccharidoses, gangliosidosis, glycogen storage disorders, and lysosomal storage disorders. QqQ methods have been developed for a selection of these disorders and

implemented in diagnostic laboratories.¹⁷ A quantitative method is particularly needed for these disorders as these disorders can have a mild accumulation. Also, a quantitative method for treatment monitoring or diet interventions can be of help to correct accumulation differences due to age, resulting in less bias and making reliable therapy threshold values possible.

As for all CDGs, management of MOGS-CDG patients requires a multidisciplinary approach as multiple systems are affected, ideally in a CDG center of expertise. A coordinating pediatrician, metabolic diseases specialist, immunologist, genetic counselor, neurologist, and allied health support including physiotherapy and speech/language therapy should be involved. Immunoglobulins should be monitored, and a low threshold for antimicrobial treatment may be warranted in case of an immune deficiency. Supportive management includes adequate seizure control and involvement of a nutritionist to ensure adequate intake and growth, if necessary via tube feeding.

Apart from symptomatic management, there is no targeted treatment available for MOGS-CDG patients. However, for future evaluation of new treatments, a quantitative method will be needed to monitor treatment efficacy. Our rapid screening method will allow for this and should be considered when determining therapeutic outcomes.

In conclusion, with this novel quantitative method we were able to diagnose five novel MOGS-CDG patients as well as quantitatively determine the tetrasaccharide accumulation in urine. Moreover, we characterized six novel cases and confirmed the typical phenotype with dysmorphic features, global developmental delay, hypotonia, seizures; and described less common (vision problems, hypogammaglobulinemia, hyporeflexia) features as well as PIND for the first time. For patients with these clinical symptoms, a diagnostic screen for the Glc₃Man tetrasaccharide in urine should be considered to diagnose MOGS-CDG and/or validate VUS found in the exome.

AUTHOR CONTRIBUTIONS

Conception and design: Clara D. M. van Karnebeek and Dirk J. Lefeber. Method development: Fokje SM Zijlstra, Merel A. Post, Arno van Rooij, and Melissa Bärenfänger. Clinical data collection and interpretation: John Christodoulou, Danqun Jin, Joy Yaplitto-Lee, Beom Hee Lee, Min Li, Karen J. Low, Andrew A. Mallick, Katrin Öunap, Tiong Yang Tan, Anna Le Fevre, James Pitt, William Reardon, Mari-Anne Vals, and Saskia B. Wortmann. Biochemical analysis and interpretation: Hans J. C. T. Wessels, Melissa Bärenfänger, Merel A. Post, Isis de

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data presented in this study are available upon reasonable request from the corresponding authors.

ETHICS STATEMENT


All patients and control subjects (or their guardians) gave their informed consent for sharing of patient information and use leftover body fluid samples from clinical diagnostics for laboratory method validation purposes in their electronic patient record, in agreement with institutional and national legislation.

INFORMED CONSENT

All procedures followed were in accordance with the declaration of Helsinki revised in 2000. Written informed consent for publishing anonymized patient information was obtained from the guardians for all individual participants included in the study. Additional written informed consent was obtained from all guardians of the patients for which identifying information is included in this study.

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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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