Toward understanding tissue-specific symptoms in dolichol-phosphate-mannose synthesis disorders; insight from DPM3-CDG

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Abstract
The congenital disorders of glycosylation (CDG) are inborn errors of metabolism with a great genetic heterogeneity. Most CDG are caused by defects in the N-glycan biosynthesis, leading to multisystem phenotypes. However, the occurrence of tissue-restricted clinical symptoms in the various defects in dolichol-phosphate-mannose (DPM) synthesis remains unexplained. To deepen our understanding of the tissue-specific characteristics of defects in the DPM synthesis pathway, we investigated N-glycosylation and O-mannosylation in skeletal muscle of three DPM3-CDG patients presenting with muscle dystrophy and hypo-N-glycosylation of serum transferrin in only two of them. In the three patients, O-mannosylation of α-dystroglycan (αDG) was strongly reduced and western blot analysis of β-dystroglycan (βDG) N-glycosylation revealed a consistent lack of one N-glycan in skeletal muscle. Recently, defective N-glycosylation of βDG has been reported in patients with mutations in guanosine-diphosphate-mannose pyrophosphorylase B (GMPPB). Thus, we suggest that aberrant O-glycosylation of αDG and N-glycosylation of βDG in skeletal muscle is indicative of a defect in the DPM synthesis pathway. Further studies should address to what extent hypo-N-glycosylation of βDG or other skeletal muscle proteins contribute to the phenotype of patients with defects in DPM synthesis. Our findings contribute to our understanding of the tissue-restricted phenotype of DPM3-CDG and other defects in the DPM synthesis pathway.

KEYWORDS
congenital disorders of glycosylation, dolichol-phosphate-mannose, DPM3-CDG, dystroglycanopathy, tissue-specific glycosylation
1 | INTRODUCTION

The congenital disorders of glycosylation (CDG) are inborn errors of metabolism characterized by aberrant glycoprotein and glycolipid glycan synthesis. CDG-I phenotypes are due to defects in the assembly of the lipid-linked oligosaccharide (LLO) in the ER, leading to a lack of complete N-glycans. Since LLO synthesis is essential for protein N-glycosylation in all tissues, patients with CDG-I present with multisystem phenotypes comprising developmental disability, hypotonia, skin and skeletal abnormalities, hepatopathy, and neurologic involvement.\(^1,2\) Defects in the synthesis of the sugar donor dolichol-phosphate-mannose (DPM) results in CDG-I with tissue-specific disease (Figure 1). Phosphomannomutase

![Figure 1](image_url)

**FIGURE 1** Defects in the dolichol-phosphate-mannose biosynthesis pathway lead to N- and O-glycosylation disorders. Dolichol-phosphate-mannose (Dol-P-Man) is the mannose donor for N-glycosylation, O-mannosylation, C-mannosylation, and GPI-anchor biosynthesis. SRD5A3-CDG patients present with structural and functional eye abnormalities, cerebellar defects, intellectual disability (ID), and muscle hypotonia.\(^3\)–\(^6\) PMM2-CDG patients have characteristic multisystem symptoms that are associated with generalized N-glycosylation abnormalities. Patients with mutations in GMPPB have dystroglycanopathy due to reduced O-mannosylation of αDG,\(^7,8\) congenital myasthenic syndrome (CMS),\(^9\) or pseudo-metabolic myopathy.\(^10,11\) Mutations in DOLK lead to abnormal serum transferrin N-glycosylation and heart αDG O-glycosylation with dilated cardiomyopathy, muscular hypotonia, neurological involvement, and/or ichthyosis.\(^12\)–\(^16\) The clinical phenotypes associated with mutations in the DPM synthase subunits DPM1, DPM2, or DPM3 are different: DPM1-CDG patients show multisystem presentations characteristic for CDG-I and muscular dystrophy,\(^17\)–\(^21\) DPM2-CDG patients have microcephaly, seizures, developmental delay, and hypotonia,\(^22\) and two DPM3-CDG patients have been reported with muscular dystrophy, of which one patient also presented with cardiomyopathy.\(^23,24\) Blue boxes: abnormal serum transferrin N-glycosylation. Red box: Abnormal O-mannosylation of αDG. Purple boxes: combined serum transferrin N-glycosylation and muscle αDG O-glycosylation abnormalities. Dashed arrow: alternative pathway suggested by Cantagrel et al\(^3\)
2 (PMM2)-CDG (MIM 601785) patients show a very broad phenotype with involvement of nearly all organs. Steroid 5alpha-reductase type 3 (SRD5A3)-CDG (MIM 611715) patients mainly present with eye, skin, and central nervous system involvement,3–6 while dolichol kinase (DOLK) deficiency (MIM 610746) results in dilated cardiomyopathy without obvious skeletal muscle abnormalities.13,14,16 DPM3-CDG (MIM 605951) and patients with mutations in guanosine-diphosphate-mannose (GDP-mannose) pyrophosphorylase B (GMPPB, MIM 615320) can present with muscular dystrophy, a symptom of the dystroglycanopathies.7,8,25 These congenital muscular dystrophies present with deficient O-mannosylation of α-dystroglycan (αDG), which also requires DPM. Indeed, reduced O-mannosylation of αDG has been found in skeletal muscle of DPM3-CDG and GMPPB-CDG patients, and in heart muscle of DOLK-CDG patients.14,16,23,24 However, it is unclear whether O-mannosylation is specifically affected in DPM synthesis disorders, or if N-glycosylation and O-mannosylation are both similarly affected, but involve tissue-specific metabolic pathways. To increase our understanding of the pathophysiology of the tissue-specific phenotypes of the DPM synthesis disorders, we studied protein N- and O-glycosylation in muscle tissue of all three known DPM3-CDG patients.23,24

2.1 | Subjects
Plasma and fibroblasts were obtained for diagnostics of CDG in the Radboudumc Expertise Center for Disorders of Glycosylation. Muscle biopsies were obtained at the University of Athens. All procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in this study or from their legal representatives.

2.2 | CDG diagnostics
Isoelectric focusing of serum transferrin and electrospray ionization mass spectrometry (ESI-MS) of immunopurified serum transferrin were performed as described.26,27

2.3 | Immunohistochemistry on muscle biopsies
Immunohistochemistry on muscle biopsies was performed according to standard procedures. The VIA41 antibody (Santa Cruz Biotechnology) was used to assess the presence of functional O-mannosyl glycans on αDG.

2.4 | DPM synthase assay
Skin fibroblasts were cultured in M199 medium (Lonza) supplemented with 10% Fetal Calf Serum (Gibco) and 1% Penicillin Streptomycin (Gibco) at 37°C and 5% CO2. Pellets were collected and sonicated in 7 mM Tris-HCl (pH 7.2), and 7 mM MgCl2; 0.1% Nonidet P-40 was added. Protein concentrations were determined using BCA assay (Pierce). The DPM synthase activity assay was performed as described previously.22

2.5 | SDS-PAGE and immunoblotting
Skeletal muscle homogenates were used for SDS-PAGE on 10% polyacrylamide gels and proteins were transferred to nitrocellulose membranes. Immunoblotting was performed using primary antibodies against glycosylated αDG (IIH6C4, 1:2500, Merck 05-593), βDG (1:250, Novacastra), Desmin (Y66, 1:20000, Abcam ab32362), GAPDH (1:10000, Abcam ab8245), and with secondary antibodies HRP-conjugated polyclonal goat anti-rabbit or HRP-conjugated polyclonal goat anti-mouse (1:5000, DAKO).

2.6 | Enzymatic deglycosylation with PNGase F
Muscle homogenates were incubated with PNGase F (New England Biolabs) or MilliQ water (negative control) for 4 hours at 37°C to the manufacturer's protocol. PNGase F was inactivated by heating the samples for 15 minutes at 95°C and glycosylation of βDG was assessed with immunoblotting.

3 | RESULTS

3.1 | Clinical description of a newly identified DPM3-CDG patient
Patient 1 is a Greek female born following an uneventful pregnancy to healthy, consanguineous parents. At 24 months, she was able to walk independently. Her medical history is unremarkable except for subclinical hypothyroidism (serum TSH: 7.95 μIU/mL; normal range 0.5-5 μIU/mL) and elevated transaminases (serum SGOT: 146 U/L, normal range 8-42 U/L; SGPT: 150 U/L, normal range 8-41 U/L) at the age of 9 years, found during a laboratory investigation for maternal hypothyroidism. One week later, TSH was 8.6 μIU/mL and FT4 was 16.7 pg/mL (normal range 8-20 pg/mL), and she received replacement therapy with thyroxine (T4). Transaminases remained elevated (SGOT: 76 U/L, SGPT: 80 U/L) and markedly increased creatine kinase levels (2680 U/L, normal 140 U/L) were found without obvious muscle symptoms. There was no family history of a neuromuscular disorder.
On admission at the age of 9.5 years, physical examination revealed a mild bilateral gastrocnemius pseudohypertrophy without any dysmorphic features or hepatosplenomegaly. The values of serum TSH, FT4, anti-thyroglobulin, anti-thyroperoxidase (under treatment with T4), vitamin D, PTH, plasma and urine amino acids, and urinary organic acids were within normal ranges. Gowers test was negative and neurological examinations, Doppler echocardiography, ECG, brain MRI, ophthalmoscopy, and X rays of chest and hips did not show any abnormalities. CK levels were further monitored and ranged from 1800 to 2600 U/L with a concomitant raise of aminotransferases (SGOT 68 U/L, SGPT 65 U/L), lactate dehydrogenase (324 U/L, normal range 120-300 U/L), and lactic acid (30 mg/dL normal range 5.7-22 mg/dL). A quadriceps muscle biopsy showed a myopathic pattern with a considerable number of regenerating fibers and a significantly increased number of internal nuclei, and a loss of functional O-mannosyl glycans on αDG (Figure 2A). One year after the initial diagnosis the patient developed dilated cardiomyopathy with decreased contractility of the left ventricle. Her impaired systolic function (ejection fraction 57%; normal >60%) was treated with captopril and furosemide. Due to episodes of hypotension, the treatment of furosemide was discontinued. The patient

**FIGURE 2**  Molecular and genetic characterization of DPM3-CDG patients. A, Immunohistochemistry of skeletal muscle biopsies. Hematoxylin and eosin (HE) staining of control (×10) and patient (×20). Patient skeletal muscle HE staining shows atrophic muscle fibers, increased central nuclei, and a regenerating muscle fiber. VIA41 staining showing loss of functional O-mannosyl glycans of αDG in patient skeletal muscle (×10). B, Schematic outline of the 92 amino acid DPM3 protein with two transmembrane domains and the C-terminal coiled-coil domain.28 The mutations in DPM3 of the patients are indicated.23,24 C, Nucleotide and amino acid conservation analysis of Leu85 (red box). D, Dolichol-phosphate-mannose (DPM) synthase activity in fibroblast lysates of DPM3-CDG patients. Relative production of dolichol-phosphate-[14C]-mannose is expressed as a percentage of control. Error bars present SD of duplicates.
now only receives captopril and her cardiac function has remained stable.

An alpha-glucosidase activity assay excluded Pompe disease, and further molecular studies excluded Limb Girdle Muscular Dystrophy 2A (*CAPN3* gene) and dystrophinopathy (*DMD* gene). Further metabolic testing included serum transferrin isoelectric focusing (TIEF), which showed a type 1 pattern. In view of the clinical symptoms, *DPM3* was sequenced, and a homozygous missense mutation Chr1GRCh38:g.155139987A>G; NM_153741.1(DPM3): c.254T>C; p.Leu85Ser was found. The same mutation has been reported in a patient originating from the same island. The mutation affects the C-terminal coiled-coil domain of DPM3 (Figure 2B), which is essential for DPM synthase activity via interaction with the catalytic subunit DPM1. This domain of DPM3 is strongly conserved from *Homo sapiens* to *Drosophila melanogaster* (Figure 2C). A summary of the clinical and genetic data of patient 1 and the two previously described patients is presented in Table 1.

To confirm that the *DPM3* mutation in patient 1 is pathogenic, DPM synthase activity was assessed in patient fibroblasts and compared with control and previously published DPM3-CDG patients. We incubated fibroblast lysates of all three patients with isotopically labeled GDP-[14C]-mannose and measured the incorporation in dolichol-P-[14C]mannose. DMP synthase activity in all patient cells was reduced by more than 70% (Figure 2D). Reduction was more severe in patients 1 (12.7%) and 2 (2.8%) that harbor the Leu85Ser mutation in the coiled-coil domain of DPM3.

### 3.2 Serum transferrin is hypoglycosylated in two of the three DPM3-CDG patients

Serum TIEF analysis of the three DPM3-CDG patients showed a type 1 pattern in patients 1 and 2 (Figure 3A). Subsequent analysis of immunopurified serum transferrin with ESI-MS confirmed a significant increase of disialotransferrin in patients 1 and 2 (respectively 30% and 16%, normal 4%) (Figure 3B). The serum transferrin glycosylation profile of patient P3 (disialotransferrin 3%) was normal. Taken together, serum transferrin is not hypoglycosylated in all three DPM3-CDG patients.

### 3.3 Alpha-dystroglycan and β-dystroglycan are hypoglycosylated in DPM3 skeletal muscle

To investigate both O-glycosylation and N-glycosylation in skeletal muscle, we performed immunoblotting of glycosylated αDG and βDG from skeletal muscle homogenates of all three DPM3-CDG patients. IIH6 labeling was strongly

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<th>TABLE 1 Clinical data summary of DPM3-CDG patients</th>
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<td><strong>Patient 1</strong></td>
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<td>Gender</td>
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<td>Serum creatine kinase (U/L, normal &lt;140 U/L)</td>
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<td>Dilated cardiomyopathy</td>
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<td>Muscle biopsy findings</td>
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<td>Serum transferrin IEF</td>
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*aIn Van den Bergh et al24 the mutation is reported as c.131T>C p.Leu44Pro, this is the position on transcript variant 1 (NM018973.3).*
reduced, in agreement with dystroglycanopathy (Figure 3C).

In addition, we observed that the mobility of skeletal muscle βDG was altered in all three patients compared to controls. We hypothesized that patient βDG is not properly N-glycosylated due to limited availability of DPM, thereby affecting the mobility of the protein. To investigate this, we incubated muscle lysates with PNGase F, an enzyme that cleaves the linkages between N-acetylglucosamine moieties of N-glycans and asparagine residues. After PNGase F treatment, the mobility of skeletal muscle βDG in DPM3-CDG was due to a

**FIGURE 3** N-glycosylation and O-glycosylation analysis in serum and skeletal muscle of DPM3-CDG patients. A, Serum transferrin isolectric focusing (TIEF) of DPM3-CDG patients. Numbers correspond to the number of sialic acids on transferrin. Patients 1 and 2 show a type 1 pattern and patient 3 a normal pattern. B, Transferrin ESI-MS profiles with disialotransferrin expressed as a percentage of tetrasialotransferrin. Patients 1 and 2 show a type 1 profile with increased disialotransferrin levels. Patient 3 transferrin profile is normal. C, control; P, patient. C, Glycosylated αDG (IIH6) and βDG immunolabeling of muscle homogenates of controls (H = hamstring, Q = quadriceps) and patients (P). Desmin was used as a muscle-specific loading control. D, Immunolabeling on muscle homogenates of control quadriceps (Q) and patients (P) treated with PNGase F. Desmin and GAPDH serve as loading controls. E, The human dystroglycan preprotein sequence (UniProt Q14118) is analyzed for Asn-Xaa-Ser/Thr sequons (blue) and potential N-glycosylation sites on asparagines (red) using the NetNGlyc tool.29 Sequence in bold is beta-dystroglycan, the autolytic cleavage site where the preprotein is cleaved into αDG and βDG is underlined.30,31 Lower panel shows a graphic presentation of the N-glycosylation site prediction. The black dashed line shows the position of the autolytic cleavage site. SP, signal peptide; TM, predicted transmembrane domain.32
N-glycosylation defect (Figure 3D). However, PNGase F treatment lowered the molecular weight of βDG in skeletal muscle of patients with mutations in DPM3 even further, demonstrating that βDG is partly N-glycosylated in DPM3-CDG. Previously, a N-glycosylation defect of βDG was consistently found in skeletal muscle from patients carrying mutations in GMPPB.7,33 The findings presented above suggest the presence of more than one N-glycan on processed βDG. Using the dystroglycan preprotein sequence, we used the NetNGlyc tool to predict potential N-glycosylation sites in Asn-Xaa-Ser/Thr sequons.29 Asparagines at positions 661 and 833 on βDG were predicted to be glycosylated (Figure 3E), in agreement with our findings.

4 | DISCUSSION

Here, we show that patients with DPM3 deficiency have deficient O-mannosylation of α-dystroglycan and reduced N-glycosylation of β-dystroglycan in skeletal muscle. These findings contribute to our understanding of the tissue-restricted phenotype of DPM3-CDG and facilitate subtyping of the dystroglycanopathies.

DPM is required for both N- and O-glycosylation. The DPM biosynthesis pathway consists of seven genes and mutations in any of these genes have been associated with a genetic glycosylation disorder (Figure 1, Table 2). However, the phenotypic presentations are remarkably different. First, SRD5A3-CDG patients have specific structural and functional eye defects.3–6 Cantagrel et al3 suggested the existence of an alternative pathway for dolichol synthesis, because residual dolichol was found in SRD5A3-deficient cells with early truncating mutations. Thus, tissue-specific dolichol synthesis (or polyprenol accumulations) could explain the eye phenotype of SRD5A3-CDG patients. DOLK-CDG results in a CDG-I profile and abnormal O-mannosylation of αDG in skeletal muscle and have muscular dystrophy, whereas DPM3-CDG patients show aberrant O-mannosylation of αDG in skeletal muscle and have muscular dystrophy, there are no indications that αDG in skeletal muscle is abnormal in DOLK-CDG. This suggests that there are alternative routes in skeletal muscle for dolichol-phosphate synthesis.

Recently, more progress has been made to understand the molecular pathogenesis of these disorders. For PMM2-CDG, it has been proposed that LLO degradation due to the accumulation of mannose 6-phosphate causes the N-glycosylation defect, rather than a limited synthesis of DPM,34,35 potentially explaining the different phenotypes between PMM2-CDG and GMPPB deficiency. Of the DPM syntheses, GMPPB deficiency, DPM1-CDG, DPM2-CDG, and DPM3-CDG are all associated with muscular dystrophy caused by aberrant O-mannosylation of skeletal muscle αDG.8,21–23 One of the three DPM3-CDG patients had normal serum transferrin glycosylation and a relatively mild phenotype without cardiomyopathy, with the first myopathic changes detected at 30 years of age.24 The p.Leu14Pro mutation of this patient is located in the first transmembrane domain on the N-terminal side, which has been considered not to be essential for DPM synthase activity and is not responsible for the interaction with the catalytic subunit DPM1.28 In line with the late-onset of the disease, the mild clinical phenotype and the location of the mutation, we found there was high residual activity of DPM synthase (30%), showing that relatively mild mutations in DPM3 can eventually lead to disease.

As in DPM3-CDG patient P3, patients with GMPPB-related dystroglycanopathy have normal N-glycosylation of transferrin, and share the muscular dystrophy phenotype due to abnormal O-mannosylation of skeletal muscle αDG. This suggests that αDG glycosylation is more sensitive to mutations in DPM synthesis, and only more severe mutations affect the N-glycosylation of serum transferrin, or lead to structural brain abnormalities as reported in DPM1-CDG and DPM2-CDG patients.17,19–22 As the availability of DPM is very important for the O-mannosylation of αDG, it is highly likely that more severe mutations in DPM3 will also cause brain abnormalities as have been reported in DPM1-CDG, DPM2-CDG, and other dystroglycanopathies.

Recently, defective N-glycosylation of skeletal muscle βDG was reported as a specific marker for GMPPB deficiency, but the N-glycosylation of βDG in other DPM synthesis defects has remained unassessed.7,33 Here, we found the same shift of βDG in all three DPM3-CDG patients. Sarkozy et al33 did not observe this shift in dystroglycanopathy.
patients with mutations in POMT1, POMT2, POMGNT1, B3GALNT2, and FKTN, which encode glycosyltransferases. These glycosyltransferases synthesize the O-mannosyl glycans on αDG, and are thus not expected to affect N-glycosylation. Taken together, we suggest that hypoglycosylated βDG in combination with hypoglycosylated αDG is indicative for a defect in the DPM biosynthesis pathway. We expect that the mobility of skeletal muscle βDG is also affected in patients carrying mutations in other DPM biosynthesis genes. Experiments in Chinese Hamster Ovary (CHO) cells treated with the N-glycosylation inhibitor tunicamycin suggested that N-glycosylation is required for a correct localization of αDG and βDG and further investigations are required to explore the clinical implications of the N-glycosylation defect of βDG.

In our study, we found an additional mobility shift of βDG after we treated DPM3-CDG muscle samples with PNGase F, suggesting more than one N-glycosylation site on βDG. Ibraghimov-Beskrovnaya et al predicted that βDG has three potential N-glycosylation sites. However, two of these sites reside before residues 653/654 where the preprotein is autolytically cleaved into αDG and βDG. Using the NetNGlyc tool, we found positions 661 and 833 as potential N-glycosylation sites. However, position 833 resides in the C-terminus of βDG predicted to be oriented toward the cytosol, and is therefore not expected to be N-glycosylated. Further glycoproteomic studies can shed light on why only a single N-glycan is lost when DPM pools are limited, thus providing new insights into the N-glycosylation machinery.

In conclusion, DPM3-CDG and GMPPB deficiency are characterized by both N-glycosylation and O-glycosylation defects of skeletal muscle dystroglycan, whereas serum transferrin N-glycosylation is not necessarily affected. Deficient O-mannosylation of αDG mostly determines the patients’ phenotype, but N-glycosylation defects of other skeletal muscle glycoproteins could contribute to the clinical outcome. Future studies are required to complete our understanding of the pathophysiology of DPM synthesis disorders.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

W.v.T. has set-up the study, performed laboratory experiments, acquired and interpreted data, and has written the manuscript. H.M. and E.G. have collected, analyzed, and reviewed clinical data and revised the manuscript. M.M. has performed TIEF and revised the manuscript. G.K.P. and C.P. have performed the histopathological investigations and critical reviewed muscle biopsy data. P.v.d.B., M.A., and M.A.W. have critically reviewed results and revised the manuscript. K.H. has performed laboratory investigations and revised the manuscript. D.J.L. contributed to the set-up of the study, has interpreted data, critically reviewed the results and the manuscript, and supervised the study.

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