Mutations in the V-ATPase assembly factor \textit{VMA21} cause a congenital disorder of glycosylation with autophagic liver disease.

Magda Cannata Serio$^{1,23,*}$, Laurie A. Graham$^2*$, Angel Ashikov$^{3,4,*}$, Lars Elmann Larsen$^{20,21}$, Kimiyo Raymond$^5$, Sharita Timal$^{3,4}$, Gwenn Le Meur$^1$, Margret Ryan$^6$, Elzieta Czarnowska$^7$, Jos C. Jansen$^8$, Miao He$^{9,10}$, Can Ficicioglu$^{11}$, Pavel Pichurin$^{12}$, Linda Hasadski$^{13}$, W. Alfredo Rios-Ocampo$^{20}$, Christian Gilissen$^{14}$, Richard Rodenburg$^{15}$, Johan W. Jonker$^{20}$, Adriaan G. Holleboom$^{21}$, Eva Morava$^{16}$, Joris A. Veltman$^{17,18}$, Piotr Socha$^{19}$, Tom H. Stevens$^6#$, Matias Simons$^{1,22}#$ & Dirk J. Lefeber$^{3,4}$#

1 - Laboratory of Epithelial Biology and Disease, Imagine Institute, Université Paris Descartes-Sorbonne Paris Cité, Paris, France.
2 - Department of Chemistry and Biochemistry, Institute of Molecular Biology, University of Oregon, Eugene, Oregon, USA.
3 - Department of Neurology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands.
4 - Department of Laboratory Medicine, Translational Metabolic Laboratory, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands.
5 - Department of Laboratory Medicine and Pathology, Mayo College of Medicine, Rochester, Minnesota, USA.
6 - Department of Chemistry and Biochemistry, Institute of Molecular Biology, University of Oregon, Eugene, Oregon, USA.
7 - Department of Pathology, The Children’s Memorial Health Institute, Warsaw, Poland.
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Contact Information
Dr. Matias Simons, Laboratory of Epithelial biology and disease, Institute Imagine - Inserm UMR 1163, 24 Boulevard du Montparnasse - 75015 Paris France, Tel: +33(0)1 42 75 44 55 matias.simons@institutimagine.org
Dr. Dirk J. Lefeber, Translational Metabolic Laboratory, Department of Neurology, Donders Center for Brain, Cognition and Behavior, Radboud university medical center, Box 9101, 6500 HB Nijmegen, The Netherlands. Tel: +31(0)243614567, Email: Dirk.Lefeber@radboudumc.nl

List of Abbreviations
ALP: Alkaline phosphatase
ALT: Alanine transaminase
ApoCIII: Apolipoprotein CIII
AST: Aspartate transaminase
ATF4: activating transcription factor 4
CDG: Congenital Disorders of Glycosylation
CHOP: C/EBP homologous protein
CK: Creatine kinase
CTSB: Cathepsin B
ER: Endoplasmic Reticulum
GGT: Gamma Glutamyl Transferase
GRP94: Glucose Regulated Protein 94
LDL: Low-density lipoprotein
LDs: Lipid droplets
MALDI-LTQ: matrix-assisted laser/desorption ionization- Linear Trap Quadropole
NPC: Niemann-Pick type C disease
PERK: PKR-like ER kinase
SREBP: sterol response element-binding protein
Abstract
The V-ATPase is a multi-subunit protein complex required for acidification of intracellular compartments. At least five different factors are known to be essential for its assembly in the endoplasmic reticulum. Genetic defects in four of these V-ATPase assembly factors show overlapping clinical features, including steatotic liver disease and mild hypercholesterolemia. An exception is the assembly factor VMA21 whose X-linked mutations lead to autophagic myopathy. Here, we report pathogenic variants in VMA21 in male patients with abnormal protein glycosylation that result in mild cholestasis, chronic elevation of transaminases, elevation of (LDL) cholesterol and steatosis in hepatocytes. We also show that the VMA21 variants lead to V-ATPase misassembly and dysfunction. As consequence, lysosomal acidification and degradation of phagocytosed materials are impaired causing lipid droplet (LD) accumulation in autolysosomes. Moreover, VMA21 deficiency triggers ER stress and sequestration of unesterified cholesterol in lysosomes, thereby activating the sterol response element-binding protein (SREBP)-mediated cholesterol synthesis pathways. Conclusion: Together, our data suggest that impaired lipophagy, ER stress and increased cholesterol synthesis lead to LD accumulation and hepatic steatosis. V-ATPase assembly defects are thus a novel form of hereditary liver disease with implications for the pathogenesis of non-alcoholic fatty liver disease.

Introduction
The vacuolar H\textsuperscript{+}-ATPase complexes, or V-ATPases, are large multisubunit protein complexes that are responsible for the pH homeostasis of cytoplasmic organelles (1). They are organized into two domains, a membrane-integral V\textsubscript{0} domain, responsible for proton translocation, and a cytosolic V\textsubscript{1} domain, that carries out ATP hydrolysis (1). For the biogenesis of the V-ATPases, studies in S. cerevisiae have shown that V\textsubscript{0} and V\textsubscript{1} domains are assembled separately in the endoplasmic reticulum (ER) and cytosol, respectively. The assembly of the V\textsubscript{0} domain was shown to depend on a set of ER-resident chaperones (2,3): Vma12p, Vma21p, Voa1p, Pkr1p and Vma22p (3–7). In the first step of the assembly, Vma21p comes into contact with subunit c’, and this interaction promotes the assembly of the proteolipid subunits into a ring (3,4). In a second parallel step, a complex of Vma12p and Vma22p interacts transiently with subunit a of the V\textsubscript{0} domain and mediates its assembly with the proteolipid ring (2). Once the V\textsubscript{0} is fully assembled, Vma21p escorts V\textsubscript{0} domain to the cis-Golgi, where it will bind the V\textsubscript{1} sector to form the functional V-ATPase (3). Owing to its ER retention motif KKXX, Vma21p is transported back to the ER in order to assist in additional rounds of V\textsubscript{0} assembly (3). Vma12p, Vma21p and Vma22p correspond to TMEM199, VMA21 and CCDC115, respectively, in mammals. Voa1p has been replaced by ATP6AP1 and ATP6AP2 (8,9) whereas Pkr1p does not have a mammalian ortholog. Whether the assembly of the mammalian V\textsubscript{0} is mechanistically similar to yeast V\textsubscript{0} assembly is currently unclear (10).

Lessons on the phenotypic consequences of V-ATPase assembly dysfunction can be learned from human genetic diseases. Patients with mutations in four of the assembly factor genes, ATP6AP1, ATP6AP2, TMEM199 and CCDC115 present with a heterogeneous spectrum of disease symptoms (8,11–13). Interestingly, the organ that is affected in all patients is the liver. Hepatic features include steatosis in hepatocytes, lipid and variable hepatic injury ranging from chronic elevations of liver transaminases to end-stage liver disease necessitating liver transplantation. All patients were also found to have abnormal serum protein N- and O-glycosylation (8,11–13). However, the cause of hepatic dysfunction is unknown.

In contrast to the phenotypes of these four V-ATPase assembly defects, pathogenic variants in VMA21 have been associated with X-linked myopathy with excessive autophagy (XMEA, MIM 310440), characterised by progressive vacuolisation and atrophy.
of skeletal muscle (14–18). XMEA can have a milder course with onset after the age of five years with slow progression (14,17,19), and a prenatal/neonatal presentation with a more severe phenotype of congenital autophagic vacuolar myopathy associated with death in infancy.

Here, we present VMA21 deficiency as autophagic hepatopathy with steatohepatitis and chronic hypertransaminasemia. Mechanistically, it is caused by defective lipophagy and can be readily diagnosed by screening for abnormal glycosylation in patient sera. Our results expand the clinical spectrum of VMA21 deficiency from a solely muscle disease to an autophagic hepatopathy and indicate V-ATPase assembly as a general mechanism that can be further studied to understand defective lipophagy in more common fatty liver disease.

**Experimental Procedures**

**Patient materials.** Blood and fibroblasts of affected individuals were obtained for diagnostics of inborn errors of metabolism and used, together with parental DNA, after written informed consent from parents and treating physicians, according to Helsinki’s declaration.

**Glycosylation studies.** Screening for Congenital Disorders of Glycosylation (CDG) was carried out as described before (12). Plasma N-glycan profiling was performed by MALDI-TOF mass spectrometry of permethylated glycans (22), using 10 μL of plasma. High resolution mass spectrometry of intact transferrin was performed on a 6540 nanochip QTOF (Agilent), according to published protocols (23).

**Cell culture.** Primary fibroblasts from patients and controls were grown from a skin biopsy and cultured in Dulbecco’s modified Eagle Medium DMEM DMEM (high glucose 4500 mg/L, Thermo Fisher) supplemented with 10 % fetal bovine serum (FBS), 1 % L-
glutamine and 1 % Penicillin/Streptomycin at 37°C under 5 % CO2. Research on patients’ cells was prospectively reviewed and approved by the Ethical Committees of the University Hospital of Nijmegen.

**Bodipy, Cathepsin B, Filipin and LysoSensor staining.** For Bodipy and Filipin labelling, fibroblasts or Huh7 cells were fixed for 10 min in 4 % paraformaldehyde and incubated with BODIPY® 493/503 (2.5 μg ml⁻¹, Molecular Probes) and Hoechst (0.5 μg ml⁻¹) diluted in PBS-Triton 0.1 % for 2 h RT, or with Filipin (0.05 mg/ml, F9765, Sigma) in PBS for 1 h RT.

For LysoSensor and Cathepsin B labelling, fibroblasts were grown on MatTek glass bottom dishes, incubated for 20 min at 37°C with Magic Red Cathepsin assay (ImmunoChemistry Technologies, LLC) or 5 min at 37°C with LysoSensor® Green DND-189 (50 nM, Molecular Probes), diluted in DMEM medium. The probe-containing medium was replaced with fresh medium and the cells observed live using a confocal microscope.

To study the effect of proton pump inhibition, fibroblasts grown on glass coverslips were incubated for 1 h in Bafilomycin A1 100 nM (Calbiochem) diluted in pre-warmed complete DMEM.

**Results**

**Unexplained hepatopathy with abnormal plasma protein glycosylation**

Index patient 1 (P1) is a 26-year old man whose newborn period and infancy were uneventful. During routine blood tests at the age of 3 years, he was found to have elevated alanine transaminase (ALT) and aspartate transaminase (AST). At the age of 6 years, chronic hepatitis had developed with elevated transaminases and high cholesterol (321 mg/dl). Wilson disease (WD) was suspected because of low serum ceruloplasmin and copper, and slightly elevated urinary copper excretion (Table S1). Treatment with zinc was initiated, resulting in reduced levels of cholesterol. After two years, treatment was stopped due to occurrence of pallor, anemia and severe neutropenia, and cholesterol levels increased again. Because of continuing liver abnormalities, a liver biopsy was performed at the age of 12 years, which revealed a normal liver architecture without features of inflammation or fibrosis. However, microvesicular steatosis was found in 10-20 % of hepatocytes (Fig. S1 E), hepatocytes were irregularly shaped and PAS
staining showed positive areas of glycogen (Fig. S1 A-D). Ultrastructural investigations in hepatocytes revealed the presence of LDs inside autolysosomes or lysosome-like structures, abundant glycogen, autophagic vacuoles and multilamellar structures similar to Niemann-Pick disease type C (NPC) (24) (Fig. 1 B, D). In the majority of the cells, the Golgi appeared dilated and occasionally fragmented, with accumulation of electron-dense material within both cisterns and vesicles (Fig. 1 A, B). Kupffer cells contained numerous lysosomes filled with electron-dense material, an expanded Golgi and large multivesicular bodies in the cytoplasm (Fig. 1 C, D). Since WD and other causes for the unexplained hepatitis, such as hemochromatosis, viral hepatitis, autoimmune hepatitis and alpha1-antitrypsin deficiency were excluded, biochemical diagnostics for metabolic causes of liver abnormalities was initiated. Routine metabolic tests were normal, including amino acids, organic acids and bile acids. However, screening for congenital disorders of glycosylation (CDG) as part of the routine metabolic investigations was abnormal. Analysis of N-glycosylation by isofocusing of plasma transferring revealed a Golgi glycosylation disorder with increased di- and trisialotransferrin (Fig. 3 A). In addition, analysis of mucin O-glycosylation by isofocusing of apolipoprotein CIII (ApoCIII) was altered, showing reduced Golgi sialic acid incorporation by increased ApoCIII-1 and decreased ApoCIII-2 isoforms (Fig. 3 A).

**Identification of variants in X-linked VMA21 causative for a novel CDG**

To identify genetic variants underlying the hepatopathy with abnormal glycosylation, whole exome sequencing (WES) was performed on P1. Genetic variants were ordered using a previously described prioritization scheme (25), by which VMA21 was identified as candidate gene. VMA21 is located on ChrXq28 and comprises three exons. The mutation c.188A>G, which is present in heterozygous form in the mother and absent in the father, results in a substitution of asparagine at position 63 for glycine (p.Asn63Gly) (Fig. 2 A-C, Table S2 and S3). p.Asn63Gly is localized in the luminal loop region of the protein just prior to the second predicted transmembrane region.

To identify additional patients with VMA21 variants, we selected a subgroup of undiagnosed male patients with a combination of liver symptoms and positive CDG screening results indicative of a Golgi glycosylation disorder. Sanger sequencing of VMA21 in this patient cohort resulted in the identification of genetic variants in VMA21 in
two patients (P2 and P3). P2 showed a hemizygous variant in the 5’ UTR of VMA21 (c.-10C>T), which was detected as heterozygous in the mother and was absent in the father, in agreement with X-linked recessive inheritance (Fig. 2 A). The c.-10C>T mutation results in a new ATG initiation codon, 11 bp upstream of the first exon, that could lead to a frameshift and a premature stop codon at position c.26-28 in the protein. P3 showed a hemizygous variant resulting in a predicted missense substitution of arginine for glycine (p.Arg18Gly). Analysis of cDNA showed the formation of a predominant alternative transcript, generated by disruption of the 5’ splice donor site and use of a cryptic splice site, which resulted in the inclusion of 93 base pairs of intronic sequence. On the protein level, the new splice variant results in a premature stop codon after the arginine to glycine substitution (p.Arg18Gly*) (Fig. S2 A-B).

With respect to the hepatic symptoms, CDG-P2 and -P3 showed mild signs of cholestasis which improved over time, chronic elevation of transaminases [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], elevated gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) (Table S1). Synthetic liver function was normal in all patients, and no evidence for other causes of hepatitis such as autoimmune hepatitis, hemochromatosis and viral hepatitis could be found. As in CDG-P1, elevated total cholesterol with increased low density lipoprotein (LDL) cholesterol was observed repeatedly for CDG-P3, which was accompanied by elevated secretion of apolipoprotein B (apoB) (Table S4). Cholesterol levels were normal for CDG-P2. Additional clinical signs and symptoms of patients P2 and P3, as well as all hepatic laboratory values are reported in Supplementary case reports and Table S1.

With respect to glycosylation of plasma proteins, CDG-P2 and -P3 showed glycosylation abnormalities that were highly similar to CDG-P1. TIEF of all three cases showed an accumulation of undersialylated isoforms, while apoCIII-IEF showed undersialylation with a slight decrease of apoCIII-2 and increase of apoCIII-1 isoforms (Fig. 3 A). To obtain more insight into the glycosylation abnormalities, we performed high resolution QTOF mass spectrometry analysis of hepatocyte-derived plasma transferrin and MALDI-TOF analysis of total plasma derived N-glycans. All three CDG patients showed transferrin isoforms with an increase of truncated glycans lacking sialic acid and/or galactose (peaks 2-7, Fig. 3 B, Fig. S3, Table S5). Similar abnormalities were seen in the total plasma derived N-glycans of the three CDG patients with a marked accumulation of a truncated
glycan lacking both galactose and sialic acid at m/z 2227 (Fig. 3 C, Fig. S4). Comparison with the other V-ATPase assembly defects ATP6AP1-, ATP6AP2-, CCDC115-, and TMEM199-CDG reveals highly similar abnormalities of a combined N- and O-glycosylation disorder and the presence of truncated glycans lacking galactose and sialic acid. Together, these results indicate VMA21 variants as the underlying cause of a novel CDG with hepatopathy with increased transaminases and LDL cholesterol as more common/subclinical features.

**Comparison of VMA21-CDG and VMA21-XMEA variants**

Previously, VMA21 deficiency has only been associated with X-linked Myopathy with Excessive Autophagy (XMEA) (14). Nevertheless, increased transaminases have been reported as incidental finding (16,20), and, recently, a previously described mutation has been reported in a patient with hepatic failure of unknown cause (26). Therefore, we studied liver parameters of an XMEA patient (XMEA-P1), previously reported with a vacuolar myopathy due to a p.Gly91Ala missense substitution in VMA21 (16). We found normal transaminases [ALT 54 (ref. 10-70 U/L), AST 35 (ref. 15-45 U/L)], increased GGT [181 (ref. 15-115 U/L)], high cholesterol [5.8 (ref. <6 mmol/L)] with increased LDL cholesterol [4.1 (ref. <3.0 mmol/L)] and low normal ceruloplasmin [206 (ref. 160-450 mg/L)]. Creatine kinase (CK) was elevated as reported [593 (ref. 40-280 U/L)] (Table S1). Together with the finding of hypercholesterolemia and increased transaminases in the three CDG patients, this indicates that the liver could be more frequently involved in VMA21 deficiency and that this clinical phenotype is more similar to the one of other V-ATPase assembly defects (11-13). By contrast, TIEF and apoCIII-IEF results for three XMEA patients (14), XMEA-P1, XMEA-P2 (c.*6A>G), and XMEA-P3 (c.164-7T>G) were normal. Similarly, more detailed studies on protein glycosylation by high resolution QTOF mass spectrometry analysis of transferrin and MALDI-TOF analysis of total plasma derived N-glycans (Fig. 3 B, C, Fig. S3, Fig. S4, Table S5) were normal for the three XMEA patients.

**VMA21 variants are hypomorphic and reduce protein expression**

To functionally validate the VMA21 variants, we employed CDG fibroblasts and compared them to fibroblasts from a healthy control and fibroblasts from XMEA patient 1.
(XMEA-P1). Strongly reduced VMA21 protein expression was found for CDG-P2 (c.10C>T) and CDG-P3 (p.Arg18Gly), while CDG-P1 (p.Asp63Gly) and XMEA-P1 showed a milder reduction (Fig. 4 A). Interestingly, analysis of mRNA expression by qPCR showed strongly reduced levels for all CDG patients, suggesting mRNA instability as a cause for reduced protein levels (Fig. 4 B). In contrast, although the c.272C>G (XMEA) mutation was reported to abolish a predicted splice enhancer site, it only slightly reduced mRNA expression in fibroblasts (Fig. 4 B).

**VMA21 variants impair V-ATPase assembly**

To study the impact of VMA21 variants on the assembly of the V-ATPase V₀ and V₁ domains, western blot analysis was performed. As expected, steady state levels of V₁ subunits ATP6V1D1 and ATP6V1B1/2 in total cell extracts remained unaffected in control, CDG and XMEA fibroblasts, confirming that the assembly of the V₁ domain, which occurs independently in the cytosol (14), is not impaired by mutations in the ER assembly factor VMA21 (Fig. 4 C). By contrast, expression of the V₀ subunits ATP6V0D1 and ATP6V0C was reduced in fibroblasts from CDG and XMEA patients, indicating impaired V₀ assembly in the ER, thus leading to an overall defect in V-ATPase assembly (Fig. 4 D).

Although any reduction in expression levels is sufficient to explain reduced assembly, we tested by overexpression whether the mutated proteins could have decreased interactions with V-ATPase components. Upon transient transfection of Myc-tagged VMA21R18G, VMA21D63G and VMA21G91A in HEK293T cells, the levels of the three overexpressed proteins were comparable to VMA21WT (Fig. S5 A, B). Yet, they showed reduced interaction with the assembly factor ATP6AP2 and V₀ subunit ATP6V0C (Fig. S5 C, D). This suggests that in addition to the lower expression levels, the missense mutations might also impair protein interactions of VMA21, interfering with proper assembly of the V-ATPase.

**VMA21 variants cause reduced proton pump function**

The function of VMA21 is best understood in yeast cells (2). VMA21 and yeast Vma21p share 30 % similarity, although VMA21 lacks the C-terminal dilysine motif necessary for ER retrieval (25) (Fig. 2 B, C). To study the effect of the identified variants on yeast V-
ATPase function, we used an established yeast assay for proton pump activity that is based on the dependence of pump activity for survival and growth in the presence of elevated divalent cations, such as zinc (2,27–29) (Fig. 5 A). Previously, it has been shown that strains lacking a functional Vma21p are unable to grow in these non-permissive conditions (30). Therefore, the human sequences carrying the appropriate amino acid changes were introduced by homologous recombination into the yeast genomic locus. While the expression of VMA21<sup>WT</sup> rescued the yeast growth under elevated zinc conditions (Fig. 5 A), CDG and XMEA variants impaired this rescue (Fig. 5 A).

To study proton pump dysfunction in patient fibroblasts, we used LysoSensor, a dye able to emit fluorescence only inside acidic cellular compartments and which fluorescence’s intensity has an inverse correlation with the pH-value (31), and Lysotracker, a dye able to label acidic compartments. Live fibroblasts from both CDG and XMEA-P1 showed a strong reduction in number and intensity of both LysoSensor (Fig. 5 B, Fig. S6 A) and LysoTracker-positive punctae compared with control fibroblasts (Fig. 5 C, Fig. S6 B). Taken together, these results indicate that human VMA21 variants reduce V-ATPase function.

**VMA21 deficiency leads to autophagic defects**

To understand if impaired organellar acidification results in decreased lysosomal degradation, we tested lysosomal Cathepsin B (CTSB) activity (31,32) using a cresyl violet fluorophore fused to a peptide sequence that can be cleaved by CTSB. By live cell imaging, we observed a strong reduction in CTSB activity for all CDG and XMEA-P1 cells (Fig. 5 D, Fig. S6 C). Use of V-ATPase inhibitor Bafilomycin A1 as positive control showed a similar reduction (Fig. 5 D, Fig. S6 C). We next tested whether reduced lysosomal acidification and degradation also affected lysosomal morphology. Immunofluorescence showed a strongly increased overall intensity of the lysosomal marker LAMP1 in VMA21-deficient fibroblasts (Fig. 6 A, Fig. S7 A). LAMP1-positive vesicles also appeared to be enlarged suggesting impairment in the turnover of these organelles (Fig. 6 A, Fig. S7 A). This was supported by the increased levels of LAMP1 in western blotting of fibroblasts from CDG and XMEA-P1 patients (Fig. 6 B, Fig. S7 B).
With regard to the autophagy pathway, we found an accumulation of the autophagosome-associated lipidated form of LC3 (LC3-II) for the CDG and XMEA-P1 patients (Fig. 6 C, Fig. S7 C). Furthermore, we found an accumulation of the autophagosomal substrate p62, suggesting decreased autophagic degradation due to a block in autophagic flux (Fig. 6 B, Fig. S7 B).

LAMP1 punctae showed an extensive co-localization with LC3 in both control and patients fibroblasts, indicating that autophagosomes were able to fuse with ATPase-deficient lysosomes (Fig. 6 D, Fig. S7 D, Fig. S8 G). Similar to studies in Drosophila fat bodies (48), the accumulation of non-functional lysosomes led to a block in autophagic flux and the formation of giant autolysosomes (Fig. 6 A, Fig. S7 A, Fig. S8 B). Importantly, siRNA-mediated knock-down of VMA21 in human Huh7 hepatocytes was able to recapitulate the LAMP1, p62 and LC3 accumulation, confirming the hypomorphic effects of the VMA21 variants on the lysosomal-autophagic pathway (Fig. S8 A-D).

**VMA21 variants lead to ER stress and lipid abnormalities in patient fibroblasts**

Because of the steatotic liver phenotypes and accumulation of intracellular LDs observed in patient hepatocytes, we next tested whether impaired lysosomal degradation could be the cause of LD accumulation. LDs are cytoplasmic organelles that have a core of triglycerides and cholesterol surrounded by a single layer of phospholipids (34). LDs are formed in the ER, where triglycerides are synthesized from fatty acids (35). LD degradation can either occur via cytoplasmic lipases (36) or in the context of autophagy (lipophagy) (37).

The number and size of LDs was assessed by BODIPY staining in patient and control fibroblasts. CDG and XMEA cells showed a clear accumulation of LDs compared to the control fibroblasts (Fig. 7 A, Fig. S9 A, Fig. S8 F). Importantly, treatment with Bafilomycin A1 increased LD number in control fibroblasts (Fig. 7 A, Fig. S9 A, Fig. S8 F), confirming that lysosomal acidification is key to LD turnover. By contrast, Bafilomycin A1 treatment in patient fibroblasts did not further decrease LD number, suggesting that in these cells an additional suppression of acidification-dependent LD turnover may not be possible. Interestingly, the increase in LDs was also accompanied by an increase of total triglycerides (TAGs) in patient cells (Fig. 7 C, Fig. S9 C).
In the lysosomal storage disease Niemann-Pick C, cholesterol accumulates inside the lysosomes due to defective extraction via the NPC1 and NPC2 proteins (38). As lysosomal cholesterol extraction occurs in a pH-dependent manner (39), we tested whether the hypercholesterolemia observed in the CDG patients could also be linked to decreased lysosomal acidification. Therefore, we labelled unesterified cholesterol with the Filipin dye. As expected, the staining revealed an accumulation of cholesterol in vesicular structures of CDG fibroblasts compared to control cells, while the XMEA fibroblasts showed only a small tendency to accumulate cholesterol (Fig. 7 B, Fig. S9 B, Fig. S8 E). Similar to the LDs, pre-treatment of the cells with the autophagy inhibitor Bafilomycin A1 led to accumulation of cholesterol in both control and XMEA fibroblasts but not in CDG cells (Fig. 7 B, Fig. S9 B, Fig. S8 E).

When cholesterol is sequestered in lysosomes, the cholesterol content in the ER may be lower, which in turn can trigger lipogenic pathways by cleavage of the sterol response element-binding protein-1 (SREBP1) (38,40). To test whether such de novo lipogenic pathways could be responsible for the observed hypercholesterolemia in the VMA21 patients, SREBP1 activation and cleavage was analysed by western blotting. This revealed an increased amount of mature SREBP1 in CDG but not in control and XMEA fibroblasts (Fig. 8 A, Fig. S10 A). Taken together, this suggests that VMA21 deficiency leads to LD and cholesterol accumulation inside lysosomal-like structures, contributing to elevated plasma (LDL) cholesterol by increasing de novo SREBP1-mediated lipogenesis.

**CDG mutations cause PERK-mediated ER stress**

As defective V₀ assembly has also been associated with ER stress, which is related to LD formation in hepatocytes (9, 45), we studied ER stress in CDG cells. We tested all three unfolded protein response (UPR) stress pathways (Fig. S11 A-E), but only found a strong activation of the PKR-like ER kinase (PERK) branch in the CDG but not in control or XMEA fibroblasts. This was demonstrated by the autophosphorylation of PERK on Thr980 (Fig. 8 B, Fig. S10 B). PERK activation led to the upregulation of several UPR target genes including the activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP) and the Glucose Regulated Protein 94 (GRP94) as demonstrated by qPCR and immunostaining (Fig. 8 C-E, Fig. S10 C-E). Together, these results suggest
that ER stress due to V-ATPase misassembly may contribute to the steatotic phenotype in the patients.

**Discussion**

Here, we identified deficiency of V-ATPase assembly factor VMA21 leading to a heterogeneous clinical spectrum with hepatic features of chronic hypertransaminasemia and mild hyperlipidemia with increased LDL cholesterol. All individuals displayed abnormal glycosylation of hepatocyte-derived proteins, suggesting that the combination of liver symptoms and CDG should guide clinicians towards genetic testing of VMA21 and other V-ATPase assembly factors.

Our functional studies demonstrated that both VMA21-CDG and VMA21-XMEA variants are hypomorphic mutations lowering mRNA and protein levels. As a consequence of VMA21 reduction, there was an impairment of V-ATPase assembly, marked by reduced V₀ subunit expression and reduced interaction with V₀ subunit themselves and the ER assembly factor ATP6AP2, that led to reduced V-ATPase activity. This in turn caused reduced lysosomal acidification and protease activation as well as the inability to execute the final steps of the (auto)-lysosomal degradation pathway. In fibroblasts, the impaired lysosomal acidification led to defective lipophagy, consistent with the presence of enlarged LD-containing autolysosomes in the hepatocytes of the liver biopsy of CDG-P1. The observed cholesterol accumulation in patient fibroblasts may have been caused by reduced lysosomal acidification. Interestingly, it has been demonstrated that the activity of lysosomal protease CTSD in the lysosomal storage disorder NPC can be impaired by both altered lysosomal pH and the accumulation of lipid material inside lysosomes (42). In analogy, the accumulated cholesterol in VMA21-deficient cells may impair lysosomal function. As we additionally observed an increased cleavage of SREBP1, it can be hypothesized that the failure to properly extract cholesterol from the lysosomes leads to an overall decline of cellular cholesterol, which in turn triggers SREBP1 cleavage and *de novo* production of cholesterol (39). As newly synthesized cholesterol can directly be secreted into the serum together with lipoprotein particles, the hypercholesterolemia observed in the patients may be a result of SREBP1 activation and *de novo* cholesterol synthesis. Interestingly, the PERK branch of the UPR, which was shown to be activated in the CDG cells, is also able to activate SREBP1-mediated *de novo* lipogenesis (43),
suggesting that the ER stress responses might further contribute to cholesterol accumulation. This could also be the case for the LD accumulation, as increased LD formation has been linked to ER stress in many instances, particularly in hepatocytes (44).

What causes the ER stress in VMA21-deficient cells is unclear. Lack of ATP6AP2, another ER assembly factor, has previously been associated with ER stress (9), suggesting that it may be directly related to the defective V₀ assembly (25, 46). However, in plants it has also been shown that VMA21 interacts with and helps Erv41p-Erv46p (45), an early secretory-localized complex involved in the protein loading into COPII vesicles for transport to the Golgi. Lack of Vma21p in ∆Vma21p strains resulted in a complete absence of the v-SNARE Bos1 in the COPII vesicles (45), suggesting an impaired ER-to-Golgi transport also in CDG patient fibroblasts. Likewise, because of the involvement of Vma21p in retrograde transport back to the ER, disturbance of ER and Golgi homeostasis might also stem from defects in this trafficking pathway.

Although the phenotypes of XMEA and CDG patients are different (14, 15), we found found mild differences in patient fibroblasts. Both CDG and XMEA variants showed reduced protein expression, V-ATPase misassembly and dysfunction, while some differences were seen with regard to ER stress and cholesterol impairment. Given that liver involvement has already been described in XMEA patients, ranging from increased liver transaminases to fatal hepatic failure (16, 20, 26), while CK elevation could be observed in one of the CDG patients (P2), it seems clear that more patients are needed to fully appreciate the phenotypic spectrum of VMA21 deficiency. The elevated LDL cholesterol levels in XMEA-P1 suggests further mechanistic overlap between the CDG and XMEA variants. Nevertheless, glycosylation abnormalities were not detected in XMEA patients, and the CDG variants had a stronger impact on VMA21 expression and PERK phosphorylation than the XMEA mutation tested. Therefore, it could be that stronger reduction of VMA21 correlates with a stronger effect on ER stress and glycosylation and that these processes seem to be more sensitive to VMA21 protein dosage compared to the lysosomal acidification. Tissue-specific expression of modulator genes, alternative transcripts of VMA21 or differential expression levels of V-ATPase assembly factors need to be investigated to further explain the tissue-specific clinical differences between the patient groups.
VMA21 deficiency is the last of five known human V-ATPase assembly factors associated with hepatic steatosis (8,11–13). In some cases, this new disease class progresses to severe fatty liver disease. Our findings of defective lipophagy as a mechanism underlying LD accumulation are therefore of broader relevance to understand the pathophysiology of hepatic steatosis in the other V-ATPase assembly defects, but also bear significant relevance to understand the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (48). As LD accumulation is a hallmark of NAFLD and autophagy has been implicated in disease pathology and as being a treatment target for NAFLD, understanding the mechanisms underlying defective lipophagy is important.

In summary, we identify three individuals with a CDG featured by autophagic defects and ER stress caused by different variants in VMA21. Our data suggest X-linked VMA21 deficiency as a novel cause of mild chronic liver disease. It is thus advised that patients with signs of steatohepatitis and chronically elevated transaminases are tested for lipid profiles and plasma protein glycosylation, followed by genetic testing of the V-ATPase assembly factors. A better understanding of how ER stress and autophagic defects contribute to liver disease in these rare diseases may aid the development of new therapeutic strategies for NAFLD. Therefore, our work underscores the notion that insights from rare genetic diseases can hold important lessons for common diseases and indicate treatment targets for both.

References


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Figure Legends

Fig. 1. Ultrastructure of hepatocytes and Kupffer cells of a liver biopsy of CDG-P1. (A-B) Hepatocytes showing dilated Golgi (g), autolysosomes (a), lysosome-like structures (L) or multilamellar bodies (MVB) and lipolysosomes (LP) with incorporated bubbles containing fibro-granular material in the bile canalicular zone (bc); (C-D) Kupffer cells with numerous deposits in form of lysosomes-like (L) or multilamellar bodies (MVB).

Fig. 2. Identification of missense mutations in \textit{VMA21}. (A) Pedigree and segregation status of \textit{VMA21} variants in families F1, F2 and F3. Partial chromatograms show X-linked segregation for all patients. On the left side, family F1 shows the segregation of the missense mutation (c.188A>G) (F1-II-2). In the middle, the pedigree for family F2 shows the segregation of the pathogenic mutation in the 5’ UTR of \textit{VMA21} (c.-10C>T) identified in patient P2 (F2-II-1). On the right side, the pedigree for family F3 shows the segregation of the missense mutation (c.52A>G) to patient P3 (F3-II-3). (B) Exon structure of human \textit{VMA21} (in yellow) and the domain structure of the encoded protein (in white and grey boxes), the numbers delimiting the different domains refer to amino acids positions. The blue dotted lines indicate the positions of the missense variants within the families, both at the nucleotide and the
protein level (blue boxes). (C) The sequence alignment shows the conservation of the affected amino acids of VMA21 in Homo sapiens, Mus musculus, Xenopus laevis, S. cerevisiae and D. melanogaster, respectively. The ER retrieval motif KKXX is present only in S. cerevisiae.

**Fig. 3. CDG screening and glycosylation analysis.**
(A) CDG screening results of CDG and XMEA patients and a control (C). Transferrin isofocusing (TIEF, left) was used for assaying N-glycosylation and apolipoprotein CIII isofocusing (apoCIII, right) was used for assaying mucin type O-glycosylation. The accompanying numbers represent the total number of sialic acids in the different protein isoforms. (B) Nanochip-C8-QTOF mass spectra of transferrin isolated from CDG-P1/2/3 and XMEA-P1/2/3 patients and a healthy control. Peak 1 (79556 amu) is the intact transferrin protein with two attached complete glycans. Any subsequent loss of sialic acid and/or galactose was calculated based on the mass difference with the main peak (e.g., loss of one sialic acid (purple diamond, peak 2)). Transferrin glycoforms of the individual peaks are indicated below the profiles. Relative levels are presented in Table S5. Double peaks for CDG-P1 and XMEA-P3 indicate the presence of a polymorphism in the transferrin protein. (C) Representative MALDI-TOF spectra of plasma derived N-glycans of CDG-P1 and XMEA-P1. Spectra of all patients and a control are shown in Fig. S4.

**Fig. 4. Characterization of the VMA21 variants.**
(A) Western blot showing the endogenous level of VMA21 in control and patients fibroblasts using anti-VMA21 antibody. Results were normalized to the loading control β-Actin. The results were calculated on three independent experiments. (B) VMA21 mRNA quantification by qPCR in control and patients fibroblasts. The results were calculated on three independent experiments. (C) Western blot showing the steady state levels of the V₁ subunits in control and patients fibroblasts using anti-ATP6V1B1/2 and anti-ATP6V1D1 antibodies. Results were normalized to the loading control β-Actin. The results were calculated on five and four independent experiments, respectively. (D) Western blot showing the steady state levels of the V₀ subunits in control and patients fibroblasts using anti-ATP6V0D1 and anti-ATP6V0C antibodies. Results were normalized to the loading control β-Actin. The results were calculated on
five and three independent experiments, respectively. All data are mean ± SEM, statistical significance was determined by an ordinary one-way ANOVA followed by a Bonferroni multiple comparisons test. Levels of significance: ns, not significant, *, P < 0.05, **P < 0.01, ****, P < 0.0001.

**Fig. 5. VMA21 variants cause reduced proton pump function.**
(A) V-ATPase dependent growth tests on permissive (pH 5) or non-permissive (4mM or 5 mM ZnCl2) conditions of strains expressing VMA21WT, VMA21R18G, VMA21D63G and VMA21G91A compared to Vma21p and ΔVma21p. (B) LysoSensor Green DND-189 staining of patients and control fibroblasts. Results were calculated on two independent experiments with >30 cells/genotype. Scale bars: 20 µm. (C) Lysotracker staining of patients and control fibroblasts. Results were calculated on two independent experiments with >10 cells/genotype. Scale bars: 20 µm. (D) Magic Red staining for Cathepsin B activity of control and patient fibroblasts, in untreated conditions or upon 1h of BafilomycinA1 100 nM treatment. Results were calculated on three independent experiments with >30 cells/genotype. Scale bars: 15 µm. All data are mean ± SEM, statistical significance was determined by an ordinary one-way ANOVA followed by a Bonferroni multiple comparisons test. Levels of significance: ns, not significant, *, P < 0.05, **P < 0.01, ***, P < 0.001, ****, P < 0.0001.

**Fig. 6. VMA21 deficiency leads to autophagic defects.**
(A) Immunofluorescence analyses of control and patient fibroblasts showing LAMP1-positive vesicles. Scale bars: 20 µm. Lower panels are magnifications of the insets demarked in upper panels. Scale bars: 10 µm. (B, C) Western blot of LAMP1, p62 and LC3 in control and patients fibroblasts. Results were normalized to the loading control β-Actin. The results were calculated on three, two and two independent experiments, respectively. (D) LAMP1 and LC3 staining in control and patients fibroblasts. Scale bars: 20 µm. Lower panels are magnifications of the insets demarked in upper panels. Scale bars: 20 µm. The analysis of the LAMP1/LC3 co-localization was performed by using the JACoP plug-in (ImageJ) and quantified by using the Pearson’s co-localization coefficient. The co-localization between LAMP1 and LC3 was calculated on three independent
experiments with 15 cells/genotype. All data are mean ± SEM, statistical significance was determined by an ordinary one-way ANOVA followed by a Bonferroni multiple comparisons test. Levels of significance: ns, not significant, *, P < 0.05, **P < 0.01.

**Fig. 7. VMA21 mutations cause impaired lipid metabolism.**

(A) Bodipy staining of LDs in control and patients fibroblasts. The staining was performed in untreated samples or upon 1h 100 nM Bafilomycin A1. The results were calculated on three independent experiments with >10 cells/genotype. Scale bars: 15 µm. (B) Immunolabelling of unesterified cholesterol by Filipin staining in control and patient fibroblasts. The intensity of the staining was calculated on three independent experiments with >10 cells/genotype. Scale bars: 20 µm. (C) Total TAG levels assayed enzymatically in control and patients fibroblasts. The results were calculated on three independent experiments. All data are mean ± SEM, statistical significance was determined by an ordinary one-way ANOVA followed by a Bonferroni multiple comparisons test. Levels of significance: ns, not significant, *, P < 0.05, **, P < 0.001. ****, P < 0.0001.

**Fig. 8. Characterization of the lipid abnormalities in patient fibroblasts.**

(A) Western blot on control and patient fibroblasts showing the full-length (precursor) and the cleaved form (mature) of SREBP1. Results were normalized to the loading control α-Tubulin. The results are representative of three independent experiments. (B) Western blot of total and phosphorylated (Thr980) PERK in control and patients fibroblasts. Results were normalized to the loading control β-Actin. The results were calculated on three independent experiments. (C) ATF4 and CHOP mRNA quantification in control and patient fibroblasts. The results are representative of three independent experiments. (D) Immunofluorescence analyses of control and patient fibroblasts showing GRP94 staining in patients and control fibroblasts. The results are representative of three independent experiments. Scale bars: 15 µm. (E) GRP94 mRNA quantification in control and patient fibroblasts. The results were calculated on three independent experiments. All data are mean ± SEM, statistical significance was determined by an ordinary one-way ANOVA followed by a Bonferroni multiple comparisons test. Levels of significance: ns, not significant, *, P < 0.05, **P < 0.01, ****, P < 0.0001.
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Author contributions
All authors contributed to parts of the paper on their specific expertise and critically reviewed the paper.
Figure 2

A

B

C

H. sapiens
M. musculus
S. cerevisae
X. laevis
D. melanogaster

1
1
1
1
1

39
39
22
42
43

82
82
55
85
87

101
101
77
104
105

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Figure 3

A

C

CDG

XMEA

TIEF

2

1

0

apoCIII

XMEA-P1

XMEA-P2

XMEA-P3

CDG-P1

CDG-P2

CDG-P3

Control

B

CDG-P1

CDG-P2

CDG-P3

XMEA-P1

XMEA-P2

XMEA-P3

C

CDG-P1

CDG-P2

CDG-P3

XMEA-P1

GlcNAc Man Gal Neu5Ac Fuc

1500 2000 2500 3000 1500 2000 2500 3000
Figure 4

A  Fibroblasts

<table>
<thead>
<tr>
<th>VMA21 expression levels (%)</th>
<th>ATP6V1B1/2 expression levels (%)</th>
<th>ATP6V0D1 expression levels (%)</th>
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<tr>
<td>control</td>
<td>-10C&gt;T</td>
<td>R18G</td>
</tr>
<tr>
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<td>D63G</td>
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<tr>
<td>XMEA</td>
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B  VMA21 mRNA expression levels (%)

<table>
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<tr>
<th>VMA21 mRNA expression levels (%)</th>
<th>ATP6V1d1 expression levels (%)</th>
<th>ATP6V0C expression levels (%)</th>
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<tr>
<td>control</td>
<td>-10C&gt;T</td>
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<tr>
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<tr>
<td>XMEA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Figure 5

A

Vma21p
VMA21
VMA21D63G
VMA21R18G
VMA1G91A
VMA21∆

pH 5
5 mM ZnCl₂

B

control
-10C>T
XMEA

LysoSensor
20 μM

C

control
-10C>T
XMEA

LysoTracker
20 μM

D

control
-10C>T
XMEA

Cathpsin B
Magic Red
15 μM

Graphs:
Intensity LysoSensor
Intensity LysoTracker
Cathpsin B Activity

ns
+Baf A1

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Figure 7

A

control BODIPY Hoechst -10C>T XMEA

+Baf A1

control BODIPY Hoechst -10C>T XMEA

+Baf A1

Filipin intensity

Normalized TAGs

1.0

0.5

0.0

Volume of LDs

Number of LDs / cell surface

8

6

4

2

0

0.4

0.3

0.2

0.1

0.0

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Figure 8

A

SREBP1 Precursor
130
100
75
55

SREBP1 Mature

α-Tubulin

control -10C>T XMEA

B

pPERK (Thr980)
250
130

Tot PERK

β-Actin

control -10C>T XMEA

C

ATF4 mRNA expression levels (%)

control -10C>T XMEA

CHOP mRNA expression levels (%)

control -10C>T XMEA

D

GRP94 mRNA expression levels (%)

control -10C>T XMEA

E

Intensity GRP94

control -10C>T XMEA

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