Biallelic variants in TAMM41 are associated with low muscle cardiolipin levels, leading to neonatal mitochondrial disease

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Summary

Mitochondrial membranes have a unique lipid composition, with low phospholipid to protein and sterol to protein ratios compared with other subcellular fractions and a high cardiolipin (CL) content in the range of 10%–15%.1 Mitochondria are able to synthesize some of their own lipids, phosphatidyllethanolamine (PE), phosphatidic acid (PA), phosphatidylglycerol (PG), and CL. CL is an acidic mitochondrial lipid that consists of three glycerol molecules bridged by two phosphates where the outer two glycerol moieties carry a total of four fatty-acid side chains. One of the key functions of CL is the mediation of protein interactions in the inner mitochondrial membrane (IMM), which in turn influences a diverse range of central processes, such as respiratory chain biogenesis, stabilization of respiratory chain supercomplexes, mitochondrial dynamics, iron-sulfur cluster (Fe-S) biogenesis, and mitophagy. CL also is involved in the mitochondrial phospholipid biosynthesis and modification and that its deficiency results in a mitochondrial disorder, though unlike families with pathogenic AGK (Sengers syndrome) and TAFAZZIN (Barth syndrome) variants, there was no evidence of cardiomyopathy.

MITochondrial disorders are clinically and genetically heterogeneous, with variants in mitochondrial or nuclear genes leading to varied clinical phenotypes. TAMM41 encodes a mitochondrial protein with cytidine diphosphate-diacylglycerol synthase activity: an essential early step in the biosynthesis of phosphatidylglycerol and cardiolipin. Cardiolipin is a mitochondria-specific phospholipid that is important for many mitochondrial processes. We report three unrelated individuals with mitochondrial disease that share clinical features, including lethargy at birth, hypotonia, developmental delay, myopathy, and ptosis. Whole exome and genome sequencing identified compound heterozygous variants in TAMM41 in each proband. Western blot analysis in fibroblasts showed a mild oxidative phosphorylation (OXPHOS) defect in only one of the three affected individuals. In skeletal muscle samples, however, there was severe loss of subunits of complexes I–IV and a decrease in fully assembled OXPHOS complexes I–V in two subjects as well as decreased TAMM41 protein levels. Similar to the tissue-specific observations on OXPHOS, cardiolipin levels were unchanged in subject fibroblasts but significantly decreased in the skeletal muscle of affected individuals. To assess the functional impact of the TAMM41 missense variants, the equivalent mutations were modeled in yeast. All three mutants failed to rescue the growth defect of the Δtam41 strains on non-fermentable (respiratory) medium compared with wild-type TAMM41, confirming the pathogenicity of the variants. We establish that TAMM41 is an additional gene involved in mitochondrial phospholipid biosynthesis and modification and that its deficiency results in a mitochondrial disorder, though unlike families with pathogenic AGK (Sengers syndrome) and TAFAZZIN (Barth syndrome) variants, there was no evidence of cardiomyopathy.

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been found to mediate the association of the TIM23 complex with its integral membrane subunit, TIMM50. 2

CL is synthesized on the matrix side of the IMM. TAMM41 (MIM: 614948; mitochondrial translocator assembly and maintenance homolog 41) encodes a phosphatidate cytididylyltransferase (CDP-DAG synthase), the first mitochondrial enzyme of CL biosynthesis pathway, that converts PA and cytidine triphosphate (CTP) into cytidine diphosphate (CDP)-diacylglycerol (CDP-DAG).3 CDP-DAG is then converted to phosphatidylglycerol phosphate, which is dephosphorylated to form PG. CL synthase then condenses PG and CDP-DAG to form CL. A CL phospholipase and the transacylase, tafazzin, are subsequently required to produce the mature fatty acid composition of CL via deacylation and reacylation remodeling.4

In humans, the only genetic disease related to a CL biosynthesis and remodeling defect is Barth syndrome (MIM: 302060), caused by variants in TAFAZZIN (MIM: 300394), which encodes tafazzin.5 Barth syndrome, an X-linked disease, is clinically characterized by cardioklental myopathy and neutropenia associated with mitochondrial dysfunction.6 Tafazzin transfers acyl chains from various phospholipids to monolysop-CL (MLCL) via transacylation, and deleterious variants in TAFAZZIN are associated with a CL profile that contains more saturated acyl chains and increased MLCL levels.7 Here, we describe the identification by whole-exome sequencing (WES) and whole-genome sequencing (WGS) of a novel cause of CL biosynthesis defect related to variants in TAMM41 in three unrelated families of various ethnic origins.

Our study adhered to the Declaration of Helsinki and was approved by the Institutional Review Boards at each site. Written informed consent was obtained from the respective parents of each subject. The three independent subjects were matched using the online GeneMatcher platform.8

Subject 1 is an 11-year-old girl born to non-consanguineous parents of European descent (birth weight: 2.8 kg). She presented with failure to thrive and lethargy from birth (Table 1). She was admitted to pediatric intensive care at 5 weeks due to aspiration pneumonia, requiring intubation and intravenous antibiotics followed by percutaneous endoscopic gastrostomy insertion for unsafe swallow. She exhibited development delay in her gross motor skills; she was sitting unsupported at the age of 5 years but remained unable to walk and was wheelchair bound. She has profound hypotonia and myopathy, which is worse in the proximal muscle groups of the neck, trunk, shoulders, and hips. She has chronic progressive external ophthalmoplegia (CPEO) and bilateral ptosis. She also exhibits mild dysmorphic facial features suggestive of myopathic disease, with a long narrow face and tented mouth. She also presented with recurrent infections that could be related to autosomal dominant chronic mucocutaneous candidiasis inherited from the mother; both subject 1 and her mother are heterozygous for a c.820C>T

Table 1. Clinical feature of subjects with biallelic TAMM41 variants

|                   | Subject 1 | Subject 2 | Subject 3 |
|-------------------|-----------|-----------|-----------|
| Sex               | F         | F         | F         |
| Age of onset      | at birth  | at birth  | at birth  |
| Onset symptom     | lethargy  | lethargy, hypotonia | lethargy |
| Age               | 7 years   | 8 years   | died at 3 months |
| Birth weight      | 2.8 kg    | 3.2 kg    | 3.4 kg    |
| Delivery          | 41 ÷ 4 weeks normal vaginal delivery | 39 weeks C-section for breech presentation | 39 weeks normal vaginal delivery |
| Motor skills      | rolling on back, head drop 6 months, unable to walk, wheelchair bound | head control at 3 months, sitting independently at 6 months, walked independently at 13 months | no contact (3 months) |
| Social and intellectual | normal | ADHD and dyslexia, in 6th grade with an individualized learning plan |
| Brain MRI         | normal at 2 years |

ADHD, attention deficit hyperactivity disorder.
Subject 2 is a now 12-year-old girl born to non-consanguineous parents of European descent at 39 weeks through normal vaginal delivery and weighed 3.4 kg (Table 1). She was lethargic from birth and developed bilateral ptosis and dysphagia in the 1st week of life. She was fed parenterally from 2 months old due to severe gastroesophageal reflux. She did not fix and follow objects with her eyes. She died from respiratory failure at the age of 3 months. She had normal blood lactate and urine organic acids. Enlarged mitochondria with irregular shape and increased lipid droplets were observed by electron microscopy (Figure S1). Respiratory chain enzyme analysis in skeletal muscle revealed decreased activities of complexes I, III, and IV, although these were normal in subject fibroblasts (Table 2). There was no evidence of mtDNA depletion in muscle.

Molecular genetic investigations were undertaken for all three subjects using EDTA-blood DNA samples. WES and WGS were performed on subjects 1–3 as family trio. WES (subjects 1 and 3) or WGS (subject 2) data were interpreted according to American College of Medical Genetics (ACMG) guidelines.9 The pathogenic variants were selected after filtering against known SNPs reported in dbSNP, 1000 Genomes, Exome Variant Server, in-house polymorphisms, and intergenic variants. This filtering resulted in the identification of compound heterozygous variants in TAMM41 (NM_001284401.1) in each affected individual. All variants were confirmed by Sanger sequencing and all variants segregated with disease in each family following a recessive pattern of inheritance.

Subject 1 was compound heterozygous for a c.410C>T (p.Pro137Leu) missense variant and a c.709–2A>G variant in intron 5 and is predicted to affect the acceptor splice site of exon 6 (Table 3). Subject 2 was compound heterozygous for c.256T>C (p.Ser86Pro) missense change and c.411+1G>T in intron 3, predicted to affect splicing of exon 3 (Table 3). Subject 3 was compound heterozygous
Table 3. Evidence of pathogenicity associated with human TAMM41 variants

| Nucleotide (NM_001284401.1) | S1 | S2 | S3 |
|-------------------------------|----|----|----|
| Nucleotide (genomic)          | c.410C>T | c.709>2A>G | c.411>1G>T | c.256T>C | c.329A>G | c.806dup |
| dbSNP                         | rs775491404 | rs934260435 | rs780204589 | rs199871047 | rs768826552 |
| Allele                        | maternal | maternal | paternal | maternal | paternal | maternal |
| Amino acid                    | p.Pro137Leu | p.Ser86Pro | p.Tyr110Cys | p.Asn269Lysfs*14 |
| CAAD                          | 33 | 34 | 34 | 13 | 28.2 | 24.7 |
| SIFT                          | 0 | 0.12 | 0 |
| PolyPhen                      | 1 | 0.02 | 1 |
| MutationTaster                | disease causing (p value: 1) | polymorphism (p value: 1) | disease causing (p value: 1) |
| gnomAD allele frequency (all) | G: 0.00001315 | G: 0.00003946 | T: 0 | G: 0.00006570 | T: 0 | AA: 0 |
| Splicing predictions          | MaxEnt: −100.0%; NNSPLICE: −100.0%; HSF: 100.0% | MaxEnt: −100.0%; NNSPLICE: −100.0%; HSF: −100.0% | MaxEnt: 0.0%; NNSPLICE: 0.0%; HSF: −7.9% |

for a c.329A>G (p.Tyr110Cys) missense change and a c.806dup (p.Asn269Lysfs*14) variant (Table 3). Tyr110Cys and Pro137Leu affect highly evolutionary conserved amino acids and are predicted to be deleterious, whereas Ser86Pro modified a weakly conserved amino acid (Figure 1A; Table 3). RT-PCR analysis of TAMM41 RNA extracted from cultured skin fibroblasts showed that the c.709>2A>G variation results in absence of exon 5 in subject 1 and that the c.411>1G>T variation of subject 2 induces absence of exon 3. In subject 3, c.806dup of maternal allele results in a frameshift. Moreover, this also showed in subjects 1 and 3 a higher proportion of the alleles carrying the missense variant, suggesting that c.709>2A>G and c.806dup, from subjects 1 and 3, respectively, result in unstable transcripts. Surprisingly, the paternal allele carrying the c.411>1G>T variation in subject 2 was proportionally higher than the maternal allele, suggesting that the c.256T>C variant induces instability of the transcript (Figure S2). The TAMM41 variants reported here have been submitted to the ClinVar database (ClinVar: SUB10296292).

To visualize the location of mutant amino acids, we used the AlphaFold machine learning algorithm that predicts protein structures and that has high confidence in the locations of the identified missense variants.\(^\text{10}\) In parallel, we also referred to the crystal structure of S. pombe TAMM41\(^\text{11}\) that has previously allowed to identify functional domains of the protein. The three affected amino acids are located in the NTase domain of the protein (Figure 1B). Tyr110 and Pro137 are adjacent to Lys109 and Lys136 CTP binding sites, respectively, and located in the CTP binding pocket formed by Arg133, Lys136, Thr199, and Asn208 that is also predicted to be the active site of the enzyme (Figure 1B).\(^\text{11}\) Ser86 is predicted by AlphaFold to be involved in hydrogen bonding (H bonds) with Asn90 (Figure 1C). Tyr110 of the human TAMM41 protein is predicted by AlphaFold to be involved in H bonds with Phe20, Asn65, and Asn98 (Figure 1D). Pro137 is predicted by AlphaFold to be involved in an H bond with Arg133 (Figure 1E). Replacement of this proline, a hydrophobic residue known to bend protein structures, will probably affect the structure of the protein and possibly modify the enzyme activity.

SDS-PAGE and western blot analysis performed on total protein revealed decreased steady-state TAMM41 levels in skeletal muscle of subjects 1 and 2 compared with controls (Figure 2A), suggesting that TAMM41 variants affect the stability of TAMM41 cDNA and/or protein. Moreover, a severe decrease of various OXPHOS subunits, including NDUFB8 (complex I), SDHA (complex II), UQCR2C (complex III), and COXI (complex IV), was observed in muscle of subjects 1 and 2 with a relative sparing of complex V subunit, ATP5A (Figure 2B). No muscle sample of subject 3 was available for western blot analysis. Unfortunately, steady-state levels of TAMM41 could not be assessed in fibroblasts samples, as the anti-TAMM41 antibody resulted in detection of weak and non-specific signal in fibroblast whole-cell lysates (data not shown). A mild decrease in NDUFB8 and COXI subunits was observed in fibroblasts from subject 1 compared with controls (Figure 2D); however, no obvious changes in OXPHOS subunits in fibroblasts from subjects 2 or 3 were detected. These data strongly suggest that there is a tissue-specific manifestation of phenotype due to TAMM41 variants, with severe consequences in skeletal muscle, but not in fibroblasts.

Blue native PAGE (BN-PAGE) analyses revealed a marked decrease in the assembly of OXPHOS complexes I–IV in muscle of subjects 1 and 2, consistent with the decreased
steady-state levels of their subunits (Figure 2C). Complex V assembly was also greatly impaired despite a relatively mild decrease of ATP5A steady-state level detected by western blot. Consistent with the SDS-PAGE experiments, subject fibroblasts samples did not show any obvious defect in the assembly of any of the OXPHOS complexes (Figure 2E), further highlighting a tissue-specific expression of the biochemical phenotype.

To assess the effect of the identified TAMM41 variants on CL levels, we performed lipidomic analysis as described previously. This demonstrated that, in skeletal muscle, the total CL and PG content was decreased in samples from affected individuals, whereas phosphatidylinositol (PI) and phosphatidylcholine (PC) levels were comparable (Figure 3A). This is consistent with a TAMM41-specific problem, as CL and PG are both synthesized from the mitochondrial pool of CDP-DAG, which is produced via TAMM41, whereas PI is synthesized in the endoplasmic reticulum (ER) and uses CDP-DAG derived from the ER-specific enzymes CDS1 and CDS2. Synthesis of PC is not dependent on CDP-DAG at all. We did not see evidence of an accumulation of PA (not shown), but this is likely due to the fact that the analysis was performed on whole-muscle extracts rather than isolated mitochondria, and PA is also present and can be metabolized in the ER. CDP-DAG was not detected in the lipidomic analysis, since it is metabolized quickly and the absolute steady-state levels are relatively low. Since whole-muscle and fibroblast samples were used for lipidomics, levels of CDP-DAG would also have included ER-derived CDP-DAG and would therefore not be as informative as the downstream, mitochondrial-specific PG and CL levels that were measured. In accordance with the impaired OXPHOS phenotype in muscle of affected individuals and the comparable levels in both control and subject fibroblasts, CL and PG levels in fibroblasts were not decreased in affected individuals (Figure 3B). This reinforces the tissue-specific nature of the defect observed and highlights the correlation between decreased TAMM41, decreased CL levels, and diminished OXPHOS protein levels, which are all observed in skeletal muscle from affected individuals and are suggestive that these

Figure 1. Identification of TAMM41 variants

(A) Domain architecture of S. pombe13 and human TAMM41 and sequence alignment of TAMM41 proteins from various species. NTase and winged helix domains are in dark and light blue, respectively. MTS, mitochondrial targeting sequence. The amino acid residues involved in CTP binding are shown in blue, and the residues involved in dimer formation are shown in red. The arrows indicate the affected amino acids (pink). Human Ser86, Tyr110, and Pro137 correspond to Thr133, Tyr157, and Pro184 in S. pombe and to Lys186, Pro236, and Tyr209 in S. cerevisiae.

(B–E) Three-dimensional representation of the human TAMM41 structure predicted by AlphaFold showing the locations and the predictions of hydrogen-bonding (H bond) of mutated amino acids. The mutated residues are highlighted in pink and the H bonds by green arrows. Amino acids in blue correspond to the CTP-binding sites. The gray circle shows the NTase domain. The colors of the 3D structure correspond to the confidence scores of the different parts of the protein (dark blue, very high [pLDDT > 90]; light blue, confident [90 > pLDDT > 70]; yellow, low [70 > pLDDT > 50]; and orange, very low [pLDDT < 50]).
TAMM41 variants have a functional consequence leading to the pathology.

To further assess the functional impact of TAMM41 missense variants, we tested mutant cDNAs for their ability to complement the growth defect in a Saccharomyces cerevisiae strain with the TAM41 gene deleted (tam41Δ). Consistent with previous studies, we observed that TAM41 is an essential gene in the BY4741 background. Expression of wild-type human TAMM41 cDNA failed to complement the growth of tam41Δ yeast strain (not shown), despite a high degree of conservation between yeast and human proteins (40% identity; Figure 1A). We introduced the equivalent various missense variants into the yeast TAM41 sequence by site-directed mutagenesis and used a plasmid-shuffling system for its expression in tam41Δ strain. Growth of the tam41Δ strain was fully complemented by wild-type yeast TAM41 as expected. However, tam41Δ variants corresponding to the human Tyr110Cys and Pro137Leu variants, respectively, completely failed to complement the tam41Δ growth defect on fermentable media (YPD), especially at 36°C (Figure 4), and on non-fermentable media (YGE and YPG), which renders the cell growth dependent on mitochondrial respiration (Figure 4).

Expression of tam41K186P (Ser86Pro in human) rescued growth on fermentable media but only partly complemented the growth on respiratory media particularly at 36°C. Taken together, these results demonstrate the deleterious nature of all subject TAMM41 missense variants. Moreover, the growth phenotype of the mutant proteins expressed in tam41Δ yeast shows a good correlation with the severity of the clinical presentation of the subjects.

We have identified three affected individuals from unrelated families presenting with myopathy, hypotonia, bilateral ptosis, and lethargy associated with compound heterozygous TAMM41 variants. The unifying clinical features of ptosis, CPEO, and failure to thrive were highly suggestive of a mitochondrial genetic defect. Brain MRI was normal in one subject, and none of them presented with elevated lactate, but respiratory chain analyses within skeletal muscle in two subjects clearly revealed abnormal activities, confirming a biochemical diagnosis of mitochondrial disease. The TAMM41 variants identified by WES resulted in a relatively mild decrease in steady-state TAMM41 protein levels in skeletal muscle (subjects 1 and 2), leading to disrupted assembly of OXPHOS subunits and, consequently, the severe impairment of respiratory chain enzyme activities. In contrast, subject fibroblasts displayed a modest decrease of OXPHOS protein abundance that did not affect mitochondrial respiratory chain enzyme activities. This was mirrored in the results from the quantitative lipidomic analyses, which demonstrated a clear decrease in CL and PG in subject skeletal muscle samples but no matching phenotype in subject fibroblasts.

This tissue-specific effect of the variants is not surprising, as there are many examples of variants causing mitochondrial...
disorders that express defects in muscle, but not in fibroblasts. In this case, we show that the OXPHOS deficiency correlates with decreased CL and it is possible that the difference between the two tissues tested could be that fibroblasts are less reliant upon TAMM41 for the CDP-DAG needed to produce CL. There is some evidence to suggest that the ER enzymes CDS2 and CDS2 can contribute toward CL synthesis, since mouse knockouts of regulatory genes Ppargc1a \(^{17}\) (encoding PGC-1α) or Prkaa2 \(^{18}\) (encoding AMPKα2) led to a decrease in CL alongside a decrease in CDS1 or CDS2 mRNA, respectively. However, these studies did not assess TAMM41 mRNA levels as TAMM41, and its CDS activity had not yet been identified. Furthermore, yeast knockouts of tam41 do have some low levels of CL synthesis, suggesting the yeast CdsA may be able to provide some of the CDP-DAG required for CL synthesis. \(^{13}\) For CDP-DAG produced in the ER to be used for CL synthesis, this would need to be transported from the ER to the inner mitochondrial membrane. No such lipid transporter has yet been identified, but it remains an interesting possibility. In our experiments, we were unable to assess the effects of the variants on TAMM41 protein levels in fibroblasts due to weak and non-specific signal in fibroblast samples (including controls). This contrasted with the strong specific signal in skeletal muscle samples, perhaps suggesting a difference in expression of TAMM41 between the tissues. However, mRNA data available on the GTEX portal (https://gtexportal.org/home/gene/TAMM41) suggests expression of TAMM41 transcript is actually higher in fibroblasts than in muscle tissue. Post-transcriptional or post-translational modification in fibroblasts could potentially explain the seemingly lower expression of TAMM41 protein in fibroblasts. As with many other mitochondrial disorders, we do not fully understand the reason for the tissue-specific expression of the biochemical and clinical phenotype caused by TAMM41 variants.

We demonstrate that variants in TAMM41 represent the second cause of disturbed CL biosynthesis and illustrate the clinical heterogeneity associated with a similar metabolic defect, Barth syndrome, which is caused by variants in TAFAZZIN. Barth syndrome is a mitochondriopathy that is mainly characterized by cardiomyopathy and myopathy based on a generalized CL-remodeling defect that is found in several tissues, including fibroblasts. Symptomatically,
TAMM41 deficiency and Barth syndrome have myopathy in common; however, the subjects with TAMM41 deficiency did not display a cardiac phenotype—all subjects had either electrocardiogram or echochography performed, which were normal. The lack of CL deficiency and OXPHOS defects in TAMM41-deficient fibroblasts suggests a tissue-specific expression of the biochemical defect that might explain the phenotypic variability of the two disorders in the same biosynthetic pathway. Interestingly, TAMM41 deletion by CRISPR in zebrafish does lead to abnormal heart development, but this was attributed to TAMM41 participating in PINK1-dependent mitophagy rather than its role in CL synthesis, as CL levels were unchanged in TAMM41-depleted zebrafish heart cell lines.19

In this report, the highlights the importance of lipid homeostasis to sustain normal mitochondrial function and adds to a growing number of lipid biosynthesis defects associated with pathogenic variants in AGK, SERAC1, and LIPT2 or numerous genes encoding CoQ10 biosynthesis enzymes that result in mitochondrial disease.20–22

Data and code availability

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplemental material or can be made available upon reasonable request.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.xhgg.2022.100097.

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Declaration of interests

The authors declare no competing interests.

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Web resources

OMIM, http://www.omim.org/
Alamut Visual, https://www.interactive-biosoftware.com/alamut-visual/
PDB, http://www.rcsb.org/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/

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