

Reduced CETP glycosylation and activity in patients with homozygous B4GALT1 mutations

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Abstract

The importance of protein glycosylation in regulating lipid metabolism is becoming increasingly apparent. We set out to further investigate this by studying the effects of defective glycosylation on plasma lipids in patients with B4GALT1-CDG, caused by a mutation in *B4GALT1* with defective N-linked glycosylation. We studied plasma lipids, cholesteryl ester transfer protein (CETP) glyco-isoforms with isoelectric focusing followed by a western blot and CETP activity in three known B4GALT1-CDG patients and compared them with 11 age- and gender-matched, healthy controls. B4GALT1-CDG patients have significantly lowered non-high density lipoprotein cholesterol (HDL-c) and total cholesterol to HDL-c ratio compared with controls and larger HDL particles. Plasma CETP was hypoglycosylated and less active in B4GALT1-CDG patients compared to matched controls. Our study provides insight into the role of protein glycosylation in human lipoprotein homeostasis. The hypogalactosylated, hypo-active CETP found in patients with B4GALT1-CDG indicates a role of protein galactosylation in regulating plasma HDL and LDL. Patients with B4GALT1-CDG have large HDL particles probably due to hypogalactosylated, hypo-active CETP.

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KEYWORDS

B4GALT1, CDG, CETP, glycosylation, HDL, LDL, lipids

1 | INTRODUCTION

Identification of novel patients and novel congenital disorders of glycosylation (CDG) is expanding rapidly.¹ These inborn defects of glycan metabolism have a wide variety of clinical features and severity with generally neurological involvement^{2,3}

Glycosylation is a crucial intracellular post-translational process that covalently attaches a glycan to proteins or lipids. The importance of protein glycosylation in regulating human lipoprotein homeostasis is increasingly being recognised.^{4,5} We found that ppGalNAc-transferase 2, a specific O-glycosylation enzyme, could specifically initiate glycan synthesis on apolipoprotein C-III (apoC-III)⁶ and others found that it also glycosylates phospholipid transfer protein⁷ and angiotensin like protein 3,^{7,8} supporting an intricate role for this transferase in glycosylation of proteins and enzymes involved in lipid remodelling. Recently, we found hypobetalipoproteinemia in patients with type I congenital disorder of glycosylation (ALG6- and PMM2-CDG) due to increased LDL-receptor.⁴ To increase insight into the role of glycosylation in lipid metabolism, we set out to study lipid pathways in patients with other types of CDG.

CDG-II is caused by mutations in genes coding for N-glycosylation enzymes located in the Golgi apparatus. Here, the high-mannose glycan structures produced in the ER are further modified and deficiencies in these enzymes result in unfinished, immature glycan structures.⁹ One of these enzymes is UDP-Gal:N-acetylglucosamine β -1,4-galactosyltransferase I (B4GALT1). B4GALT1 is responsible for the galactosylation of N-linked glycans. There are three reported patients with B4GALT1-CDG¹⁰⁻¹² and a new patient reported here. All patients have an identical, homozygous insertion mutation (1031-1032insC) causing a premature translation stop with loss of the C-terminal 50 amino acids of B4GALT1. The patients have a mild clinical presentation compared to other CDG with dysmorphism, transient hypotonia and decreased blood coagulation factors and increased serum transaminases (the highest aspartate aminotransferase [AST] measures between 200 and 300 IU/L; upper limit of normal is 40 IU/L).^{10,13}

Here, we report specific plasma lipid abnormalities in B4GALT1-CDG patients. Notably, these were clearly

distinct from the hypobetalipoproteinemia found in CDG-I patients. Plasma cholesterol was sequestered mostly in the HDL fraction, and glycosylation and activity of cholesteryl ester transfer protein (CETP) were reduced. CETP is a highly glycosylated protein that transfers cholesteryl esters from high density lipoprotein (HDL) particles to other lipoproteins. CETP deficiency leads to increased levels of HDL-c and decreased levels of LDL-c. The lipid phenotype found in the B4GALT1-CDG patients are similar to that found in patients with CETP deficiency, which prompted us to explore of CETP glycosylation and function in these patients.

2 | METHODS

2.1 | Patients

Plasma of patient B1 was obtained from the blood plasma bank at Radboud University Medical Center in Nijmegen, the Netherlands. From B2 and B3, venous blood plasma samples were collected after overnight fast from patients and their parents at the Medical University of Vienna, Austria. Plasma samples of 11 age- and gender-matched, healthy, unaffected children from our own plasma databank were used as controls (unaffected siblings of children with familial hypercholesterolemia, proven to carry no mutations in known lipid genes). Plasma from a patient with CETP deficiency due to a mutation in *CETP*—also from our plasma biobank—was used as a positive control. The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and informed consent was obtained from the parents. Due to the nature of the study, no ethical approval was requested from the local ethics committee.

2.2 | Plasma lipid measurements

We analysed plasma lipids in venous blood samples collected after an overnight fast in EDTA coated tubes. Plasma was isolated after centrifugation at 3000 rpm for 15 minutes at 4°C and stored at -80°C until further analyses. Total cholesterol (TC), LDL-c, HDL-c, TG, apolipoprotein A-I (apoA-I) and apoB were measured using commercially available assays (DiaSys) on a Selectra analyser (Sopachem, the Netherlands). TC-to-HDL-c ratios

were calculated from these results. Fast protein liquid chromatography (FPLC) profiling for cholesterol distribution across lipoprotein fractions was carried out as described.¹⁴

2.3 | Deglycosylation treatment

Venous blood plasma samples (30 μ L) were treated with 15 μ L neuraminidase (Roche, 5 U dissolved in 500 μ L 0.1 M Tris/HCl, pH 7.0) for 3-4 hours at 37°C to remove the terminal sialic acids from the glycans.

2.4 | Isoelectric focusing of transferrin and CETP glyco-isoforms

Isoelectric focusing (IEF) of transferrin was performed and quantified as described.¹⁵ IEF followed by a western blot of plasma CETP was performed similar to the transferrin IEF protocol. In short, plasma samples were diluted 1:1 in a physiological NaCl solution and run in the same system as for transferrin IEF with slight adjustments to the running protocol. Blotting occurred at 60°C in the same system using nitrocellulose membranes. The membrane was subsequently blocked in a 5% enhanced chemiluminescence (ECL) solution. After washing, the membrane was incubated for at least 3 hours at room temperature (or at 4°C when overnight) with the first antibody TP1 in phosphate buffered saline supplemented with Tween 20 (PBST) with 1.5% bovine serum albumin (BSA). After a second wash, the secondary antibody (goat anti-mouse) in PBST with 1.5% BSA was added for at least 1.5 hours at room temperature. Imaging was performed after ECL reaction. Quantification of the different isoforms was performed as described for transferrin IEF.¹⁵

2.5 | Endogenous plasma CETP Activity

Determination of endogenous cholesteryl ester (CE) transfer from HDL to apoB-containing lipoproteins was assayed using the method of Guérin et al.^{16,17} that estimates net physiological CE transfer between lipoprotein donor and acceptor particles in plasma. Radiolabelled HDL particles were obtained from the $d > 1.063$ g/mL plasma fraction by ultracentrifugation at 100000 rpm for 3.5 hours at 15°C with a Beckman TL100 centrifuge. Then, the $d > 1.063$ g/mL fraction was labelled with [³H]-cholesterol (4 μ Ci/mL) at 37°C overnight. The radiolabelled [³H]-HDL were then isolated from the $d > 1.063$ g/mL plasma fraction by

centrifugation at 100000 rpm for 5.5 hours at 15°C after adjustment of the density at 1.21 g/mL by addition of dry solid KBr. CETP-mediated cholesteryl ester transfer was determined after incubation of whole plasma from individual subjects at 37°C and 0°C for 3 h in the presence of radiolabelled ³H-HDL (25 μ g HDL-CE) and iodoacetate (final concentration 1.5 mmol/L) for inhibition of lecithine-cholesterol-acyltransferase (LCAT). After incubation, apolipoprotein B-containing lipoproteins were precipitated using the dextran sulphate-magnesium procedure. The radioactive content of the supernatant was quantified by liquid scintillation spectrometry with a Trilux 1450 (Perkin Elmer). Endogenous plasma CETP activity (expressed as percentage) was calculated as the amount of the label recovered in the supernatant after incubation and divided by the label present in the supernatant before incubation. The CETP-dependent CE transfer was calculated from the difference between the radioactivity transferred at 37°C and 0°C.

2.6 | Statistical analysis

Data were compared between groups with a two-tailed Students *t* test and presented as means \pm SD or, for non-parametric parameters, tested with a Mann-Whitney *U* test and presented as medians with interquartile ranges. Categorical variables were tested with a Chi-square test. All statistical analyses were done using SPSS software (version 22.0, SPSS Inc., Chicago, Illinois). Error bars indicate standard deviations. Probability values $<.05$ were considered statistically significant.

3 | RESULTS

3.1 | Patients

Patient B1 and B2 with a homozygous insertion in exon 5 (c.1031-1032insC) of *B4GALT1* were previously identified and described (¹⁰). Patient B3 is a sibling of patient B2 and has the same homozygous mutation. She was born to consanguineous parents and the pregnancy proceeded normally. In the 13th week ultrasound examination showed an increased neck fold measurement (5.6 mm, reference 1.6-2.4 mm). Chorion biopsy was performed. Intrauterine MRI showed a hypoplastic cerebellum, a small liver sinus, splenomegaly, hydrocolpus, and subcutaneous edema. The delivery was uncomplicated and the Apgar score was 8/9/10. At 2 months of age, pulmonary artery banding was performed successfully for a large ventricle septal defect with left-to-right shunt, pulmonary hypertension and heart failure. Because of the

known coagulopathy, coagulation factors were supplied. Later she needed a drain for pericardial effusion. Over the course of childhood, she had some developmental disability, especially in language development; this is in contrast to her older sister, who has a normal IQ. She has dysmorphic features with low-set ears, saddle nose, thin lips, and fat pads. Serum transaminases are mildly elevated.

3.2 | Lipids

Table 1 shows clinical characteristics and serum lipids of the patients and 11 age- and gender-matched controls. Patients have significantly lowered plasma TC (115 ± 13 vs 173 ± 27 mg/dL, $P = .004$), LDL-c (47 ± 19 vs 88 ± 14 mg/dL, $P = .001$), apoB (35 ± 9 vs 69 ± 12 mg/dL, $P < .001$) and TC-to-HDL-c ratio (2.27 ± 0.11 vs 3.12 ± 0.56 , $P = .025$). HDL-c, apoA1 and TG were comparable between patients and controls. FPLC cholesterol profiles of two B4GALT1-CDG patients and of the controls confirm the lower cholesterol content in the LDL fraction in patients and also the distribution of most cholesterol into a larger, buoyant HDL fraction, attested by a shifted HDL peak size fraction. Of note, these profiles were found to be similar to the FPLC profile of a CETP deficient patient (Figure 1A).

3.3 | Patients have hypoglycosylated CETP

CETP was analysed on IEF followed by western blot to detect possible charge changes in CETP glyco-isoforms.

CETP has four predicted glycosylation sites, suggesting eight possible bands on IEF. Figure 1B shows a representative normal pattern of isoforms in healthy controls (lane 1 and 5) and a profound loss of negative charges in patient B2 and B3 (lane 3 and 4), with bands ranging from asialo- to pentasialo-CETP, similar to the glyco-isoform pattern seen in controls treated with neuraminidase (lane 2).

3.4 | CETP activity

To study whether the hypoglycosylated plasma CETP is also less active and thus could explain the observed lipoprotein abnormalities, endogenous CETP activity was measured. Indeed, B4GALT1-CDG patients had a 26% reduction in CETP activity compared with controls ($20 \pm 3\%$ vs $27 \pm 5\%$, $P = .019$, Figure 1C).

4 | DISCUSSION

In the present study, we demonstrate that patients with B4GALT1-CDG have increased cholesterol content in larger HDL particles, with lower cholesterol residing in the LDL fraction. IEF of CETP showed a marked loss of the more negatively charged CETP glyco-isoforms in the B4GALT1-CDG patients compared to healthy controls. There was not a complete loss of negatively charged CETP, as one would see after a long enough incubation with neuraminidase, similar to the transferrin IEF profile of B4GALT1-CDG patients. The hypoglycosylation of CETP was accompanied by a significant reduction of CETP lipid transfer activity compared to controls. The

TABLE 1 Characteristics and plasma lipids of the B4GALT1-CDG patients and controls

	Controls (n = 11)	B1	B2	B3	B4GALT1-CDG patients pooled (n = 3)	P
Age (year)	8 ± 1	4	14	3	7 ± 6	.664
Gender, n male (%)	7 (64%)	M	F	M	2 (67%)	
TC (mg/dL)	173 ± 27	128	113	103	115 ± 13	.004
LDL-c (mg/dL)	88 ± 14	67	41	32	47 ± 19	.001
HDL-c (mg/dL)	57 ± 13	58	51	43	51 ± 8	.459
TG (mg/dL)	74 ± 26	124	54	139	106 ± 45	.136
ApoA1 (mg/dL)	171 ± 57	149	162	130	147 ± 16	.493
ApoB (mg/dL)	69 ± 12	45	28	31	35 ± 9	< .001
LDL-c/HDL-c ratio	1.63 ± 0.38	1.15	0.8	0.74	0.90 ± 0.04	.005
TC/HDL-c ratio	3.12 ± 0.56	2.20	2.20	2.40	2.27 ± 0.11	.025

Note: Characteristics and plasma lipids for the B4GALT1-CDG patients and their age- and gender-matched controls. B1, B2, and B3 are B4GALT1-CDG patients.

Abbreviations: TC, total cholesterol, TG, triglycerides.

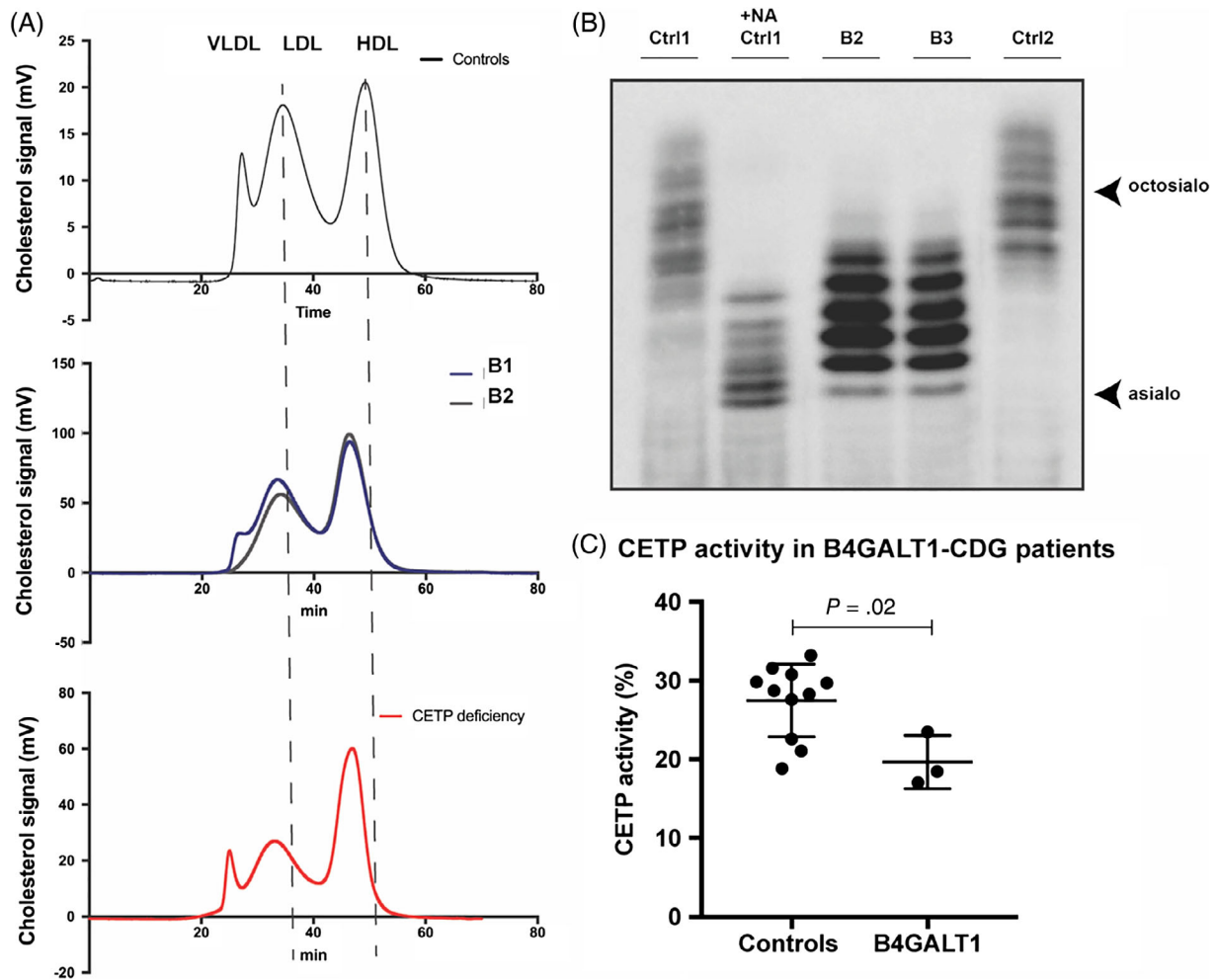


FIGURE 1 Hypoglycosylated hypo-active CETP in B4GALT1-CDG patients. A, Pooled FPLC trace of child controls (*top panel*), representative traces of patients B1 and B2 (*middle panel*) and a CETP deficient patient (*bottom panel*). The vertical line illustrates the clear HDL peak shift to the left in B4GALT1 and CETP patients, indicating the HDL size increment. B, Representative blot of CETP isoelectric focusing of a control (Ctrl1), a control treated with neuraminidase (Ctrl + NA), patient B2, patient B3, and a different control (Ctrl2). C, CETP activity in B4GALT1 patients vs age- and gender-matched controls. CETP, cholesteryl ester transfer protein; FPLC, fast protein liquid chromatography

latter finding implies that glycosylation of CETP is essential for its activity, that is, shuttling cholesteryl esters from the HDL fraction to the LDL fraction in exchange for triglycerides.

This notion may bear relevance to the clinical application of CETP inhibitors. These experimental drugs effectively lowered LDL and raised HDL in multiple phase 3 clinical trials. In most phase 3 trials, these effects failed to translate into cardiovascular benefit, leading to discontinuation of the drug development. Yet, in the recently published REVEAL study, anacetrapib added to intensive statin therapy resulted in significantly less major coronary events than statin therapy alone (10.8% vs 11.8% with a rate ratio of 0.91).¹⁸

The relative paucity of cardiovascular benefit with CETP inhibition may be related to concomitant use of

statins. ApoB, reflecting the total number of atherogenic lipid particles, has a stronger link with atherosclerotic cardiovascular disease than LDL-c.¹⁹ When used in combination with statins, CETP inhibitors reduce apoB to a smaller extent than LDL: delta apoB/delta LDLc ratio ~15%. When used without statins, CETP inhibition reduces apoB proportionately to LDL: delta apoB/delta LDLc is ~100%. The mechanism behind this discrepancy in apoB lowering and LDL lowering when CETP inhibitors are used on top of statin is unknown, but the discrepancy was supported by a recent mendelian randomisation study with genetic CETP and HMCGR variants, the latter gene being the target of statins.²⁰ Supporting a sole CETP effect in the B4GALT1 deficient patients, who did not use statins, we see a comparable proportionate reduction of LDL and apoB, ratio 92%.

Besides a reduction in CETP activity, other factors may have contributed to the observed HDL-c increase in our patients. First, B4GALT1-CDG patients have a global glycosylation defect and consequently other proteins known to regulate HDL-c levels may also contribute to the observed lipid changes. Recently, two different mutations in the genes encoding scavenger receptor class B type 1 (SR-B1), P376L²¹ and T175A,²² have been described. Both cause impaired N-glycosylation of SR-B1 with subsequent reduced molecular weight and SR-B1 protein expression, causing high plasma HDL-c and reduced selective uptake of cholesteryl esters from HDL. Indeed, FPLC profiles of these mutation carriers show large buoyant HDL particles as well. These studies describe variants in SR-B1 leading to complete loss of glycans on the protein; it remains unstudied whether hypogalactosylation, as seen in B4GALT1-CDG patients, also affects SR-B1 function. In addition, endothelial lipase deficiency also leads to increased HDL-c concentrations with larger HDL particles.²³ However, endothelial lipase activity has been shown to increase after (complete) removal of N-glycosylation sites,²⁴ which would lead to decreased HDL-c and is therefore less likely to play a role in the lipid phenotype of the patients of our study. This discrepancy might be explained by the fact that in B4GALT1-CDG patients there is not a complete loss of glycans, but increased immature glycans due to hypogalactosylation.

Interestingly, the lipid phenotypes found in CDG patients are very specific to the particular glycosylation defect. CDG-I subtypes are hallmarked by hypobetalipoproteinemia.⁴ In contrast, patients with *TMEM199*²⁵ and *CCDC115*²⁶ deficiency—resulting in a combined N- and O-linked glycosylation defect—exhibit very high levels of plasma non-HDL in combination with fatty liver disease. Therefore, even though all these patients have a generalised glycosylation defect due to mutations in factors involved in protein glycosylation, they show different and specific lipid abnormalities. These differences again indicate the intricate influences of protein glycosylation on lipid pathways.

In conclusion, our study provides further specific insight into the role of protein glycosylation in human lipoprotein homeostasis. The hypogalactosylated, hypoactive CETP found in patients with B4GALT1-CDG indicates a distinct role of protein galactosylation in regulating plasma HDL-c and LDL-c.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

M.vdB. coordinated and performed all clinical and experimental studies and analyses, and wrote the article. V.K. and D.J.L. provided patient material and clinical information. S.D.K. and G.S. performed CETP IEF on plasma samples. M.G. performed all CETP activity assays. A.W.M. performed lipid analyses of plasma samples. J.J.L. performed FPLC analysis of plasma samples. All authors reviewed the article. G.M.D.T., D.J.L., E.S.G.S., and A.G.H. oversaw all studies and the writing of this manuscript.

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