Circulation

ORIGINAL RESEARCH ARTICLE

N-Glycosylation Defects in Humans Lower Low-Density Lipoprotein Cholesterol Through Increased Low-Density Lipoprotein Receptor Expression

BACKGROUND: The importance of protein glycosylation in regulating lipid metabolism is becoming increasingly apparent. We set out to further investigate this by studying patients with type I congenital disorders of glycosylation (CDGs) with defective N-glycosylation.

METHODS: We studied 29 patients with the 2 most prevalent types of type I CDG, ALG6 (asparagine-linked glycosylation protein 6)–deficiency CDG and PMM2 (phosphomannomutase 2)–deficiency CDG, and 23 first- and second-degree relatives with a heterozygous mutation and measured plasma cholesterol levels. Low-density lipoprotein (LDL) metabolism was studied in 3 cell models—gene silencing in HepG2 cells, patient fibroblasts, and patient hepatocyte-like cells derived from induced pluripotent stem cells—by measuring apolipoprotein B production and secretion, LDL receptor expression and membrane abundance, and LDL particle uptake. Furthermore, SREBP2 (sterol regulatory element-binding protein 2) protein expression and activation and endoplasmic reticulum stress markers were studied.

RESULTS: We report hypobetalipoproteinemia (LDL cholesterol [LDL-C] and apolipoprotein B below the fifth percentile) in a large cohort of patients with type I CDG (mean age, 9 years), together with reduced LDL-C and apolipoprotein B in clinically unaffected heterozygous relatives (mean age, 46 years), compared with 2 separate sets of age- and sex-matched control subjects. ALG6 and PMM2 deficiency led to markedly increased LDL uptake as a result of increased cell surface LDL receptor abundance. Mechanistically, this outcome was driven by increased SREBP2 protein expression accompanied by amplified target gene expression, resulting in higher LDL receptor protein levels. Endoplasmic reticulum stress was not found to be a major mediator.

CONCLUSIONS: Our study establishes N-glycosylation as an important regulator of LDL metabolism. Given that LDL-C was also reduced in a group of clinically unaffected heterozygotes, we propose that increasing LDL receptor–mediated cholesterol clearance by targeting N-glycosylation in the LDL pathway may represent a novel therapeutic strategy to reduce LDL-C and cardiovascular disease.

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Key Words: cholesterol ■ congenital disorders of glycosylation ■ glycosylation ■ hypobetalipoproteinemias ■ receptors, LDL ■ sterol regulatory element binding protein 2

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Clinical Perspective

What Is New?

- Patients with type I congenital disorder of glycosylation have hypobetalipoproteinemia through increased low-density lipoprotein (LDL) receptor expression.
- Carriers of mutations in glycosylation enzymes affected in type I congenital disorder of glycosylation have decreased LDL cholesterol levels compared with control subjects.
- Defects in glycosylation enzymes can play an important role in LDL cholesterol metabolism.

What Are the Clinical Implications?

- Heterozygous carriers of type I congenital disorder of glycosylation mutations have none of the clinical features found in patients homozygous for type I congenital disorder of glycosylation; this warrants exploration of therapeutic targeting of N-linked glycosylation in the LDL receptor pathway to reduce LDL cholesterol in patients at increased cardiovascular risk.

The importance of protein glycosylation in regulating lipid metabolism is becoming increasingly apparent.[1] This intracellular process covalently attaches glycans to proteins and is an essential modification for protein function, referred to as the third language of life after nucleic acids and proteins.[2] We and others previously reported a link between glycosylation and plasma lipids based on genome-wide association studies. Single nucleotide polymorphisms in *GALNT2*, encoding ppGalNAc-transferase 2, a specific O-glycosylation enzyme, are associated with elevated plasma high-density lipoprotein (HDL) cholesterol (HDL-C) and decreased triglycerides.[3,4] We subsequently demonstrated that GALNT2 could specifically initiate glycan synthesis on apolipoprotein (apo) CIII,[5] whereas others found that it also glycosylates ANGPTL3 (angiopoietin-like protein 3)[6] and phospholipid transfer protein.[7] Collectively, these findings implicate a role for this glycosyltransferase in modifying proteins involved in lipid metabolism.

To further investigate this, we focused on lipid metabolism in patients with congenital disorders of glycosylation (CDGs). CDGs are a rare group of inborn defects of glycan metabolism with autosomal recessive inheritance and a rapid expansion of identified subtypes in recent years.[8] CDGs are associated with a broad spectrum of clinical features and severity, typically involving neuromuscular defects and psychomotor retardation.[9] Numerous gene defects resulting in abnormal glycosylation have been documented.[8] Defects are located in diverse biological pathways, ranging from glycosylation enzymes located in the endoplasmic reticulum (ER) or the Golgi apparatus to a general disruption of Golgi trafficking and homeostasis. The nomenclature for CDGs is based on the mutated gene and can be grouped according to the cellular localization of the affected protein. In type I CDG (CDG-I), the affected enzymes are located in the ER, early in the asparagine-linked or N-glycan assembly.[10]

On the basis of the observed link between plasma lipids and glycosylation and case reports of hypocholesterolemia in patients with CDG-I,[11–14] we studied plasma lipids in a large cohort of the 2 most prevalent CDG-I subtypes: ALG6-CDG, characterized by asparagine-linked glycosylation protein 6 deficiency, and PMM2-CDG, characterized by phosphomannomutase 2 deficiency. ALG6 and PMM2 are enzymes located in the ER and cytosol, respectively, and both are required for the earliest steps of N-glycan assembly. Deficiency of these enzymes disrupts assembly of dolichol-linked glycans or the transfer of these glycans onto proteins, resulting in unoccupied glycosylation sites and consequently potentially altered folding and protein function.[9]

In this study, we report analyses of plasma lipid levels in a cohort of patients with ALG6-CDG and PMM2-CDG and their clinically unaffected, heterozygous family members. We found that these patients have a new form of primary hypobetalipoproteinemia, defined by very low plasma levels of total cholesterol (TC), low-density lipoprotein (LDL) cholesterol (LDL-C), and apo B. Experiments in a human liver cell model, patient fibroblasts, and hepatocyte-like cells (HLCs) derived from induced pluripotent stem cells (iPSCs) demonstrate an increased LDL receptor (LDLR) abundance on the cell surface mediating the observed plasma lipid phenotype.

METHODS

The online-only Data Supplement provides a detailed description of the performed experiments. The data, analytical methods, and study materials for purposes of reproducing results or replicating procedures can be made available on request to the corresponding author.

Subjects

First, we reviewed medical records of 17 patients with CDG-I (diagnosed by standard methods[15]) from the CDG database of the Radboud University Medical Center (UMC) in Nijmegen, the Netherlands, for plasma lipids to assess whether there was a lipid phenotype in these patients.

Then, we collected plasma samples of 29 patients (15 patients with ALG6-CDG and 14 with PMM2-CDG, diagnosed with transferrin isoelectric focusing and genetic testing, confirming homozygosity or compound heterozygosity for *ALG6* or *PMM2* mutations), and a set of 30 healthy age- and sex-matched (child) control subjects from the plasma biobank at

the Amsterdam UMC, location AMC, the Netherlands. These were unaffected siblings of patients with dyslipidemia, without mutations in genes known to affect apo B and LDL-C (*APOB*, *LDLR*, and *PCSK9*) or in *ALG6* or *PMM2*, from our lipid clinic with plasma stored in our blood bank.

In addition, we included 23 first- and second-degree family members of patients with CDG-I (10 carriers of PMM2-CDG mutations, 7 carriers of ALG6-CDG, 2 carriers of ALG12-CDG, and 4 carriers of mannose-6 phosphate isomerase-CDG mutations, the last 2 being 2 other subtypes of CDG-I[16,17]). In line with the recessive mode of inheritance, heterozygous carriers were clinically unaffected; none had the characteristic neurological defects found in patients with CDG-I. Heterozygosity for the mutations was assessed by DNA sequencing for the known mutations. Because the heterozygous carriers were mostly obligate heterozygous parents of the patients with CDG-I and thus significantly older (mean age of heterozygous carriers, 45.5 years; mean age of patients with CDG-I, 9 years), we have included a separate set of 23 healthy age- and sex-matched (adult) control subjects without mutations in genes known to affect apo B and LDL-C (*APOB*, *LDLR*, and *PCSK9*) or in *ALG6* or *PMM2*. These adult control subjects were unaffected family members of patients with dyslipidemia from our clinic with plasma stored in our blood bank.

Furthermore, we assessed all available exomes of subjects in the biobank of our lipid clinic and found a heterozygous carrier of a relatively common pathogenic *PMM2* mutation. She was referred to our clinic for having a plasma HDL-C above the 95th percentile, and expansion of her family was done. On further investigation, she also had low LDL-C levels, and her family was included for segregation analysis of the *PMM2* mutation and low plasma LDL-C levels.

None of the subjects included in these studies used medication that could affect lipid metabolism.

Plasma Lipids

We analyzed 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.

TC, LDL-C, HDL-C, triglycerides, apo AI, and apo B were measured with commercially available assays (DiaSys and WAKO) on a Selectra analyzer (Sopachem, the Netherlands). Proprotein convertase subtilisin/kexin type 9 (PCSK9) plasma levels were measured with a commercially available ELISA (R&D Systems).

Study Approval

Written informed consent was obtained from all participants before they enrolled in the study, according to a protocol approved by the Medical Ethical Committee of the Amsterdam UMC, location AMC (protocol NL46676.018.13), in compliance with the Declaration of Helsinki.

Cell Lines

RNA silencing was done in human hepatoma (HepG2) cells to mimic the ALG6 and PMM2 deficiency. Patient fibroblasts from 6 patients with CDG-I (3 with ALG6-CDG and 3 with PMM2-CDG) were obtained from the Radboud UMC, and 2 healthy control fibroblast cell lines were obtained from subjects in the biobank at our lipid clinic in Amsterdam. Compared with cell lines such as the HepG2 model, the use of patient fibroblasts with their inherent glycosylation defect represents an improvement in terms of clinical relevance; however, LDL metabolism occurs mainly in the liver. Therefore, we also studied patient-derived HLCs. Easily accessible differentiated cells such as fibroblasts can be reprogrammed to iPSCs and subsequently redifferentiated to HLCs.[18] This enables the study of liver metabolism in patients noninvasively. Finally, we used an HepG2 cell line stably expressing LDLR–green fluorescent protein under a cytomegalovirus promoter.[19]

In Vitro Experiments

The HepG2 cell model was used for a wide quantitative polymerase chain reaction screen of expression levels of important players in plasma lipid and lipoprotein metabolism (see Table I in the online-only Data Supplement for primer sequences), apo B secretion assays with or without oleic acid stimulation, ER degradation inhibition (with MG-132 for 4 hours), or autophagy inhibition (with bafilomycin A for 4 hours). In addition, HepG2s, fibroblasts, and HLCs were used to assess cellular and surface LDLR protein expression levels with Western blot and flow cytometry, and LDL uptake was assessed with Dylight-labeled LDL particles combined with flow cytometry.[20] In HLCs, apo B secretion was assessed by ^{35}S steady-state protein labeling and precipitation in methionine-free hepatocyte basal medium as described by Ota et al.[21] Cholesterol efflux assays were performed on the patient fibroblasts as described by Franssen et al.[22] Cellular SREBP2 (sterol regulatory element-binding protein 2) protein levels and mRNA expression of SREBP2 targets were assessed in siALG6-treated HepG2s with Western blot and quantitative polymerase chain reaction. Furthermore, SREBP2 activation was assessed with cyclodextrin cholesterol (methyl-β-cyclodextrin, which inhibits SREBP2 by delivering cholesterol that directly binds to SCAP [SREBP cleavage-activating protein]) and 25-hydroxycholesterol (an oxysterol that is shown to bind insulin-induced gene [INSIG] to SCAP[23]) for 4 hours. Intracellular cholesterol levels were measured quantitatively with filipin staining combined with flow cytometry. Lipid droplets were isolated and assessed for lipid content with high-performance liquid chromatography. ER stress was assessed by measuring mRNA and protein levels of certain indicators of ER stress such as CHOP (C/EBP homologous protein). Tunicamycin and thapsigargin, both known inducers of ER stress and the unfolded protein response, were used as positive controls. For the Myc-tagged INSIG1 overexpression experiments, the small interfering (si) RNA–treated HepG2 cells were cotransfected with INSIG1-Myc and used for RNA and protein isolation after 48 hours. All in vitro cell experiments were performed at least twice with triplicate measurements per condition.

Statistical Analyses

Data are compared between groups with a Student *t* test and presented as mean±SD or, when appropriate, tested with a Mann–Whitney *U* test and presented as medians with interquartile ranges for nonparametric parameters. Categorical variables were tested with a χ^2 test. All statistical analyses were done with SPSS software (version 22.0, SPSS Inc,

Chicago, IL). Error bars indicate SDs. Values of $P<0.05$ were considered statistically significant.

RESULTS

The medical records of 17 patients (mean age, 10 years; SD, 9.7 years) with CDG-I (diagnosed by established methods[15]) were reviewed for plasma lipids. Analysis revealed markedly decreased TC and LDL-C, with normal HDL-C and triglycerides levels (Figure I in the online-only Data Supplement). On average, TC was 31% lower and LDL-C was 61% lower in patients with CDG-I compared with reference values for children in the relevant age category, that is, 0 to 14 years of age, as measured in our lipid clinic. Available data on liver and thyroid function, nutritional status, and enteric absorption did not show signs of secondary causes of hypocholesterolemia such as malabsorption or malnutrition, hyperthyroidism, or renal or liver failure (Table II in the online-only Data Supplement).

To validate these findings, we retrieved plasma samples of 29 patients of the 2 most prevalent CDG-I subtypes (15 ALG6-CDG and 14 PMM2-CDG) from the plasma biobank at the Radboud UMC and 30 healthy age- and sex-matched control subjects. The patients with ALG6-CDG and PMM2-CDG had an average age of 9.3±10.8 and 8.6±10.7 years, respectively, and were comparable to the also prospectively collected control subjects (age, 10.2±2.6 years; $P=0.750$ and $P=0.576$).

As depicted in Figure 1A, homozygous or compound heterozygous carriers of *ALG6* or *PMM2* mutations had marked hypocholesterolemia: plasma TC and LDL-C were half that of control subjects (TC: 107±34 mg/dL in ALG6 and 93±25 mg/dL in PMM2 versus 171±22 mg/dL in control subjects; LDL-C: 51±23 and 48±22 mg/dL versus 87±10 mg/dL in control subjects; all $P<0.0001$). Apo B was 59% and 44% lower in patients with ALG6-CDG and PMM2-CDG, respectively (29±13 and 39±21 mg/dL versus 70±8 mg/dL in control subjects; both $P<0.0001$). In fact, TC, LDL-C, and apo B were below the fifth percentile for age and sex, thereby qualifying as hypobetalipoproteinemia.[24] In addition, HDL-C was lower in patients: 36% and 45% lower in patients compared with control subjects (36±10 and 29±11 mg/dL, respectively, versus 57±14 mg/dL; $P<0.0001$ for both). Plasma apo AI and triglycerides levels were comparable to those of control subjects. The discrepancy between HDL-C and apo AI may indicate reduced maturation of HDL particles resulting from reduced efflux of cholesterol from ABCA1 to apo AI (in support, see Figure II in the online-only Data Supplement) or reduced cholesterol esterification driven by lecithin-cholesterol acyltransferase, both of which could explain the reduced HDL-C.

Potential secondary causes for the hypobetalipoproteinemia phenotype in this validation cohort were not found (Table III in the online-only Data Supplement).

Heterozygous Carriers of CDG-I Mutations Have Decreased Plasma LDL-C and Apo B

To determine whether clinically unaffected heterozygous carriers of CDG-I mutations had altered plasma lipids, these were analyzed in first- and second-degree family members of patients with CDG-I. For this analysis, 23 heterozygous carriers were available: 10 carriers of PMM2-CDG mutations, 7 carriers of ALG6-CDG, 2 carriers of ALG12-CDG, and 4 carriers of MPI-CDG mutations (the last being 2 other subtypes of CDG-I[16,17]). Their mean age was 45.5 years (SD, 13.6 years). In congruence with the observation in the patients with CDG-I, plasma LDL-C (118±35 mg/dL versus 136±22 mg/dL in control subjects; $P=0.038$) and apo B (84±28 mg/dL versus 106±20 mg/dL; $P=0.007$) in heterozygous carriers (n=23) were significantly lower compared with age- and sex-matched control subjects (Figure 1B). No significant differences in HDL-C or apo AI were observed between heterozygous carriers and control subjects. When assessed per CDG-I subtype, this difference remained significant for heterozygous carriers of *PMM2* mutations (n=10) and *ALG12* mutations (n=2); a trend toward lower LDL-C was observed in carriers of *MPI* mutations (n=4; Figure 1C).

In a complementary approach, we screened for heterozygous carriers of CDG-I mutations among the subjects in a cohort of 150 subjects recruited from our outpatient clinic with available exome sequence data. This approach resulted in the identification of 1 family with 4 carriers of a previously described pathogenic *PMM2* mutation, p.Pro113Leu.[25] The heterozygotes (n=4) tended to have lower TC (182±25 mg/dL versus 227±41 mg/dL; $P=0.115$) and LDL-C (100±25 mg/dL versus 131±27 mg/dL; $P=0.137$) than their unaffected family members (n=4; Table IV in the online-only Data Supplement), and compared with a larger group of 12 age- and sex-matched unrelated control subjects, the heterozygote carriers of CDG-I mutations had significantly lower LDL-C (100±25 mg/dL versus 132±17 mg/dL; $P=0.012$; Table IV in the online-only Data Supplement).

Molecular Explanations of Hypobetalipoproteinemia in CDG-I

We subsequently set out to unravel the potential cellular mechanisms underlying the effect of CDG-I mutations on LDL metabolism.

First, we used siRNAs to silence *ALG6* and *PMM2* expression in human hepatoma cells (HepG2) using nontargeting siRNAs as control. We achieved effective silencing of *ALG6* and *PMM2* (>95% on average for *ALG6* and 67% for *PMM2*; Figure 2A), which was accompanied by a corresponding decrease in ALG6 (Figure 2B) and PMM2 (Figure 2C) protein levels.

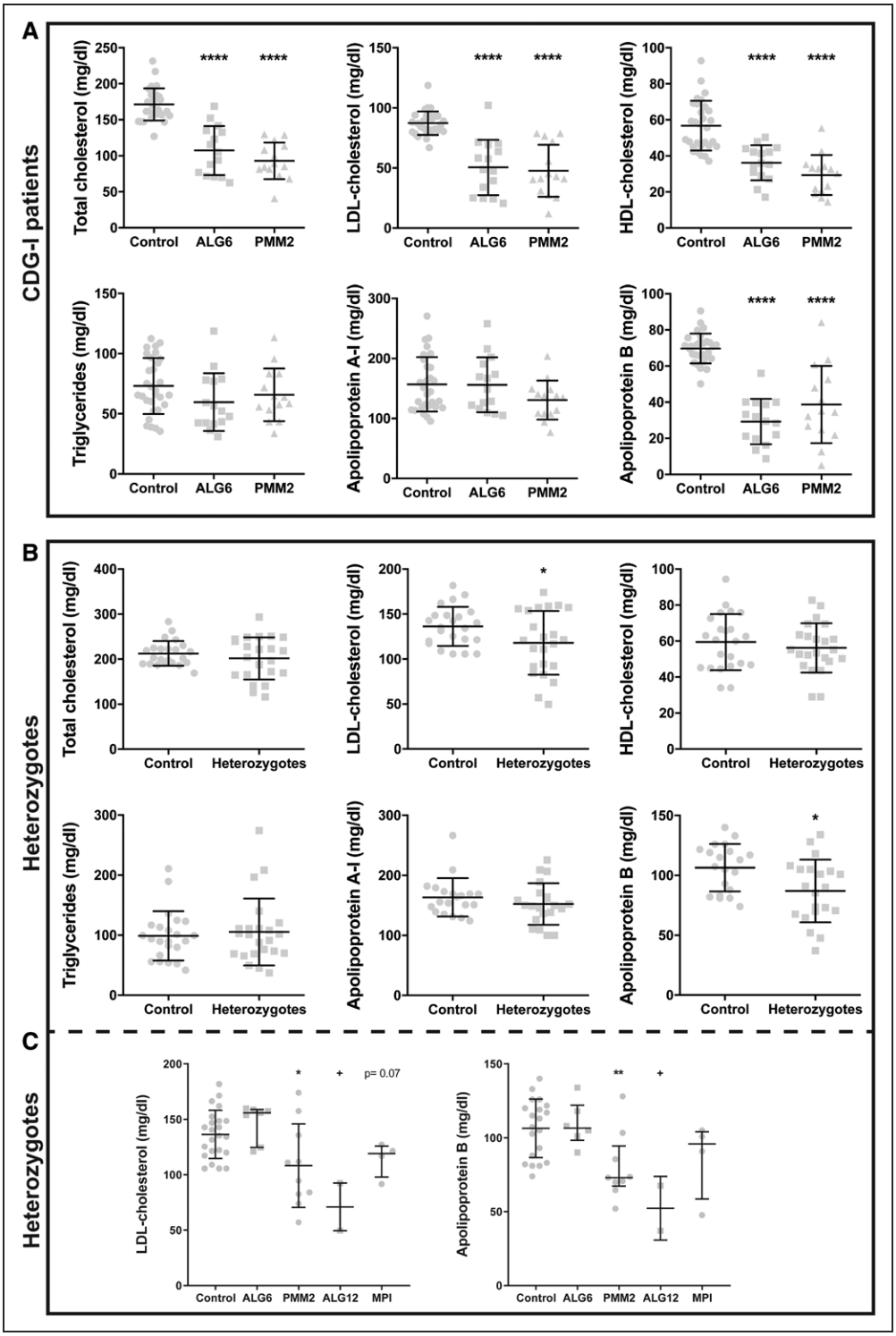

Figure 1. Patients with type 1 congenital disorder of glycosylation (CDG-I) and mutation carriers exhibit a hypobetalipoproteinemia phenotype. **A**, Plasma samples from patients with CDG-I (15 with asparagine-linked glycosylation protein [ALG] 6–CDG and 14 with phosphomannomutase 2 [PMM2]–CDG) were collected from the plasma biobank. Plasma total cholesterol (TC), low-density lipoprotein (LDL) cholesterol (LDL-C), high-density lipoprotein (HDL) cholesterol (HDL-C), apolipoprotein (apo) AI, apo B, and triglycerides were measured in EDTA plasma after an overnight fast and compared with those of 30 age- and sex-matched control subjects. **B**, Twenty-three first- or second-degree relatives of patients with CDG-I proven to be heterozygous for known CDG-I mutations were included. Plasma TC, LDL-C, HDL-C, apo AI, apo B, and triglycerides were analyzed after an overnight fast and compared with those from 23 age- and sex-matched control subjects. **C**, Plasma LDL-C and apo B of the 23 heterozygotes per CDG-I gene (here the statistical comparisons for the ALG6, ALG12, and mannose-6 phosphate isomerase heterozygotes are done with Mann–Whitney *U* tests because of the low number of subjects per group, and for those, median and interquartile range are shown). All measurements are shown as a scatterplot with mean±SD, and each data point depicts a single measure from an individual subject plasma sample. ****P<0.0001, **P<0.01, *P<0.05 as calculated with Student unpaired 2-sided *t* tests. +P<0.01 as calculated with Mann–Whitney *U* test.

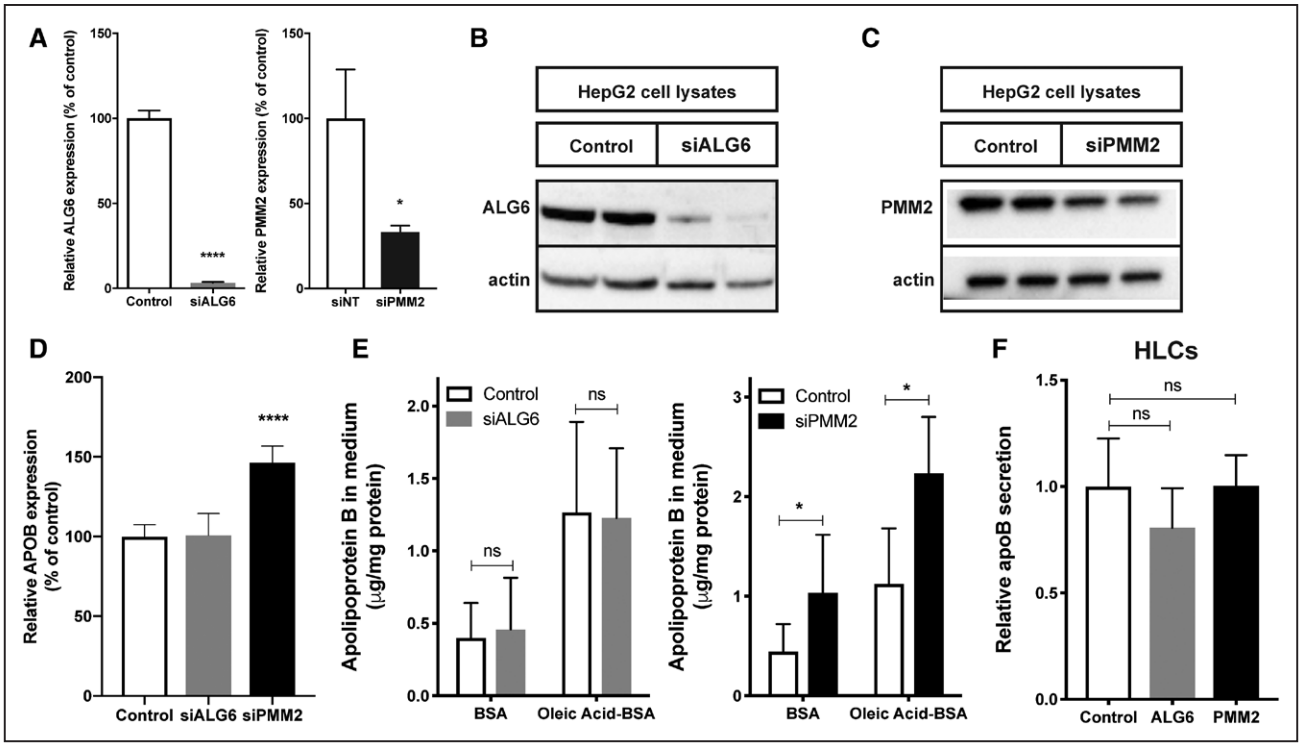

Figure 2. Apolipoprotein (apo) B production and secretion are unaffected in small interfering asparagine-linked glycosylation protein 6 (siALG6)– and small interfering phosphomannomutase 2 (siPMM2)– treated HepG2 cells and type 1 congenital disorder of glycosylation (CDG-I) hepatocyte-like cells (HLCs).

siRNA knockdown of *ALG6* and *PMM2* in HepG2 cells. Shown are ALG6 (**left**) and PMM2 (**right**) mRNA expression levels (**A**), ALG6 protein levels (**B**), PMM2 protein levels (**C**), apolipoprotein (apo) B mRNA expression (**D**), and secretion of apo B into the medium (normalized for total protein) in normal and oleic acid–stimulated conditions (**E**) for siALG6-treated cells (**left**) and siPMM2-treated cells (**right**). **F**, Relative ^{35}S apo B secretion into the medium in patient-derived HLCs. Representative Western blots or mean±SD values from 3 experiments with triplicate measurements per experiment are shown. Oleic acid-BSA is oleate complexed to BSA. ****P<0.0001, *P<0.05, and nonsignificant (NS) results as calculated with Student unpaired 2-sided t tests. siNT indicates non targeting siRNAs.

Apo B Production and Secretion Are Unaffected in siALG6- and siPMM2-Treated HepG2 Cells and Patient-Derived HLCs Redifferentiated From iPSCs

Decreased apo B production can limit its secretion.[26] In addition, ER-associated degradation and autophagy of apo B are major rate-limiting steps of its secretion, such that experimental inhibition of these degradation pathways generally increases apo B secretion.[27] To address apo B production in siRNA-treated cells, APOB mRNA expression was assessed with quantitative polymerase chain reaction (Figure 2D); there was no difference in *APOB* expression in siALG6-treated cells compared with controls. A significant increase in APOB expression was observed in the siPMM2-treated cells. Secretion of apo B protein into the medium was measured with ELISA (Figure 2E). Basal and oleic acid–stimulated apo B secretion was not affected by siALG6. In siPMM2 cells, apo B secretion was significantly increased, analogous to the increased APOB mRNA expression.

Inhibiting degradation pathways with the proteasomal inhibitor MG-132 or with bafilomycin A, an inhibitor of autophagy, for 4 hours did not affect apo B secretion in siALG6-treated cells more than in control cells (Figure III in the online-only Data Supplement). Lastly, we also assessed apo B secretion by ^{35}S steady-state protein labeling in patient-derived HLCs and found no significant differences between the ALG6-CDG or PMM2-CDG patient cells compared with control cells (Figure 2F).

Cell Surface LDLR Expression Is Increased in siALG6-Treated HepG2 Cells, CDG-I Fibroblasts, and Patient-Derived HLCs Redifferentiated From iPSCs

The presence of hypobetalipoproteinemia in the absence of steatosis and unaltered apo B secretion strongly suggests an increased clearance of apo B–containing lipoproteins from the circulation. Thus, we determined LDLR protein abundance after sterol depletion in whole-cell lysates of siALG6- and siPMM2-treated HepG2 cells (Figure 3A), specifically on the cell surface (Figure 3B). Indeed, we found both to be markedly increased in siALG6-treated cells; LDLR expression on the cell membrane was on average 145% that of controls in siALG6 (P=0.005), and this was accompanied by a 153% increase (P<0.0001) in the uptake of fluorescently labeled LDL (Figure 3C). In siPMM2 cells, we observed almost

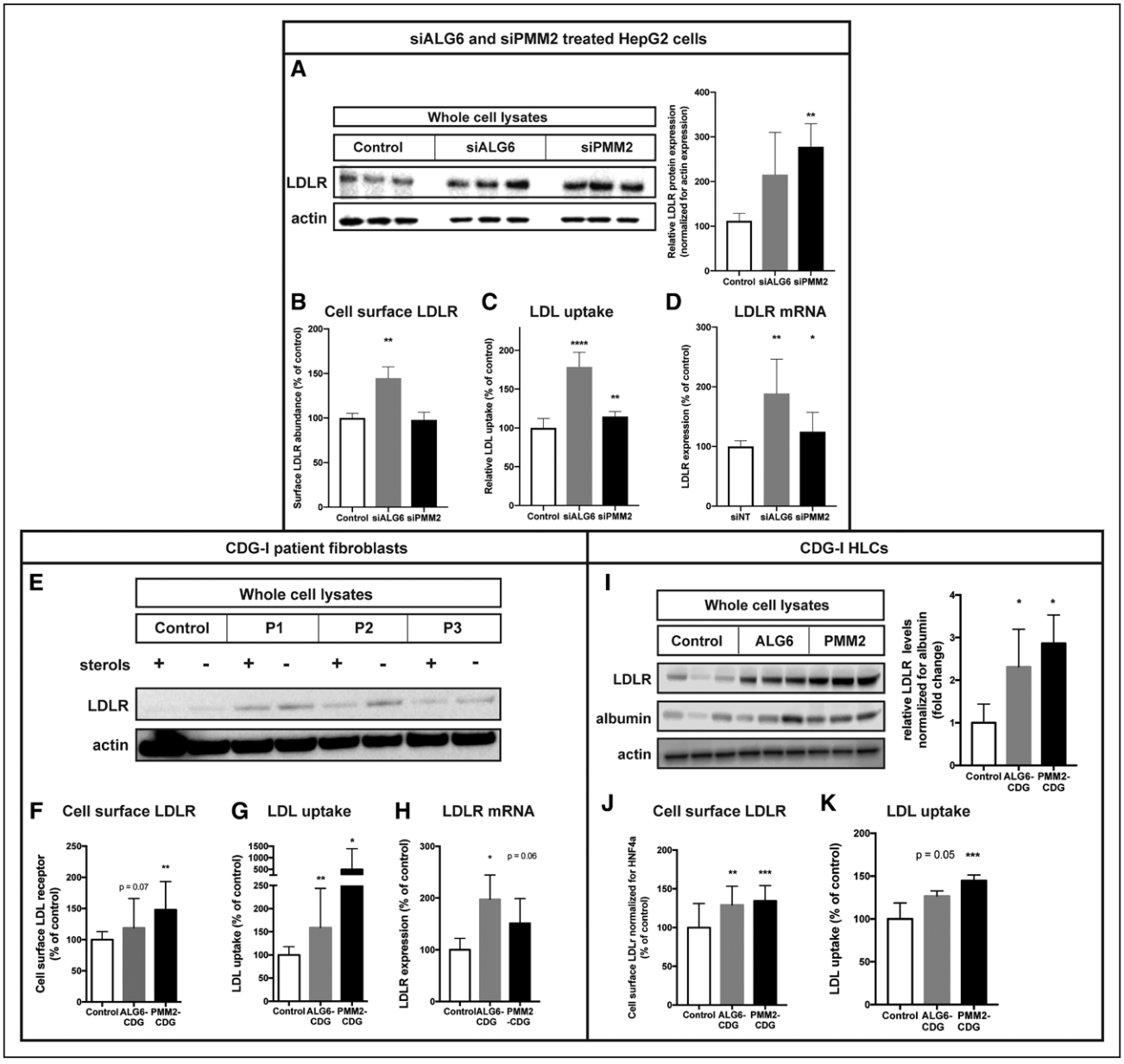

Figure 3. Low-density lipoprotein (LDL) receptor (LDLR) expression is increased in small interfering asparagine-linked glycosylation protein 6 (siALG6)– and small interfering phosphomannomutase 2 (siPMM2)–treated HepG2 cells, fibroblasts from patients with type 1 congenital disorder of glycosylation (CDG-I), and hepatocyte-like cells (HLCs) from a patient with CDG-I.

Shown are representative blots of analysis of LDLR protein expression in whole-cell lysates before and after sterol depletion, cell surface LDLR abundance measured with fluorescence-activated cell sorting (FACS), Dylight488-labeled LDL uptake, and LDLR mRNA expression in siALG6-treated HepG2 cells (**A** through **D**) and in fibroblasts from patients with CDG-I (**E** through **H**). **I**, LDLR protein expression in whole-cell lysates after sterol depletion and relative LDLR expression normalized for albumin in patient-derived HLCs. **J**, Cell surface LDLR measured with FACS. **K**, ^{125}I LDL uptake for the different HLC lines. Representative Western blots or mean±SD values from 3 experiments with triplicate measurements per experiment are shown. P1 through P3 are 2 patients with ALG6-CDG and 1 patient with PMM2-CDG. GFP indicates green fluorescent protein; and HNF4α, hepatocyte nuclear factor 4α. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 as calculated with Student unpaired 2-sided t tests.

tripled LDLR protein in whole-cell lysates (Figure 3A); however siPMM2 did not seem to affect surface LDLR expression (Figure 3B) and was accompanied by only a slight yet significant increase in LDL uptake (114% of control subjects; P=0.003; Figure 3C).

To verify these findings, we studied patient-derived fibroblasts, an established model to study LDLR functionality in patients with familial hypercholesterolemia.[28] Fibroblasts of 6 patients with CDG-I were analyzed (3 patients with ALG6 and 3 with PMM2 versus 2 healthy control subjects). In this model, there was also markedly increased LDLR protein abundance, both in cell lysates (Figure 3E) and on the cell surface (Figure 3F), which was again accompanied by enhanced uptake of LDL (on average 159%, P<0.001 in ALG6-CDG; and a 5-fold increase in PMM2-CDG, P=0.011; Figure 3H), together with an increased LDLR mRNA expression in both siALG6-

and siPMM2-treated HepG2 cells and in ALG6-CDG fibroblasts. In PMM2-CDG fibroblasts, this failed to reach significance (P=0.06; Figure 3D and 3H), arguing against a solely posttranscriptional effect on LDLR abundance.

Moreover, in both HepG2 cells and ALG6-CDG and PMM2-CDG fibroblasts, the LDLR protein migrated at approximately its expected molecular weight on SDS-PAGE gels (≈160 kDa) with no apparent difference compared with controls (Figure 3A and 3E). This also argues against a direct posttranscriptional effect on LDLR.

To further establish this, we used HepG2 cells that stably overexpress an LDLR–green fluorescent protein fusion protein. Indeed, when we silenced *ALG6* expression in these cells, the endogenous, untagged LDLR protein increased, as observed in our other cell models. However, the green fluorescent protein–tagged LDLR was refractory to *ALG6* silencing; its levels remained unchanged (Figure IV in the online-only Data Supplement). This reinforces the notion that ALG6 influences a pretranslational step in LDLR production.

We replicated these findings in patient-derived HLCs and again found increased LDLR protein levels in whole-cell lysates of the patient HLCs compared with controls (Figure 3I). When normalized for cellular albumin, a marker of well-differentiated HLCs, there was a 2- to 3-fold increase in LDLR protein compared with controls. Cell surface LDLR on hepatocyte nuclear factor 4α–positive HLCs was also significantly increased in HLCs of both patients with ALG6 and patients with PMM2 (129% and 134% of control subjects, respectively; P<0.01 and P<0.001; Figure 3J), which was also accompanied by increased ^{125}I LDL uptake (Figure 3K). Of note, the cells derived from patients with PMM2-CDG differentiated less efficiently compared with those from control subjects; however, all cell lines reached >70% redifferentiation efficiency on average.

Collectively, the results from 3 cell models convincingly show increased LDLR protein expression and function in patients with CDG-I.

In our cohort, total and free PCSK9 plasma levels were comparable between the patients with CDG-I and matched control subjects. In the carriers, total and free PCSK9 plasma levels were higher than in adult control subjects (Figure VA in the online-only Data Supplement). Furthermore, we found increased mRNA expression of *PCSK9* (Figure 4B) and comparable levels of secreted PCSK9 in siALG6-treated HepG2 cells compared with controls (Figure VB in the online-only Data Supplement).

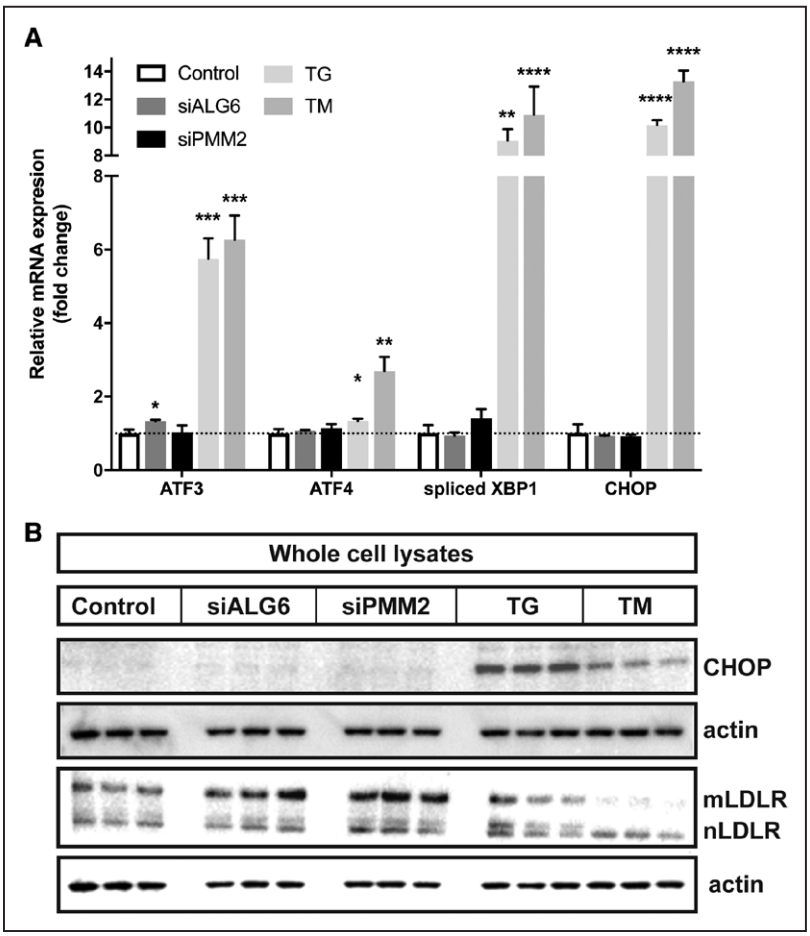

Figure 4. Endoplasmic reticulum (ER) stress markers in small interfering asparagine-linked glycosylation protein 6 (siALG6) and small interfering phosphomannomutase 2 (siPMM2) HepG2 cells. **A**, mRNA levels of ER stress markers ATF3, ATF4, spliced XBP1, and C/EBP homologous protein (CHOP). **B**, Representative blot of CHOP protein expression and low-density lipoprotein receptor in cells treated with siALG6, siPMM2, thapsigargin (TG), and tunicamycin (TM). Representative Western blots or mean±SD values from at least 2 experiments with triplicate measurements per experiment are shown. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 as calculated with Student unpaired 2-sided t tests.

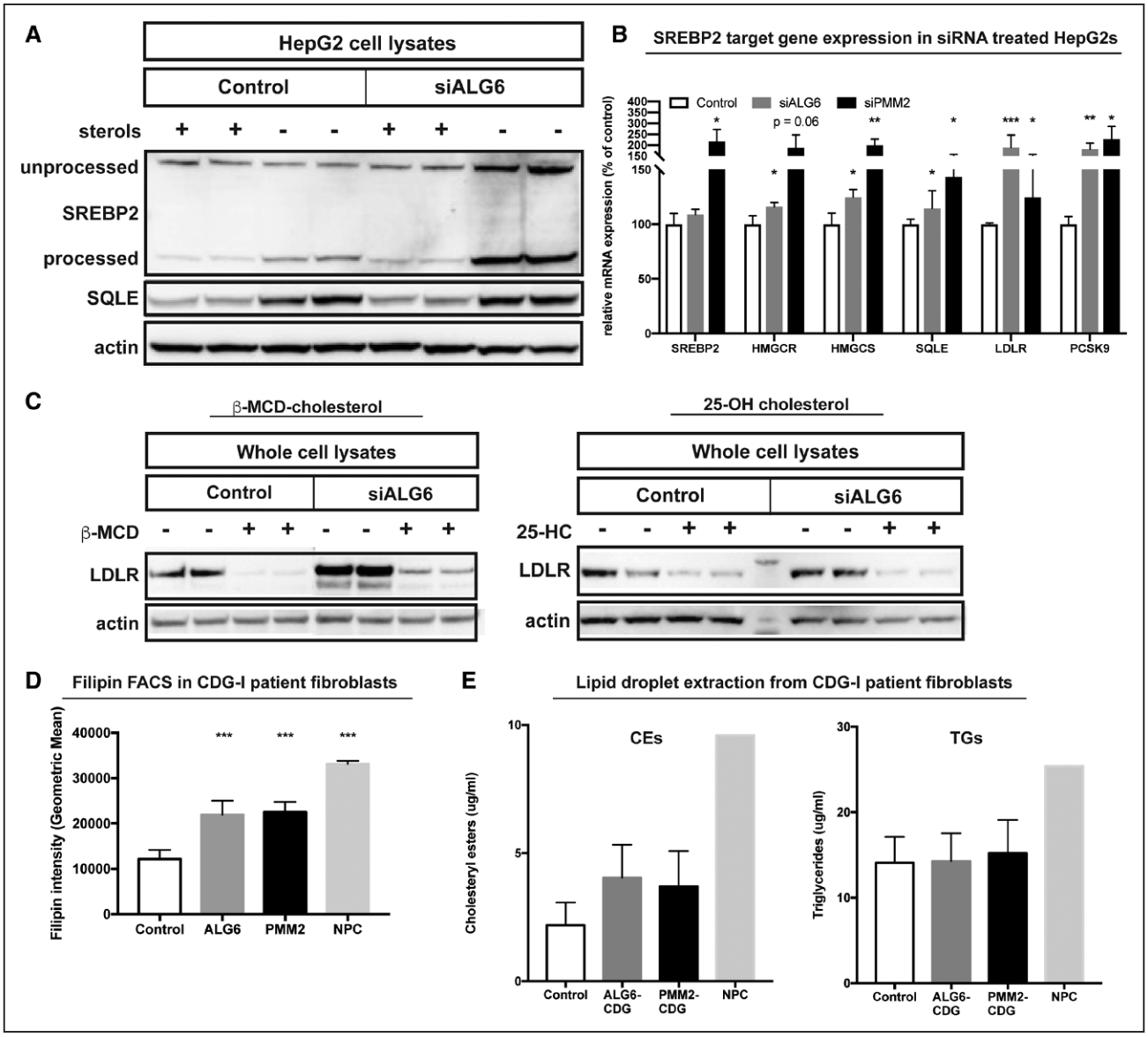

Figure 5. Increased sterol regulatory element-binding protein 2 (SREBP2) levels with intact cholesterol sensing in cells with decreased asparagine-linked glycosylation protein 6 (ALG6) or phosphomannomutase 2 (PMM2) activity.

Shown are unprocessed and processed SREBP2 and SREBP2-target squalene epoxidase (SQLE) protein expression in HepG2 cells before and after sterol depletion (**A**), mRNA expression of other SREBP2 targets (**B**), whole-cell lysate low-density lipoprotein receptor (LDLR) protein levels before and after 4 hours of β-MCD cholesterol or 25-hydroxycholesterol (25-OH) incubation (**C**), intracellular cholesterol levels measured with filipin staining in patient fibroblasts (**D**), and cholesteryl ester (CE) and thapsigargin content of lipid droplets in patient fibroblasts (**E**). Representative Western blots or mean±SD values from at least 2 experiments with triplicate measurements per experiment are shown. CDG-I indicates type I congenital disorder of glycosylation; FACS, fluorescence-activated cell sorting; NPC, Niemann-Pick C, PCSK1, proprotein convertase subtilisin/kexin type 9; si, small interfering; and TG, triglycerides. ***P<0.001, **P<0.01, *P<0.05 as calculated with Student unpaired 2-sided t tests.

No Major Role for ER Stress in the LDLR Upregulation in siALG6- and siPMM2-Treated Cells

Several studies have shown a role for ER stress in activating SREBP2[29–31] and subsequent *LDLR* transcription.[32] To investigate this in our CDG-I cell models, we studied mRNA and protein expression levels of ER stress markers in siALG6- and siPMM2-treated HepG2 cells. mRNA levels for *ATF3*, *ATF4*, spliced *XBP1*, and *CHOP* were not elevated in siPMM2-treated HepG2 cells, and only *ATF3* was slightly increased in siALG6 cells (Figure 4A), but not nearly to the degree observed when the cells were treated with the known ER stress inducers thapsigargin and tunicamycin. Thapsigargin and tunicamycin significantly increased mRNA levels of the ER stress markers (Figure 4A). Protein levels of the ER stress marker CHOP were also not increased in the siALG6- and siPMM2-treated cells compared with control cells, whereas thapsigargin and tunicamycin induced protein expression of CHOP, but this was not accompanied by a concomitant LDLR increase (Figure 4B).

Increased SREBP2 Levels With Intact Sterol Sensing

The observed higher LDLR mRNA and cell surface protein led us to study SREBP2, the major transcriptional regulator of LDLR expression.[33] SREBP2 protein expression in siALG6-treated HepG2 cells was markedly increased (Figure 5A). Both unprocessed and processed SREBP2 protein levels were significantly higher in siALG6-treated HepG2 cells, suggesting an increased SREBP2 protein abundance at a basal level, not an increased cleavage. In both control and siALG6 cells, SREBP2 protein was also increased on sterol depletion, and SREBP2 appeared functional because SQLE (squalene epoxidase) protein, a downstream target of SREBP2, was also increased (Figure 5A). In addition, mRNA expression of several other SREBP2 targets such as *HMGCR*, *HMGCS*, and *PCSK9* was increased (Figure 5B), although not to the same extent as *LDLR* mRNA. To test whether sterol sensing in the ER was intact, we evaluated the ability of β-MCD and 25-hydroxycholesterol to inhibit the SREBP2 pathway. Despite higher basal LDLR levels in siALG6-treated HepG2 cells compared with control cells, a similar response on SREBP2 inhibition was found (Figure 5C). Collectively, these results suggest that both SREBP2 activation and sterol sensing are intact in our CDG-I cell model.

Because SREBP2 activation and expression of cholesterogenic enzymes are elevated in CDG-I cells, we measured intracellular cholesterol levels in fibroblasts. As a positive control, we used fibroblasts of a patient with genetically proven Niemann-Pick C disease because of the known accumulation of free cholesterol in the cells of these patients (Figure 5D). Filipin staining of siALG6-treated HepG2s showed similar amounts of intracellular cholesterol compared with controls. In contrast, all patient fibroblasts showed significantly increased intracellular cholesterol (Figure 5D). Furthermore, lipid droplets in CDG-I fibroblasts showed a 2-fold increase in cholesteryl esters without affecting triglyceride content (Figure 5E), possibly suggesting higher esterification activity resulting from increased free cholesterol.

Combined, the increased LDLR and SREBP2 despite high intracellular cholesterol and the intact sterol sensing without significant ER stress led us to investigate whether the ER resident protein INSIG1 could link the disturbed N-glycan synthesis in the ER to increased levels of SREBP2 and LDLR. To this end, we overexpressed Myc-tagged INSIG1 in siALG6 HepG2 cells, which abolished the upregulation of LDLR (Figure XI in the online-only Data Supplement).

DISCUSSION

This study provides evidence for a novel molecular origin of low plasma LDL-C levels and describes a new primary hypobetalipoproteinemia phenotype without hepatic steatosis or steatorrhea. This evidence was collected in a cohort of patients with biallelic mutations in *ALG6* or *PMM2*, the 2 most common forms of autosomal recessive CDG-I, who demonstrated hypobetalipoproteinemia and their clinically unaffected heterozygous relatives who also had decreased LDL-C plasma levels, as well as in vitro studies with patient-derived cells demonstrating increased LDLR expression and LDL particle uptake. Furthermore, the hypobetalipoproteinemia phenotype in the patients was not associated with signs of secondary causes of low plasma cholesterol.

Primary hypobetalipoproteinemia comprises a group of rare genetic dyslipidemias characterized by plasma levels of TC, LDL-C, and apo B below the fifth percentile.[34] Physiologically, there are 2 potential explanations for profound reductions in plasma apo B–containing lipoproteins: decreased production or secretion of apo B/very-low-density lipoprotein particles, as observed in patients with *APOB* or *MTTP* mutations, and increased clearance of apo B–containing lipoproteins through increased LDLR, as is observed in patients with *PCSK9*[35] or *ANGPTL3* loss-of-function mutations.[36] In homozygous apo B- or MTP (microsomal triglyceride transfer protein)-deficient patients, decreased hepatic secretion of very-low-density lipoprotein causes hepatic steatosis and, in some cases, fat malabsorption, leading to severe steatorrhea. Heterozygosity for *APOB* mutations displays a milder clinical phenotype,[24] as well as patients with hypobetalipoproteinemia caused by loss-of-function mutations in *PCSK9*,[35,37] in whom increased cell surface LDLR expression leads to increased LDL clearance without hepatic steatosis.

The underlying mechanism we identify in the development of hypobetalipoproteinemia in patients with CDG-I involves a pathway that results in increased LDLR mRNA and higher LDLR membrane abundance via increased basal levels of processed SREBP2. To date, the mentioned loss-of-function mutations in *PCSK9* are the only other known cause of hypobetalipoproteinemia resulting from increased LDLR[35,37] and increased LDL-C clearance, as seen in patients with CDG-I. The role of PCSK9 in LDLR membrane abundance and LDL clearance is well established.[38] Benjannet and colleagues[39] showed that impaired glycosylation of PCSK9 did not significantly alter its secretion or function at least in Huh7 cells. Indeed, we did not find a role for PCSK9 in the hypobetalipoproteinemia phenotype in patients with CDG-I.

Our in vitro studies demonstrate that cell surface LDLR and LDL particle uptake were significantly increased when ALG6 or PMM2 activity is decreased, without affecting apo B secretion. In contrast, others reported increased apo B degradation on inhibition of glycosylation with tunicamycin[40,41] or site-specific mutagenesis of N-glycosylation sites in truncated apo B con-

structs.[42] The discrepancy with the latter study might lie in a difference in the apo B isoform studied (apo B_{37} versus apo B_{100}) and in the different cell model (rat McArdle cells versus human HepG2 cells). We have no specific explanation for the discrepancy with the tunicamycin model in the former study. Taken together, our observations do not support a significant role for reduced production or increased degradation of apo B in the hypobetalipoproteinemia phenotype in ALG6-CDG and PMM2-CDG.

The main driver of LDLR transcription is decreased intracellular cholesterol through SREBP2 activation. SREBP2 is located in the ER and held in place by the association with SCAP and INSIG.[33] When cells are depleted of sterols, a conformational change in SCAP allows SREBP2 to translocate to the Golgi apparatus to undergo sequential proteolytic activation by site-1-protease and site-2-protease. The cleaved product, nuclear SREBP2, is responsible for transcription of the ≈30 genes involved in cholesterol synthesis such as the rate-limiting enzymes HMGCR and SQLE, as well as those involved in cellular cholesterol uptake such as LDLR and PCSK9. In our study, SREBP2 protein levels and expression of SREBP2 target proteins were significantly increased in cell models for CDG-I despite elevated intracellular cholesterol, and intact SREBP2 activation through sterol sensing was observed.

Several studies have shown a role for ER stress in activating SREBP2[29–31] and subsequent *PCSK9* and *LDLR* transcription.[32] Lebeau et al[32] also showed that tunicamycin increased LDLR mRNA expression and reduced plasma LDL-C in mice. Thus, it could be hypothesized that ER stress caused by an accumulation of unfolded proteins resulting from deficient glycosylation plays a role in the SREBP2 activation in the CDG-I models. However, we did not find elevated ER stress markers in the siALG6- and siPMM2-treated HepG2 cells; we therefore concluded that ER stress does not underlie the increased LDLR function in our CDG-I cells.

Taking these results together, we observed increased LDLR and SREBP2 function despite high intracellular cholesterol, together with intact sterol sensing, but in the absence of significant ER stress. This led us to postulate that in CDG-I the defective N-glycan assembly in the ER may affect INSIG1, the ER resident protein that controls proteolytic activation of SREBP2 via retention of SCAP.[43] In our hands and in those of others, endogenous INSIG1 protein levels are below detection limit. Thus, we overexpressed Myc-tagged INSIG1 in our siALG6 HepG2 model. In support of our postulated mechanism, overexpression of INSIG1 in siALG6 HepG2 cells abolished the upregulation of the LDLR, which we so strongly observed in the 3 complementary cell models of our study.

Four aspects merit closer consideration. First, patients with CDG-I have a general glycosylation defect early in the glycan tree assembly machinery, causing empty glycosylation sites on a wide variety of glycoproteins. This evidently poses a challenge to the identification of the causal pathway underlying the hypobetalipoproteinemia in CDG-I. This is further compounded by biological and technical aspects related to N-glycosylation; the sequon (amino acid consensus motif) for N-glycosylation enzymes to initiate and attach N-glycans is identical for each individual N-glycosylated protein. In addition, no N-glycoproteomics technology is available at the moment that allows proper normalization to allow quantification of glycan site occupancy at the proteome scale. Yet, all in vitro studies point to 1 distinct pathway, increased LDLR expression, without indications of disrupted apo B secretion or very-low-density lipoprotein assembly.

Second, the hypobetalipoproteinemia phenotype seems to be specific to CDG-I. We have studied plasma lipids in other CDG types: CDG type II, affecting editing of N-glycans in the Golgi apparatus, and Golgi trafficking defects, affecting ER to Golgi transport and the N- and O-glycosylation enzymes located within these compartments. These patients with CDG have different yet still global protein glycosylation defects, but they show completely different plasma lipid abnormalities from the hypobetalipoproteinemia observed in patients with CDG-I. Patients with CDG type II sequester all their plasma cholesterol in the HDL fraction.[44] Patients with Golgi trafficking and mixed glycosylation defects such as CCDC115 or TMEM199 deficiency have marked hypercholesterolemia, in the range of familial hypercholesterolemia.[45,46] These distinctions again suggest a specific influence of N-linked glycosylation on LDL pathways.

Third, the observation that heterozygous carriers also display a hypolipidemic phenotype without other clinical features of the CDG-I syndrome underscores the more general notion that the influence of genetic variation in protein glycosylation on metabolic pathways in the general population is understudied.[47]

Finally, it is well established that low plasma LDL-C levels reduce atherosclerotic cardiovascular disease. Whether patients with CDG-I are protected from atherosclerosis cannot yet be determined. The oldest patients known to date are in their 40s; the prognosis and life expectancy of patients with CDG-I are determined predominantly by their neuromuscular pathology.[48] Promising in this respect is our finding that most heterozygous carriers also show the beneficial lipid phenotype of low LDL-C but none of the clinical symptoms found in patients with CDG-I.

Conclusions

We have shown that mutations in *ALG6* and *PMM2* can cause a hypobetalipoproteinemia phenotype. Mecha-

nistically, these changes can be explained by increased LDLR protein abundance with concomitant increased hepatic LDL clearance, driven by increased SREBP2 in the absence of major ER stress. Our data thus establish a major impact of protein glycosylation on LDL metabolism. Heterozygous carriers of CDG-I mutations exhibit marked hypocholesterolemia in the absence of the adverse clinical symptoms observed in the homozygous patients. In this light, our findings warrant exploration of therapeutic targeting of N-linked glycosylation in the LDLR pathway to reduce LDL-C in patients at increased cardiovascular risk, especially because therapeutic targeting of protein glycosylation is already being ventured in virology, immunology, and oncology.[49,50]

ARTICLE INFORMATION

Received June 15, 2018; accepted April 22, 2019.

The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/circulationaha.118.036484.

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Acknowledgments

The authors thank all the patients and their families for participating in this study. They thank Mayda Hernandez, Deepti Abbey, Wen Li Yang, and the Penn iPSC core for helpful discussions and advice in the HLC differentiation experiments. They are very grateful to Kobie Los for her invaluable help in expanding families for this study. They thank Prof C.E. Hollak and Dr G. Linthorst at the Amsterdam UMC, location AMC, for their assistance in the inclusion of patients. They also thank Lynne Wolfe and Christina Lam at the National Institutes of Health for their assistance in the inclusion of heterozygous family members.

Sources of Funding

This work was financially supported by the Dutch Organization for Scientific Research (Veni grants 91613031 and 91619098 to Drs Holleboom and Kroon, respectively; Vidi grant 91713359 for Dr Lefeber), the Amsterdam UMC Fellowship 2018 (to Dr Holleboom), the AMC Graduate School (AMC PhD Scholarship for Dr van den Boogert), the Netherlands CardioVascular Research Initiative (Genius2 CVON2017-2020), and the European Union (Transcard FP7-603091–2). Dr Kuivenhoven is Established Investigator of the Netherlands Heart Foundation (2016T068). Dr Loregger is supported by a Dekker grant from the Dutch Heart Foundation (2016T015). Dr Zelcer is an Established Investigator of the Dutch Heart Foundation (2013T111) and is supported by an ERC Consolidator grant (617376) from the European Research Council and a Vici grant from the Netherlands Organization for Scientific Research (016.176.643).

Disclosures

None.

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