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Elevated Oxysterol and *N*-Palmitoyl-*O*-Phosphocholineserine Levels in Congenital Disorders of Glycosylation

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Conflict of Interest

Informed Consent

Animal Rights

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Abstract

Congenital disorders of glycosylation (CDG) and Niemann-Pick type C (NPC) disease are inborn errors of metabolism that can both present with infantile-onset severe liver disease and other multi-systemic manifestations. Plasma bile acid and *N*-palmitoyl-*O*-phosphocholineserine (PPCS) are screening biomarkers with proposed improved sensitivity and specificity for NPC. We report an infant with ATP6AP1-CDG who presented with cholestatic liver failure and elevated plasma oxysterols and bile acid, mimicking NPC clinically and biochemically. On further investigation, PPCS, but not the bile acid derivative *N*-(3β,5α,6β-trihydroxy-cholan-24-oyl) glycine (TCG), were elevated in plasma samples from individuals with ATP6AP1-, ALG1-, ALG8-, and PMM2-CDG. These findings highlight the importance of keeping CDG within the diagnostic differential when evaluating children with early onset severe liver disease and elevated bile acid or PPCS to prevent delayed diagnosis and treatment.

Keywords

1 INTRODUCTION

Congenital disorders of glycosylation (CDG) are a rapidly expanding, clinically and genetically heterogenous group of diseases caused by different abnormalities in the glycan synthesis or modification pathways. CDGs affect multiple organs including the brain, eyes, musculoskeletal, liver, and hematologic systems.[1] Hepatopathy has been described in numerous CDG including phosphomannomutase deficiency (PMM2-CDG), the most commonly identified CDG, MPI (phosphomannose-isomerase), ALG8 (glucosyltransferase II), ALG1 (mannosyltransferase I), and COG (conserved oligomeric Golgi complex) deficiencies.[2-4] More recently, ATP6AP1-CDG is a combined N- and O-linked glycosylation disorder that has been reported to cause early onset acute cholestatic liver failure and variable other features. Diagnosis for CDG relies on biochemical testing (carbohydrate deficient transferrin assay, glycan analysis, and/or enzymatic assays if available), and molecular testing.

Niemann-Pick disease type C (NPC) is a neurodegenerative lysosomal disorder of intracellular cholesterol transport with multisystemic manifestations that may overlap with CDG. Typical findings of NPC in the neonatal and early childhood period include visceral symptoms such as abdominal ascites, cholestasis, conjugated hyperbilirubinemia, and progressive hepatosplenomegaly. Affected individuals harbor pathogenic variants in the *NPC1* or *NPC2* genes, which encode protein products that direct cholesterol

trafficking among subcellular organelles. As a result, unesterified cholesterol and other lipids accumulate in the central nervous system and various tissues throughout the body. Glycosylation of NPC2 is required for correct subcellular targeting and function.[5] Within the past decade, blood-based metabolites have been added to the traditional cell-based diagnostic methods. These metabolites include oxysterols [7-ketocholesterol (7KC) and cholestane- 3β,5α,6β-triol (3β,5α,6β-triol)], *N*-palmitoyl-*O*-phosphocholineserine (PPCS), and a bile-acid derivative *N*-(3β,5α,6β-trihydroxy-cholan-24-oyl) glycine (TCG).[6,7]

N-acyl-*O*-phosphocholineserines belong to a relatively new lipid class characterized from biological samples, of which PPCS is the most abundant species.[7] PPCS was previously mis-classified as lyso-sphingomyelin-like compound (lyso-SM509).[8] Sterols (steroid alcohols) are biosynthetic compounds produced by plants (phytosterols) and animals (zoosterols such as cholesterol) that can be oxidized through non-enzymatic or enzymatic reactions to form oxysterols.[9] Non-enzymatic oxidation of cholesterol produces the following oxysterols along various steps of the reaction pathways: 25-hydroxycholesterol (25HC), 7-KC, and 3β,5α,6β-triol. The 3β,5α,6β-triol is further converted to bile acids (e.g., TCG) from cholesterol precursors.[6] While plasma oxysterols (3β,5α,6β-triol and 7-KC) have been proposed to be sensitive and specific diagnostic biomarkers for NPC,[10,11] these cholesterol oxidation products can be elevated in other conditions that result in oxidative stress, such as atherosclerosis, neurodegenerative diseases, and liver dysfunction.[12] Specifically, plasma oxysterol levels can be elevated in individuals with cholestatic liver disease.[13] Recent efforts have explored PPCS and TCG as more specific markers for NPC.[14]

We report a case of an infant presenting with neurologic and hepatic failure leading to early death, along with foamy macrophages on liver biopsy and elevated plasma oxysterols suggestive of NPC. Cholestasis gene panel and sequencing of *NPC1* and *NPC2* were negative, and the child was diagnosed post-mortem with ATP6AP1-CDG via exome sequencing. The elevated oxysterol levels in this index child prompted us to evaluate more NPC-specific markers, PPCS and TCG, in CDGs.

2. MATERIALS and METHODS

2.1 Clinical Case

The study received exempt status by the Institutional Review Board (IRB) of Seattle Children's Hospital.

2.2 Clinical Samples

Samples were collected and analyzed on a clinical basis or per study protocols approved by the respective IRB at each institution as followed: Clinical and basic investigations into known and suspected CDG (NCT02089789, NHGRI, NIH), STUDY00001831 (Seattle Children's Hospital), Molecular genetic causes and biochemical consequences of Congenital disorders of glycosylation, approval no. 18/21 (grant AZV VES 2022 1. LF UK , 20.5.2021), Analysis of lysoSM-509 and bile acid B (Washington University in Saint Louis), Evaluation of Patients with Genetic Disorders (NCT02769949, NICHD, NIH) and Investigations into

Inborn Errors of Cholesterol Synthesis and Related Disorders (NCT00046202, NICHD, NIH).

2.3 Exome Sequencing (GeneDx)

Using genomic DNA from the clinical case proband and parents, the exonic regions and flanking splice junctions of the genome were captured using IDT xGen Exome Research Panel v1.0. Massively parallel (NextGen) sequencing was done on an Illumina system with 100bp or greater paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19 and analyzed for sequence variants using a custom-developed analysis tool. Additional sequencing technology and variant interpretation protocol has been previously described.[15] The general assertion criteria for variant classification are publicly available on the GeneDx ClinVar submission page (http://www.ncbi.nlm.nih.gov/clinvar/submitters/26957/).

2.4 PPCS and TCG Assays

Plasma or serum samples from CDG participants were prepared and analyzed by LC-MS/MS as previously described for *N*-palmitoyl-*O*-phosphocholineserine (PPCS)[7] and *N*-(3β,5α,6β-trihydroxycholan-24-oyl) glycine (TCG).[6]

3   RESULTS

3.1   Clinical Case

The proband was a term male who presented at 28 days of age with jaundice and unconjugated hyperbilirubinemia (total 20 mg/dL, direct 5.2) that were unresponsive to phenobarbital and ursodiol. His prenatal and neonatal course were unremarkable. He was admitted at 3 months of age for jaundice, hepatosplenomegaly, unconjugated hyperbilirubinemia (14.7 mg/dL (normal range 0-1.1)), and hemolytic anemia. At 4 months, he had progressive hepatosplenomegaly, increased hepatic parenchymal echogenicity and ascites on exam and abdominal ultrasound. Laboratory findings showed hepatic dysfunction with elevated transaminases, coagulopathy, and hemolysis. Percutaneous liver biopsy showed fibrosis and lipid-laden (foamy) macrophages on electron microscopy. Further diagnostic testing showed elevated plasma oxysterols [cholestane-3β,5α,6β-triol 0.06 nmol/mL (0.02) and 7-ketocholesterol 0.2 nmol/mL (0.05)], and normal PPCS [0.01 nmol/mL (0.02)]. Acid sphingomyelinase level was normal. Additional testing showed normal or negative results (Supplemental Data). Molecular testing with a cholestasis gene panel was sent, but based on clinical and laboratory findings, the care team adopted a working diagnosis of Niemann-Pick disease type C. His clinical condition further destabilized with liver failure refractory to treatment, hyperammonemia, and hepatic encephalopathy. After emergency Investigational New Drug (eIND#142656) approval, he received two doses of 1000 mg/kg IV VTS-270 (2-hydroxypropyl-β-cyclodextrin; cyclodextrin) at 5 and 7 days premortem. His neurologic signs unfortunately progressed to diffuse cerebral edema, uncal herniation, and death at 5.5 months of age.

The cholestasis gene panel with deletion and duplication testing including the *NPC1* and NPC2 genes did not identify any pathogenic variants. TCG bile acid level was initially

below the NPC diagnostic cut-off (10.4 ng/mL, cut-off 18.5 ng/mL), then peaked at 32.5 ng/mL shortly premortem. Postmortem trio clinical exome sequencing revealed that the proband was hemizygous for a maternally inherited, disease-associated c.1036 G>A (p.Glu346Lys) variant in ATP6AP1, confirming the diagnosis of X-linked ATP6AP1-CDG.

3.2 Biochemical and clinical findings in CDG participants

To evaluate NPC-related biomarker levels in individuals with CDG, we measured PPCS and TCG in serum or plasma samples from participant with various CDG types. Table 1 contains information on demographics, genotype, liver enzyme, bilirubin, TCG, and PPCS levels. Additional clinical information and samples used in this work is in Supplemental Table 1. PPCS level above cut-off for diagnosing NPC1 was present in ATP6AP1-, ALG1-, ALG8-, and PMM2-CDG samples (Fig. 1). No CDG samples have TCG level above the diagnostic cut-off for NPC. Elevated PPCS levels do not correlate with levels of the liver enzymes ALT [Pearson correlation $r(25) = -0.07$, $p = 0.71$], AST [$r(25) = -0.0002$, $p = 1$], or alkaline phosphatase [$r(25) = -0.11$, $p = 0.59$] (Fig. 2A-C). PPCS levels (raw or log10 transformed) in samples do not differ significantly {non-parametric Kruskal-Wallis raw [$\chi^2(3) = 4.2$, $p = .24$] or log10 transformed [$\chi^2(3) = 4.1$, $p = .25$]} among group of samples with different number of liver enzymes that are above the reference ranges in the same individual (Fig. 2D). Aside from the proband sample, only one other sample had elevated bilirubin and PPCS levels (Table 1).

4   DISCUSSION

Congenital disorders of glycosylation and Niemann-Pick disease, type C disease with infantile presentation are multisystemic diseases with overlapping clinical features including severe infantile-onset cholestatic liver disease. Timely and accurate diagnosis of these conditions with clinical, histologic, biochemical, and molecular evidence is imperative for early and appropriate treatment. The presentation of the index child provided the impetus for further evaluation of NPC-related markers in CDGs. False positive plasma oxysterol results may occur in the setting of acute liver disease, CDG, and other disorders.[16] Results from this collection of samples suggest PPCS level in CDGs can be above the cut-off for NPC, and this may not necessarily relate to concurrent liver processes as transaminases, alkaline phosphatase, and bilirubin levels were not associated with elevations in PPCS. TCG is more specific for NPC, as its level in all evaluated CDG samples remain below the cut-off for NPC diagnosis. Depending on local accessibility variable combinations of diagnostic testing may be available, and in some cases molecular testing may be more feasible and informative as in the index case described.

ATP6AP1 deficiency, also known as ATP6AP1-CDG, is an X-linked congenital disorder of glycosylation characterized by a broad phenotypic spectrum of severity including hepatopathy, immunodeficiency, variable neurocognitive symptoms, and an abnormal transferrin glycosylation pattern.[17-20] Affected males can have normal development and non-emergent health issues in their 20-30's, or die within the first few years of life from severe liver disease as in our index case and others previously reported.[20] Presence of elevated liver enzymes, low serum copper and ceruloplasmin in an affected individual has

invoked comparison to Wilson disease, an abnormal copper metabolism condition with hepatic involvement.[17] ATP6AP1 encodes an accessory subunit to the transmembrane proton pump V0 component of vacuolar H^+-ATPases, which serve important roles in endocytosis, intracellular transport and acidification, and Golgi targeting of lysosomal enzymes.[21,22] In yeast, Vma13p, a vacuolar H^+-ATPase subunit, regulates the activity of Ynd1p, a nucleoside triphosphate diphosphohydrolase with a role in maintaining the level of sugar nucleotides used in glycosylation reactions in the Golgi.[23]

ATP6AP1-CDG fibroblasts harbor abnormalities in myriad protein and lipid metabolic pathways.[17] In addition, fibroblasts with defective ATP6AP1 or another accessory subunit such as ATP6AP2 or Vacuolar ATPase Assembly Factor (VMA21) display decreased autophagic clearance evidenced by increased presence of autophagosomal markers LAMP-1/2 and LC3, and increased endoplasmic reticular (ER) stress.[20,24,25] The lysosomal abnormalities and filipin staining of unesterified cholesterol in VMA21 fibroblasts are similar to the cellular phenotype in NPC. Altered lysosomal acidification likely contributes to the disturbance in cholesterol transport. However, the connection between defects in the glycosylation and cholesterol transport pathways may involve other mechanisms. For example, NPC2 interacts with different ER-resident proteins involved in membrane trafficking[26] and dolichol synthesis[27] and requires glycosylation to be fully functional[5]. Further delineation of how ATP6AP1 affects cholesterol transport is necessary.

PPCS belongs to a class of newly characterized lipids that has been detected in cerebrospinal fluid, blood, and liver sources.[7] The biosynthetic pathway of this compound is currently unknown. Oxysterols and TCG, a bile acid derivative, have been strongly linked to defects in cholesterol metabolism and transport. As liver is a source of oxysterols and bile acid derivatives, other pathologic liver processes are associated with elevations in various forms of these markers.[28] The lack of correlation between liver enzymes and PPCS levels in this sample group, however, suggests that liver dysfunction is not the only explanation for the increased level in PPCS. The observations reported here will need to be evaluated in a larger cohort with measurement of liver enzymes and bilirubin with PPCS and TCG concurrently from the same samples. Assessment of the same measurements in a cohort of pediatric individuals with cholestatic liver diseases would also be informative.

In summary, we report a male infant with molecularly confirmed ATP6AP1-CDG presenting with severe liver disease, lipid-laden macrophages in hepatocytes, and elevated plasma oxysterols. Biochemical findings in this index case and additional samples from individuals with various other types of CDG show elevation of oxysterols and PPCS, proposed markers for NPC but not of the bile acid derivative TCG. Thus, in individuals presenting with early childhood severe liver disease and elevation of oxysterols or PPCS, CDGs should be included in the differential and broad molecular testing such as exome or genome sequencing should be considered for timely diagnosis. Specifically, ATP6AP1-CDG should be added to gene panels for cholestatic liver disease and genetic testing should be performed to confirm the molecular diagnosis for these individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SYNOPSIS

Congenital disorders of glycosylation can present with liver pathologies and elevated oxysterols, bile acid, or *N*-palmitoyl-*O*-phosphocholineserine, similar to findings seen in Niemann-Pick type C disease.
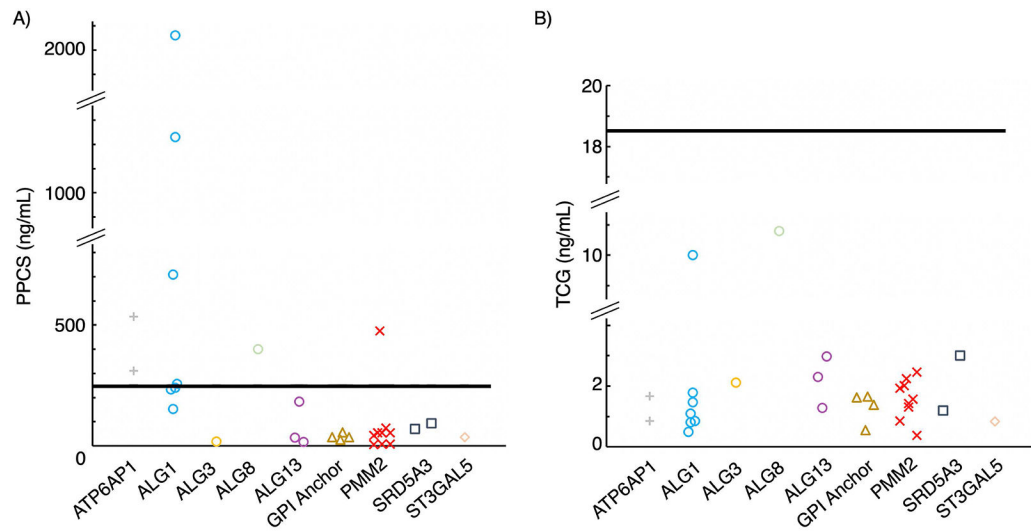
Figure 1.
Levels in plasma or serum from individuals with congenital disorders of glycosylation of A) *N*-palmitoyl-*O*-phosphocholineserine (PPCS), B) *N*-(3β,5α,6β-trihydroxy-cholan-24-oyl) glycine (TCG). Solid lines depict the cut-off where values above which suggest a diagnosis of NPC (PPCS 248 ng/mL, TCG 18.5 ng/mL).
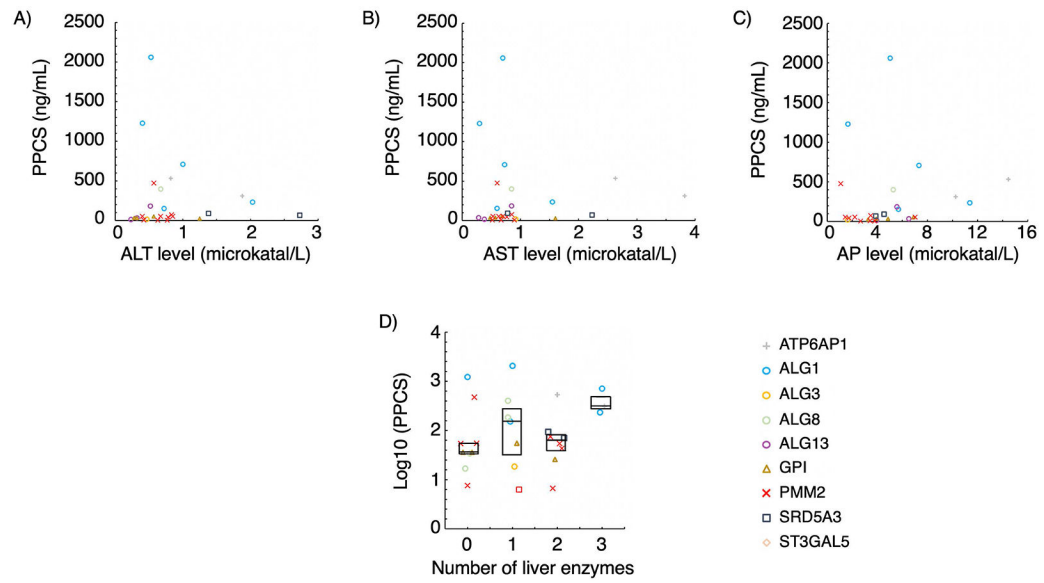
Figure 2.
PPCS in relation to liver enzyme levels. A) Alanine transaminase (ALT) level [Pearson correlation r(25) = −0.07, p = 0.71]. B) Aspartate transaminase (AST) level [r(25) = −0.0002, p = 1]. C) Alkaline phosphatase (AP) level [r(25) = −0.11, p = 0.59]. D) Log transformed PPCS level in relation to number of liver enzymes outside of the reference ranges [non-parametric Kruskal-Wallis $\chi^2(3) = 4.11$, p = .250 across the four groups]. Rectangular boxes denote 1st quartile, median, and 3rd quartile. The same symbols are used in A-D.

Table 1.

Demographic and clinical features of study participants diagnosed with congenital disorder of glycosylation (both clinical case presented and samples). CDG: congenital disorders of glycosylation. TCG: bile acid derivative N-(3β,5α,6β-trihydroxy-cholan-24-oyl) glycine; cutoff for Niemann-Pick type C diagnosis > 18.5 ng/mL. PPCS: N-palmitoyl-O-phosphocholineserine; cutoff for Niemann-Pick type C diagnosis > 248 ng/mL. ALT: alanine transaminase. AST: aspartate transaminase. AP: alkaline phosphatase. D/T: direct/total bilirubin. µkat/L: microkatal/L. Liver enzyme levels at the time of PPCS/TCG measurements. NA: not available/applicable. Bolded text: values above reference range.

Participant	Age (years) at		Sex	CDG			Liver enzyme levels				TCG	PPCS
Study ID	presentation	sample collection		Gene	Variant 1	Variant 2	ALT	AST	AP	Bilirubin	(ng/mL)	(ng/mL)
							(µkat/L)	(µkat/L)	(µkat/L)	(D/T, mg/dL)		
Proband	0.25	0.46	M	ATP6AP1	c.1036G>A (Glu346Lys)	NA	**3.5**	**2.2**	**31**	**4.7/**	see text	see text
3198	0.17	0.17	M	ATP6AP1	c.221T>C (Leu74Pro)	NA	0.82	**2.63**	**14.47**	**7.3/ 28.1**	0.847	**534**
5121	0.01	0.58	M	ATP6AP1	c.221T>C (Leu74Pro)	NA	**1.88**	**3.82**	**10.26**	0.7/1.2	1.67	**311**
CDG-0286	0.5	10.67	F	ALG1	c.841G>T (Val281Phe)	c.1057T>G (Tyr353Glu)	0.53	**0.7**	5.03	0.4/	1.78	**2060**
CDG-0320	NA	0.58	M	ALG1	c.1079C>T (Ala360Val)	c.212C>T (Ser71Phe) c.221A>T (His74Leu)	**0.72**	0.6	5.68	/0.2	0.814	153
CDG-0330S	NA	NA	NA	ALG1	c.149A>G (Gln50Arg)	c.773C>T (Ser258Leu)	NA	NA	NA	NA	1.47	240
CDG-0330Z	NA	NA	NA	ALG1	c.149A>G (Gnl50Arg)	c.773C>T (Ser258Leu)	NA	NA	NA	NA	0.859	**256**
CDG-0377	0.83	11.5	F	ALG1	c.773C>T (Ser258Leu)	c.342G>C (Leu114Phe)	**1**	**0.73**	**7.33**	3/	1.1	**709**
CDG-0424	0.06	0.33	F	ALG1	c.1188-2A>G	c.262T>G (Leu88Val)	**2.03**	**1.55**	**11.38**	/0.2	0.499	234
CDG-1081	0	4	F	ALG1	c.877T>C (Ser293Pro)	c.876C>G (Phe292Leu)	0.4	0.3	1.67	<0.2/0.2	10	**1230**
BCGBB009	0.06	17	F	ALG3	c.656T>C (Leu219Pro)	c.749T>A (Leu250Gln)	0.47	**0.92**	1.63	0.2	2.11	18.4
CDG-1026	0.5	3	M	ALG8	c.584T>C (Leu195Pro)	c.1334T>C (Leu445Pro)	0.67	**0.85**	5.27	<0.2/0.3	10.8	**400**
CDG-1017	0	8	F	ALG13	c.320A>G (Asn107Ser)	NA	0.23	0.38	3.95	<0.2/0.2	2.97	16.9
CDG-1044	0.04	11	F	ALG13	c.320A>G (Asn107Ser)	NA	0.32	0.28	6.52	<0.2/0.2	2.3	34.2
CDG-1082	0.25	1	F	ALG13	c.320A>G (Asn107Ser)	NA	0.52	**0.85**	5.58	<0.2/<0.2	1.29	184
CDG-1078	0	1	M	GPI Anchor	c.355C>T (Arg119Trp)	NA	0.57	0.68	**6.88**	<0.2/0.3	1.65	55.2
CDG-1085	0.06	4	M	GPI Anchor	c.2284-1G>C	c.2091_2093 delTGT	0.28	0.48	3.92	<0.2/<0.2	1.62	35.7
CDG-1091	1	6	M	GPI Anchor	c.395C>G (Ser132Cys)	NA	0.32	0.57	4.87	<0.2/<0.2	1.39	35.9
CDG-1110	0.33	7	M	GPI Anchor	c.391T>C (Phe131Leu)	NA	**1.25**	**1.6**	3.28	<0.2/0.3	0.554	25.9
BCGBB004	0.25	6	M	PMM2	c.470T>C (Phe157Ser)	c.531G>C (Gln177His)	0.4	0.53	2.2	NA	1.31	54.2

| Participant | Age (years) at | | Sex | CDG | | | Liver enzyme levels | | | | TCG | PPCS |
Study ID	presentation	sample collection		Gene	Variant 1	Variant 2	ALT (μkat/L)	AST (μkat/L)	AP (μkat/L)	Bilirubin (D/T, mg/dL)	(ng/mL)	(ng/mL)
BCGBB005	0.42	12	M	PMM2	c.323C>T (Ala108Val)	c.710C>G (Thr237Arg)	0.85	0.63	7.02	0.4/	2.01	54.2
BCGBB007	0.33	38	M	PMM2	c.338C>T (Pro113Leu)	c.422G>A (Arg141His)	0.67	0.7	1.45	NA	2.46	55
BCGBB008	0	33	M	PMM2	c.338C>T (Pro113Leu)	c.422G>A (Arg141His)	0.57	0.6	1.08	NA	2.24	476
CDG-1022	0	3	M	PMM2	c.98A>C (Gln33Pro)	c.140C>T (Ser47Leu)	0.77	0.90	2.68	0.2/0.8	0.385	6.64
CDG-1075	0.04	3	F	PMM2	c.415G>A (Glu139Lys)	c.422G>A (Arg141His)	0.42	0.67	3.43	<0.2/0.5	1.42	7.59
CDG-1079	0.33	7	M	PMM2	c.395T>C (Ile132Thr)	c.422G>A (Arg141His)	0.78	0.77	1.75	<0.2/0.2	1.93	43.2
CDG-1113	0.17	7	F	PMM2	c.686A>C (Tyr229Ser)	c.710C>G (Thr237Arg)	0.83	0.85	3.47	<0.2/0.3	1.57	74.5
CDG-1119	0.08	6	F	PMM2	c.563A>G (Asp188Gly)	c.691G>A (Val231Met)	0.63	0.52	3.80	<0.2/<0.2	0.851	6.24
BCGBB001	0.33	15	F	SRD5A3	c.645_670del26 (His216ArgfsX7)	c.617G>T (Gly206Val)	2.73	2.23	3.88	0/0.3	1.19	70.5
BCGBB002	Prenatally	21	M	SRD5A3	c.645_670del26 (His216ArgfsX7)	c.617G>T (Gly206Val)	1.38	0.78	4.54	NA	3	93.6
BCGBB006	0.25	2	F	ST3GAL5	c.719T>A (Ile240Lys)	c.353delA (Lys118ArgfsX70)	NA	NA	NA	NA	0.839	35.3