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Abstract

Subcellular membrane systems are highly enriched in dolichol, whose role in organelle homeostasis and endosomal-lysosomal pathway remains largely unclear besides being involved in protein glycosylation. *DHDDS* encodes for the catalytic subunit (DHDDS) of the enzyme *cis*-prenyltransferase (*cis*-PTase), involved in dolichol biosynthesis and dolichol-dependent protein glycosylation in the endoplasmic reticulum. An autosomal recessive form of retinitis pigmentosa (retinitis pigmentosa 59) has been associated with a recurrent *DHDDS* variant. Moreover, two recurring *de novo* substitutions were detected in a few cases presenting with neurodevelopmental disorder, epilepsy, and movement disorder.

We evaluated a large cohort of patients (*n*=25) with *de novo* pathogenic variants in *DHDDS* and provided the first systematic description of the clinical features and long-term outcome of this new neurodevelopmental and neurodegenerative disorder. The functional impact of the identified variants was explored by yeast complementation system and enzymatic assay.

Patients presented during infancy or childhood with a variable association of neurodevelopmental disorder, generalized epilepsy, action myoclonus/cortical tremor, and ataxia. Later in the disease course they experienced a slow neurological decline with the emergence of hyperkinetic and/or hypokinetic movement disorder, cognitive deterioration, and psychiatric disturbances. Storage of lipidic material and altered lysosomes were detected in myelinated fibers and fibroblasts, suggesting a dysfunction of the lysosomal enzymatic scavenger machinery. Serum glycoprotein hypoglycosylation was not detected and, in contrast to retinitis pigmentosa and other congenital disorders of glycosylation involving dolichol metabolism, the urinary dolichol D18/D19 ratio was normal. Mapping the disease-causing variants into the protein structure revealed that most of them clustered around the active site of the DHDDS subunit. Functional studies using yeast complementation assay and *in vitro* activity measurements confirmed that these changes affected the catalytic activity of the *cis*-PTase and showed growth defect in yeast complementation system as compared with the wild-type enzyme and retinitis pigmentosa-associated protein.

In conclusion, we characterized a distinctive neurodegenerative disorder due to *de novo DHDDS* variants, which clinically belongs to the spectrum of genetic progressive encephalopathies with myoclonus. Clinical and biochemical data from this cohort depicted a condition at the intersection of congenital disorders of glycosylation and inherited storage diseases with several features akin to of progressive myoclonus epilepsy such as neuronal ceroid lipofuscinosis and other lysosomal disorders.

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Abbreviations: CDGs=congenital disorders of glycosylation; cis-PTase=*cis*-prenyltransferase; DHDDS=dehydrodolichyl diphosphate synthase; Dol-P=dolichyl monophosphate; ER=endoplasmic reticulum; GDD=global developmental delay; GPI= glycosylphosphatidylinositol; GTCS=generalized tonic-clonic seizure; ID=intellectual disability; LC-MS/MS=tandem mass spectrometry; MD=movement disorder; NgBR=Nogo-B Receptor; TF=transferrin; WES= whole exome sequencing; WGS=whole genome sequencing

Introduction

DHDDS (MIM*608172) encodes for the dehydrodolichyl diphosphate synthase subunit DHDDS, which in a complex with NgBR (also termed Nogo-B Receptor) encoded by the *NUS1* gene, constitutes the *cis*-prenyltransferase (cis-PTase), a branch point enzyme of the mevalonate pathway. Cis-PTase is located in the endoplasmic reticulum (ER) and is involved

in the biosynthesis of dolichyl monophosphate (Dol-P), an essential lipid serving as a glycosyl moiety carrier for protein N-glycosylation and as a carrier of dolichol-linked monosaccharide donors (Dol-P-Glc and Dol-P-Man) for O-mannosylation, C-mannosylation, and glycosylphosphatidylinositol (GPI) anchor formation.[1,2] The structure of the eukaryotic enzyme as well as the mechanism through which DHDDS and NgBR stabilize each other, which is crucial for cis-PTase activity,[3-5] has recently been clarified.[2,6] Dolichols size varies between species and is determined by the given *cis*-PTase. In humans, DHDDS determines the number of isoprenoid units,[6] that is commonly between 17 and 20, with a preponderance of dolichol-19 (D19).[7]

Protein N-glycosylation is a post translational modification crucial to protein folding, oligomerization, and intracellular sorting and transport. Molecular defects in genes involved in N-glycans biosynthesis belong to a family of metabolic disorders, collectively known as congenital disorders of glycosylation (CDGs).[8,9] These diseases share an abnormal pattern of protein or lipid glycosylation and major nervous system involvement in the context of multisystemic disease. CDG subtypes with autosomal recessive defects in dolichol biosynthesis (DHDDS-CDG, SRD5A3-CDG, NUS1-CDG, and DOLK-CDG)[10-12] have been reported, and most are detectable by routine CDG screening of serum transferrin via isoelectric focusing or mass spectrometry.

Defects in the dolichol oligosaccharide assembly pathway cause accumulation of underglycosylated misfolded glycoproteins driving a chronic induction of the unfolded protein response pathway and ER stress, along with apoptosis and regeneration.[13,14] A marked induction of ER stress and impaired N-linked glycosylation has also been observed in *NUS1* mutant fibroblasts, in association with intralysosomal cholesterol storage as seen in Niemann Pick type C disease.[4]

Despite the crucial role of dolichol synthesis in protein glycosylation in several tissues, germline mutations in *DHDDS* have only recently been associated with a complex multisystem phenotype in an individual exhibiting intrauterine growth deficiency, micropenis, hepatomegaly, renal failure, axial hypotonia, increased appendicular tone, severe global developmental delay (GDD) and refractory epilepsy, who died at eight months in *status epilepticus*. Analysis of skin fibroblasts revealed variable amounts of truncated dolichol and protein-linked N-glycans. This condition was associated with biallelic loss-of-function mutations and a CDG type I pattern of glycosylation.[15]

A recurrent homozygous variant in *DHDDS* (c.124A>G; p.Lys42Glu) was found in consanguineous families with non-syndromic retinitis pigmentosa (RP type 59).[16-19] In these patients, D18 becomes the dominant species, with altered plasma and urinary D18/D19 ratios.[20]

Two additional recurrent pathogenic variants, *i.e.* p.Arg37His and p.Arg211Gln, have been reported in 7 individuals from unrelated families with intellectual disability (ID), epilepsy, tremor, myoclonus, and movement disorder (MD).[21-23] The p.Arg37His variant falls in an evolutionary conserved stretch of 5 amino acid residues (pos. 34-38), which corresponds to the catalytic domain of the enzyme.[2,21] Crystal structure and mutagenesis studies demonstrated that Arg^{211} is critical in homoallylic binding to the isopentenyl diphosphate (IPP) substrate.[2] Although generalized epilepsy, tremor, and ID seem to be the main features associated with de novo *DHDDS* missense mutations, available clinical information is very limited and detailed genotype-phenotype correlation is lacking. A dominant-negative mechanism has been proposed to explain the allelic heterogeneity associated with *DHDDS*-related disorders.[23]

Given the shared metabolic pathway, *de novo* variants in *NUS1* have recently been associated with a clinical phenotype that overlaps with that described for *de novo DHDDS* pathogenic variants, namely ID, well controlled generalized epilepsy, prominent tremor/myoclonus, ataxia, and parkinsonism with a slowly progressive course.[21,24,25] *NUS1* variants were reported in isolated early-onset Parkinson's disease.[26] A homozygous *NUS1* variant was described in a patient with severe neurological impairment, refractory epilepsy and congenital disorder of glycosylation.[4]

In this study we delineated the clinical phenotype associated with dominant *DHDDS* variants through the long term clinical observation of a large cohort of patients. We provided the first genotype-phenotype correlation analysis through functional characterization of reported *DHDDS* variants using yeast complementation and enzymatic assays. We also confirmed the role of the NgBR-DHDDS complex dysfunction in early onset neurodevelopmental and neurological disorders including myoclonus syndromes.

Materials and methods

Patients

We collected clinical and molecular data of 25 patients from 24 unrelated families with likely pathogenic/pathogenic variants in *DHDDS* from different clinical centers in Europe, United

States, and Canada through international clinical collaborations and networking (GeneMatcher).[27] Three patients (Pts 1-3) were previously reported with limited clinical data in Hamdan *et al*.[21]

For each confirmed case, the referring physician completed a case review and returned pseudo-anonymized data including developmental, neurological, behavioral, and epilepsy medical history, metabolic studies, electroencephalograms (EEGs), and neuroimaging data. Seizure types were classified using the International League Against Epilepsy (ILAE) criteria,[28] or in more descriptive terms when the seizure phenomenology did not fit this classification terminology. Patients were evaluated according to developmental and cognitive functioning scales routinely used in the participating centers. Written informed consent was locally obtained for all participants.

Molecular genetic investigations and *in silico* modelling

The *DHDDS* (NM_024887) pathogenic variants reported herein were all identified by clinical (19/25) or research (5/25) whole exome sequencing (WES), except for one which was identified by research whole genome sequencing (WGS) (Supplementary Table 1). With the exception of a single-residue deletion (Lys42) documented in a single patient, they were mostly missense. In 22 of the 23 tested families, direct Sanger sequencing confirmed the de novo origin of the *DHDDS* variant. In family 4, segregation analysis documented mosaicism (p.Arg211Gln) in the father. Parental genomic DNA specimens were not available in family 12 (p.Arg37His). In both cases, the identified variants were recurrent and demonstrated to occur as de novo events in multiple instances. The clinical relevance of the identified variants was assessed using the American College of Medical Genetics and Genomics (ACMG) guidelines for variant interpretation (Supplementary Table 1A).[29,30]

Mapping of the *de novo DHDDS* mutations was achieved using the previously solved crystal structure of human NgBR/DHDDS enzyme in complex with Mg^{2+} and IPP substrate (PDB ID: 6W2L). PyMOL software was used to model the location and binding interactions of individual mutations.

Functional characterization of *DHDDS* missense variants

Detailed methods for expression and purification of the NgBR/DHDDS complex, *cis*-PTase activity assay, and yeast complementation assay are available in the supplementary methods and materials.

Metabolic studies

Detailed methods for glycosylation profiling through liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) analysis of serum transferrin (TF) and dolichol content analysis in urine by LC-MS/MS are available in the supplementary methods and materials.[31-33]

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary data.

Results

Clinical description

We studied 25 patients (17 males and 8 females) aged 4 to 59 years. Eleven were young adults or adults (age range 18-59 years). Most patients were born from normal pregnancies and deliveries, with normal growth parameters and neonatal examinations. Detailed descriptions of neurodevelopmental, epileptic, and motor features are reported in Table 1 and Supplementary Table 1. Extensive clinical reports for 14/25 patients are available in the supplementary materials.

Presenting features and disease evolution

Initial signs of neurodevelopmental and/or neurological impairment were noticed during infancy in 22/25 patients (age at symptom onset: range 1-24 months, median 12 months). Patients 17 and 23, now 44 and 16 years old, who manifested their initial clinical symptoms at age 5 and 3, respectively, had the latest disease onset and mildest phenotype.

GDD with or without hypotonia was the most frequent presenting symptom (23/25 pts). Additional features frequently reported at onset included tremor and seizures. Ataxia was an early symptom in 6/25 patients. In two patients, parents reported a neurological regression beginning from early disease stages.

The combination of GDD and tremor occurred in 11/25 patients, four of whom also experienced seizures, thus manifesting the core triad of the condition from onset.

Neurological deterioration was described in most patients (17/25), with no obvious genetic, demographic, or clinical factor influencing prognosis. Seven patients (Pts 1,6,9,10,13,19, and 21) exhibited prominent cognitive decline. Progression occurs over years or decades. In some patients, there were periods of rapid deterioration while in others progression was gradual. Stepwise neurological deterioration with multiple phases of regression was observed in patients 1,9, and 12. The outcomes of patients 1 and 19, respectively 39 and 17 years, were particularly severe with intense fatigue, autonomic dysregulation (bladder, temperature, heart rate, breathing), and dementia with akinetic mutism.

Neurodevelopmental, cognitive and behavioral phenotype

All patients except one had ID, which was severe in 12, moderate in 6 and mild in 5. Patient 8 had normal development up to 18 months of age, after which developmental issues were first noticed in relation to epilepsy onset. Patients were able to sit between 9-18 months, walk between 13-36 months, pronounce their first words after 3 years and, in those with milder cognitive impairment, speak in sentences after 4-5 years of age.

Patient 17 graduated from high school and attended community college, achieving above-average grades. Gross motor functioning was normal and he was an excellent swimmer and skier. He exhibited absence seizures since age of 5 years, myoclonus at 16 years and rare GTCS in adulthood. Patient 12 complained of tremor from the age of 10 years and had his first epileptic seizures at the age of 19 years. He showed borderline intellectual functioning, tough it was compatible with a normal employment in adulthood.

Relevant behavioral and/or neuropsychiatric comorbidities were reported in the majority of patients (18/25) (Fig.1, Table 1, Supplementary results). Four patients presented a severe

psychiatric outcome with psychosis and/or catatonia in advanced disease stage (adolescence to adulthood) (Pts 1,6,13,and 17).

Epilepsy

Epilepsy, reported in 22/25 patients, was a core feature of the disorder. Age at seizure onset ranged from 6 months to 10 years. Thirteen/25 patients had infantile onset epilepsy (birth-2 years), 8/25 had childhood onset epilepsy (3-12 years), and one patient (Pt 12) had GTCS onset at 19 years. Febrile seizures (myoclonic, absence, or GTCS) were reported in 7/25 and were part of the presenting features (11-25 months of age) in 6/25 patients (Pts 3,7,8,10,17,24,and 25), preceding afebrile seizure onset. In patient 7 seizures started after vaccination. Photosensitivity was observed in 5 patients (Pts 1,18,19,24,and 25).

Generalized epilepsy including myoclonic-atonic epilepsy was reported in 21/25 patients with absence (15/25), GTC (13/25), myoclonic (8/25), and atonic (6/25) being the most frequent seizure types. Distribution of seizure types is summarized in Table 1 and Fig.1. Absence seizures included typical absences, atypical absences, myoclonic absences, and absences with eyelid myoclonia (Supplemental table 1). Patients 9, 18 and 20 had frequent nocturnal seizures, which were tonic in patients 9 and 20, whereas patient 18 had GTCS. Twenty out of 25 patients presented multiple seizure types, typically 2-4.

Epilepsy was well controlled with anti-epileptic drugs (AEDs) in 13/25 patients, while 9 patients had drug resistant seizures. All patients with childhood onset epilepsy (Pts 1,6,9,10, and 13-17) were well controlled under AED therapy. Among patients with infantile onset epilepsy, 9/13 had drug-resistant epilepsy non-responsive to combinations of several conventional AEDs, and 4/13 were well-controlled using valproate alone or in combination with levetiracetam, or clobazam.

Generalized EEG abnormalities were reported in 16/22 epileptic patients. EEG recordings in 12/22 patients showed bursts of ictal and interictal generalized slow spike and wave complexes (2-4 Hz), sometimes predominantly on the frontal region, in association with generalized slow activity, and/or diffuse slowing of the posterior dominant rhythm, or abnormal background activity. Ictal slow spike-wave complexes correlated with absences, eyelid myoclonia, myoclonic seizures, and atonic events (head drops, falls) (Pts 1,3,4,7,8,10,18,19,21,22, and 25) (Fig.2).

Epilepsy usually improved or resolved from adolescence and epileptiform abnormalities disappeared over the disease course (Supplementary results). The most effective AEDs, alone or in combination were valproate (11/22), levetiracetam (8/22), clonazepam (6/22), and clobazam (6/22); other drugs were less effective, including lamotrigine (2/22), zonisamide (3/22), topiramate (2/22), ethosuximide (1/22), phenobarbital (1/22), primidone (1/22), lacosamide (1/22) and brivaracetam (1/22). Levetiracetam led to seizures worsening in patient 8.

Movement disorder

Movement disorder, generally as bilateral distal intention and postural tremor/myoclonus of the upper limbs (Pts 1-5,8,15,18,19,and 23), was part of the clinical presentation in most of patients (14/25), detected in the first months of life in some patients (Pts 3,5,8,15, and 18), and sometimes associated with ataxia (Pts 1-5,18,19,and 21) and/or hypotonia (6/25). Patients 1, 2 and 23 underwent neurophysiological studies with back averaged EEG which demonstrated a cortical origin of tremor.

Tremor tended to change over time in terms of distribution and type with involvement of the trunk and lower extremities and onset of rest tremor superimposed to the already present action and postural components (Pts 1,6,8,12,15,19, and 21) (videos 1-5, available from figshare https://doi.org/10.6084/m9.figshare.15106032.v1). Tremor became continuous over time, exacerbated by sudden lights, loud noises, stress, and movement (pts 1, 8, 12, and 16; videos 1,2, and 5). Facial involvement, particularly of the eyebrows and mouth, was observed in 8/25 patients (Pts 1, 6-8, and 11-14) (videos 1-5). Periodic exacerbations of tremor/myoclonus lasting hours to days (Pts 1 and 2) (video 1) and episodic weakness requiring hospitalization (Pts 1 and 9) were reported.

At follow up 24/25 patients had a MD. In most of them (14/25), a complex pattern of MD emerged over time (Pts 1,6-9,11,12,15,18-21,24,and 25) including ataxic features (Pts 2-5,8,12-16,18- 23, and 25), dystonic posturing of the upper and/or lower limbs (Pts 1, 6,8,11,15, and 18-20) (video 2), parkinsonism (Pts 1, 6,11, 12,18-20, and 24), and chorea (Pts 7, 9,16, and 22) (Fig.1 and Table 1). Fluctuations in tremor and other MDs severity were observed in patients 1,8,11,18,19, and 22. Parkinsonism with rigidity, rest tremor, bradykinesia and hypomimia was a late disease feature, which developed gradually from adolescence (video 1 and 2). Over the disease course 8/25 patients developed speech abnormalities such as hypophonic and slow speech (Pts 1,20, and 23), dysarthria (Pts 3,8,11,12, and 19), and loss of

speech/anarthria (Pts 1 and 6). Pyramidal signs were reported in 5/24 patients (Pts 5,6,12,19, and 21).

Most patients did not receive treatment for MD (21/25) and epilepsy treatment had a relatively mild effect on tremor and MDs. Piracetam was ineffective for cortical tremor in patient 1, while a partial response was observed with high doses of clonazepam (up to 9 mg/day). Parkinsonism didn't respond to levodopa which instead increased impulsivity and hyperactivity. Interestingly, very low doses of risperidone (0,5 mg/day) or tetrabenazine (6.25 mg/day) were able to control the severity of myoclonic episodes in advanced disease stage, while higher dosages induced akinetic status. In patient 17 tremor responded to propranolol while in patient 24 a mild effect of benzodiazepines on cortical tremor was observed.

Associated features

Dysmorphisms were reported in a subgroup of patients (Fig. 3). The most recurrent features were altered dental architecture with wide spaced or missing teeth (Pts 2,4, and 7) and generalized hypertrichosis with dark hair and thick eyebrows (Pts 1,6,9, and 15). Patient 15 developed axillary and pubic hair at age 4. Other dysmorphic features were wide-set almond-shaped eyes (Pts 7 and 9), upslanted palpebral fissures (Pts 4 and 7), flat/concave nasal bridge (Pts 4 and 7), prominent supraorbital ridge (Pts 7,9, and 12), high frontal airline (Pt 7), large mouth with full lips (Pt 7), high palate (Pt 2), bifid uvula (Pt 9), thick ear lobes (Pt 7), fetal pads (Pt 7), and first toe clinodactyly (Pt 4). Sagittal craniosynostosis was surgically repaired at 3 months of age in patient 15. Hyperpigmentation at the right side of the thorax was seen in patient 2.

Gastrointestinal issues with periods of severe constipation were reported in patients 1 and 4, while recurrent abdominal pain and vomiting were reported in patient 20. At the age of 14, patient 9 was diagnosed with Crohn disease and successfully treated with infliximab. Patient 17 underwent resection of a dysplastic rectal polyp at the age of 42 years with negative genetic testing for hereditary cancer predisposition. Patient 1 had fatty liver disease with hypercholesterolemia, increased levels of Gamma-glutamyl transferase and milder increase of serum transaminase.

Conjunctival telangiectasias was observed in patients 1 and 20. Patient 20 was affected by a complex autoinflammatory disorder characterized by recurrent arthralgia of the knees,

episcleritis, intermittent fever, and suffered from an acute episode of purpura with erythrocyturia/hematuria, and increased CRP, and IgA, which significantly improved after steroid treatment. Skin biopsy was diagnostic for small vessel vasculitis demonstrating perivascular leucocyte infiltrates.

Brain imaging

Brain MRIs, available for 24/25 patients, were normal in 23/24, with 6 patients (Pts 1,2,9, 15,17, and 21) scanned more than once at different disease stages. Patient 1 had normal brain imaging up to the age of 27. At the age of 37, he started to severely deteriorate. Brain MRI showed T2/FLAIR subcortical hyperintense non-enhancing foci localized to the frontal, temporo-occipital and visual cortices bilaterally with prominent involvement of calcarine cortex (Fig. 4A). A follow-up study at 10 months demonstrated severe and diffuse cortical and subcortical atrophy (Fig. 4B) including previously hyperintense areas, the dorsal striatum with prominent caudate involvement, and cerebellum. ^{1}H-MRS demonstrated decreased N-acetylaspartate (NAA) peak and NAA/choline (Cho) ratio at the level of the caudate lesions, thus confirming neuronal degeneration. ^{1}H-MRS was performed and resulted normal in 4/24 patients. Stable corpus callosum thickening was documented in patient 21. MRI of the spinal cord of patient 17 showed a T5-T11 syrinx, which was stable on serial imaging from ages 35-43 years.

Metabolic features

CDG screening was performed by isoelectric focusing of plasma transferrin (TF) and resulted normal in 12/25 patients. Mass spectrometry analysis of plasma TF was subsequently performed in 5/25 patients, two of which showing marginally abnormal glycosylation (Table 1). Urinary dolichol isoforms (D18/D19 ratio) were evaluated and resulted normal as compared with age-matched controls in 4/25 patients. Hypercholesterolemia without hypertriglyceridemia was reported in 3/25 patients.

Ultrastructural analysis of the skin biopsy from patient 1 showed osmiophilic material deposits in myelinated fibers. Numerous single membrane-surrounded vacuoles containing lamellated membrane structures resembling phospholipids or other lipid-like material were observed in

the axons of myelinated fibers (Fig. 5A-D). Cholesterol-like deposits associated with glycogen were evident in the cytoplasm of Schwann cells. In a small percentage of stroma fibroblasts large secondary lysosomes filled with different electron-density substances were found in the cytoplasm (Fig. 5E-F). Axillary skin biopsy revealed PAS positive bodies in eccrine glands in patients 6.

Genetic findings and genotype-phenotype correlation

Fifteen individuals carried two previously reported *DHDDS* pathogenic variants (p.Arg37His and p.Arg211Gln),[21] while 10 harbored new pathogenic/likely pathogenic variants (p.Gly35Glu, p.Arg37Cys, p.Lys42del, p.Arg205Gln, p.Ser213Asn, and p.Pro233Arg) (Supplementary Tables 1, 1A). Twenty-three variants were validated as *de novo* events, while parental mosaicism was demonstrated in family 4, in which the asymptomatic father was found to be mosaic.

The most recurrent variant was p.Arg211Gln, which was detected in 11/25 patients (patients 1 and 2 were previously reported with limited clinical information by Hamdan *et al*.).[21] Four out of 25 patients had the previously reported p.Arg37His change[21,22] (patient 3 was previously reported previously with limited clinical information by Hamdan *et al*.).[21] Newly identified variants were distributed as follows: four subjects carried the p.Arg37Cys substitution, two patients carried the p.Gly35Glu, while p.Pro233Arg, p.Ser213Asn, p.Arg205Gln, and Lys42del were identified in single individuals.

None of these variants was predictive of disease severity in terms of cognitive functioning, epilepsy, and MD phenotype. Moreover, the phenotype associated with the three most frequent variants detected in this cohort, namely p.Arg37His, p.Arg37Cys, and p.Arg211Gln, did not differ significantly in terms of age of disease onset, clinical presentation pattern, or disease course. No specific variants were associated with a better cognitive outcome (Pts 3,12,17,19,20,23), while disease course in patients less cognitively compromised (Pts 12,17, 23) seemed to be characterized by a later onset and milder epilepsy and MD, regardless of the specific variant. Indeed, the p.Arg37His and p.Arg211Gln were found to occur with a substantially variable clinical phenotype.

Some genotype-phenotype correlations, however, emerged in our cohort. Patients harboring the p.Arg37His change all suffered from febrile seizures as a presenting feature or during the

disease course and epilepsy remained well controlled in 3/4 patients. The p.Arg211Gln variant was also associated with a severe phenotype, with stepwise deterioration in 2/11 patients and fluctuations or exacerbations of MD in 3/11 patients. MD in this subgroup tended to become particularly severe over the disease course. Four out of 8 patients of this cohort who developed parkinsonism harbored the p.Arg211Gln variant. Among the 5 patients with severe psychiatric manifestations, 3 carried the p.Arg211Gln variant (Pts 1,6, and 17) while 2 carried the p.Arg37Cys variant (Pts 8 and 13).

Structural data

The recently resolved structure of the human NgBR/DHDDS complex[2,6] was used to explore the structural and functional consequences of the identified disease-causing variants. The mutated residues are localized within the previously established *cis*-PTase catalytic domain of DHDDS (residues 25-265). As shown in Fig.6A, most amino acid changes affect residues that cluster around the active site of DHDDS and are involved in allylic (farnesyl diphosphate, FPP) and homoallylic (IPP) substrate binding. The β-phosphate group of the FPP substrate is hydrogen-bonded to the backbone amide of Gly^{35}, which is localized on the P-loop, and is well established to be involved in FPP binding. Therefore, replacing Gly^{35} with Glu^{35} would result in steric clashes with nearby residues and hence perturb packing of the P-loop and potentially disrupt Arg^{38} conformation on the proceeding α1 helix, which is also involved in FPP binding. In addition, the side chain of Arg^{37} is stabilized by a network of salt bridges with the β-phosphate group of FPP, Glu^{89} on DHDDS and the main chain carboxylate group of Lys^{293} on the C-terminus of NgBR (Fig.6B). Introducing a histidine residue at this position would abolish binding interactions with the β-phosphate group of FPP substrate as well as with the C-terminus of NgBR. The introduction of a cysteine at this position could be more detrimental due to the loss of a positive charge. Similarly, the side chains of Arg^{205} and Arg^{211} form salt bridges with the pyrophosphate of IPP (Fig. 6C), and replacement of either residue with Gln would eliminate binding interactions with IPP. Although substituting Ser^{213} with Asn can still maintain hydrogen bonding with the β-phosphate group of IPP, introducing a bulky side chain at this region would result in steric clashes with nearby residues, hence leading to structural destabilization. While the aforementioned residues are directly involved in substrate binding, Pro^{233} is an exception to this pattern. Pro^{233} is localized within a loop that connects βF with α7 and seems to be involved in hydrophobic packing against Tyr39 on α1, harboring FPP binding

residues (Fig. 6D). Accordingly, substituting Pro with a charged residue like Arg could disrupt packing of the α1 helix and binding to the FPP substrate. Finally, previous structural and functional studies on the p.K42E substitution have already established its indirect role in FPP binding by stabilizing the α1-helix through a salt bridge formed with a conserved E^{234}.[2] Similarly, a deletion at this position (**Δ**K42) could affect the stability of the structure as well as FPP substrate binding.

Functional validation of de novo DHDDS missense variants: yeast complementation assay and enzymatic activity measurement

Yeast complementation assays were performed to functionally validate the identified variants.[4] As seen in Fig 7*A*, mutants containing $DHDDS^{\Delta K42}$ were able to support the growth of *rer2*Δ/*srt1*Δ/*nus1*Δ yeast cells (lacking endogenous genes critical for *cis*-PTase activity) in a manner similar to that of wild type (WT) *cis*-PT and $DHDDS^{K42E}$ (a mutation underlying RP).[5] $DHDDS^{S213N}$ causes slight growth defect suggesting that enzyme activity is below 20% and that $DHDDS^{R211Q}$ severely impairs yeast growth. Finally, $DHDDS^{G35E}$, $DHDDS^{R37C}$, $DHDDS^{R37H}$, $DHDDS^{R205Q}$ and $DHDDS^{P233R}$ did not support yeast growth, further confirming their predicted essential role in enzymatic activity. To expand the detection limit of our yeast complementation assay, we modified our system by expressing DHDDS from a single-copy plasmid under the native yeast *RER2* promoter. Since NgBR is stable in yeast cells only when co-expressed with DHDDS and its co-expression from the single plasmid does not support the growth (data not shown), we generated yeast strains of interest co-expressing DHDDS from single-copy plasmid and NgBR from multi-copy plasmid.

We compared the growth rates of *yeast expressing either WT or one of the DHDDS* mutants using the yeast spot test (Fig. 7B) and kinetic growth assay (Supplemental Fig. 1) on yeast extract peptone dextrose (YPD) medium or YPD supplemented with lovastatin, an inhibitor of HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway responsible for substrate synthesis. The results showed that yeast cells harboring the mutated version of DHDDS displayed different degrees of growth delay in YPD medium, with a more pronounced phenotype at 37 °C. As expected, these cells were also more sensitive to lovastatin due to the low substrate levels.

Next, we compared the steady-state activities of purified mutants to those of the WT complex. As shown in Fig. 4C, all mutants analyzed displayed lower enzymatic activity compared to the WT enzyme. In addition, mutations that did not support yeast growth ($DHDDS^{G35E}$, $DHDDS^{R37C}$, $DHDDS^{R37H}$, $DHDDS^{R205Q}$ and $DHDDS^{P233R}$) were able to incorporate radioactive IPP substrate within the detection limit range, suggesting that they formed a catalytically dead enzyme. Overall, enzymatic activity changes were proportional to the level of growth defect observed in our yeast studies. In summary, the data presented herein further support the pathogenicity of the identified *de novo DHDDS* variants.

Discussion

DHDDS phenotypic spectrum

Here, we report a first comprehensive description of the phenotype associated with *de novo DHDDS* variants. We demonstrated that heterozygous *DHDDS* mutations are associated with a complex slowly progressive central nervous system disorder with broad functional consequences including cognitive impairment, abnormal motor control (myoclonus, cortical tremor, ataxia, MD), and epilepsy.

We depicted a distinctive disorder presenting during infancy or childhood with neurodevelopmental and neurological findings including GDD/ID, epilepsy, myoclonic movements, and postural and intention tremor (with or without ataxia). Starting from the second decade of life, the disease followed a slow or stepwise pattern of progression, at times, punctuated by episodes of acute deterioration. Cortical tremor generalized, and further signs of diffuse neurological impairment appeared, including epilepsy (when not already present), hyperkinetic and hypokinetic MD, cognitive decline, and psychiatric disturbances.

The epileptic phenotype was characterized by febrile seizures at presentation, coexistence of multiple generalized seizure types, and an EEG pattern of ictal and interictal generalized slow spike-wave complexes, as also described in two previously reported patients with dominant mutations in *DHDDS* [22,23] and in a patient with heterozygous *NUS1* pathogenic variants.[25]

Neuroimaging studies do not contribute to diagnosis or clinical monitoring. No metabolic biomarkers have been associated with the disease. The electrophysiological investigations of the abnormal movements are in support of an exaggerated cortical hyperexcitability state as reported in other myoclonic syndromes. [23,40,41]

Consistent with this hypothesis, short latency somatosensory evoked potentials (SEPs) showed exaggerated cortical responses (giant SEPs) in a previously reported 15 year old girl with the most recurrent p.Arg211Gln variant and cortical myoclonic tremor,[23] and back averaged EEG indicated a cortical origin of tremor in 3 patients of this cohort thus suggesting cortical tremor.

Functional impact of *DHDDS* pathogenic variants

We provided a first genotype-phenotype correlation analysis supported by functional studies using yeast complementation assay and *in vitro* activity measurements.

Mapping the disease-causing *de novo DHDDS* variants into the protein structure revealed that most cluster around the active site of the DHDDS subunit and likely directly affect enzymatic activity and/or substrate binding. Our findings indicated that all the identified variants formed *cis*-PTase with impaired/reduced enzymatic activity and show growth defects in yeast complementation assays. DHDDS$^{\Delta K42}$ and DHDDS^{S213N} had a less severe phenotype compared to the other mutants. The impact of these changes on yeast growth became evident only in spot test (37°C) and kinetic growth assay performed in the presence of lovastatin, but was significantly higher compared to that of the recessive RP-causing DHDDS^{K42E} mutant.

Molecular mechanisms of DHDDS-related diseases

DHDDS encodes a protein involved in dolichol biosynthesis on the cytoplasmic face of the ER, where N-glycosylation, O-mannosylation, C-mannosylation and GPI-anchor synthesis occurs. Besides the presence of glycosylated dolichol species in the ER, dolichol is present in all

subcellular membrane systems. Free alcohol and phosphorylated and esterified dolichol, are detected in peroxisomes and are highly enriched in lysosomes, the plasma membrane and Golgi vesicles.[34]

Mitochondrial membranes and nuclei also contain a limited amount of dolichol. Furthermore, dolichol plays a more general role in membrane trafficking, particularly between the ER and the lysosomal-endosomal system.[35] Given the ubiquity of dolichol in different cellular compartments, dolichol biosynthesis defects and accumulated dolichol precursors can presumably affect many other cellular processes independent of protein post-translational modifications. Interestingly, dolichol accumulation has been reported in neuronal ceroid lipofuscinosis (NCLs),[36-38] in which retinal degeneration occurs, while alterations in dolichol/Dol-P levels have been reported in patients with Alzheimer disease.[39]

Understanding the molecular mechanisms of DHDDS-related diseases is not trivial. Before the recent description of autosomal dominant forms[21-23], recessive inheritance was described in a fatal case with severe multi-organ involvement[15] as well as in isolated RP.[16-18] Assuming that human *cis*-PTase forms a heterodimer under physiological conditions, variants with a loss-of-function/hypomorphic behavior may lead to dolichol levels that are below the threshold necessary to fulfil its various biological roles in cells. The dolichol threshold concept could explain why heterozygous parents and siblings of patients carrying the p.Lys42Glu substitution underlying RP or the p.Trp64*/p.Cys148Glufs changes (DHDDS) and p.Arg290His (NgBR)[4] associated with CDG type I appear to be healthy individuals.[15-18] Indeed, the probability of being loss-of-function intolerant (pLI score, gnomAD ver 2.1.1), a metric used to determine the tolerance of a gene for LOF variants, supports *DHDDS* is not haploinsufficient (pLI= 0.25).

Interestingly, a recent study on the human NgBR/DHDDS complex suggested a higher oligomerization state of the complex whereby the two heterodimers were depicted to form a tetramer.[6] This observation introduces the possibility of a dominant-negative mechanism that may reduce *cis*-PTase activity of certain mutants to lower levels than expected. Based on these findings, we suggest the homozygosity for loss-of-function or strong hypomorphic variants underlies CDG type I (both DHDDS and NgBR), while *de novo* pathogenic variants are likely to have a dominant negative effect on heterodimer/tetramer assembly and function.

On the other hand, it has been suggested that p.Lys42Glu may affect only retinal photoreceptors because the highest level of DHDDS/NgBR enzymatic activity is required in

this tissue for opsin N-glycosylation.[16] Consistent with zebrafish studies indicating that a slight downmodulation of the endogenous gene is sufficient to cause photoreceptor degeneration, whereas a stronger knockdown causes a more complex phenotype, this change might be tolerated outside the retina.[17] This model, however, does not explain why the retina is not affected in subjects carrying dominant *DHDDS* pathogenic variants, suggesting a more complex scenario for these substitutions. In line of principle, single nucleotide changes at codon 42, not encompassing a CpG site, are predicted to lead to multiple amino acid substitutions, *i.e.* Gln and Glu (first position), Thr, Arg and Met (second position), and Asn (third position). The invariant association of RP with p.Lys42Glu suggests a neomorphic effect for this change or, more likely, a deleterious effect restricted to the retina (*e.g.*, altered interaction of defective DHDDS with a photoreceptor-specific protein or a tissue-specific toxic effect of accumulating isoprenoid compounds)[16,19]. In line with this hypothesis, in patients with DHDDS-related RP as well as in other CDGs involving dolichol metabolism,[32] D18 becomes the dominant species, with altered plasma and urinary D18/D19 ratios and a possible accumulation of damaging precursors in the retina.[20] Of note, the urinary dolichol D18/D19 ratio determined by LC-MS/MS was normal in patients carrying *de novo DHDDS* variants.

Finally, it is also worth noting that the broad phenotypic spectrum amongst patients harboring the common p.Arg211Gln mutation suggests additional factors, including environmental and genetic background, that may contribute to the final clinical picture.

Aberrant NgBR/DHDDS complex functioning is associated with a neurodegenerative disorder in the cortical myoclonus spectrum

The association between generalized epilepsy and cortical tremor or other myoclonic phenomena, which patients with AD pathogenic *DHDDS* and *NUS1* variants share, places the two disorders in the differential diagnosis of several syndromes in the cortical myoclonus spectrum, including progressive myoclonus epilepsy-ataxia (PME/PMA), and, for patients with milder presentations, benign adult familial myoclonus epilepsy (BAFME).[22,25,41]

Furthermore, the relatively high MD incidence, including parkinsonism in patients with *de novo DHDDS* and *NUS1* pathogenic variants, is indicative of a possible contribution of the NgBR-DHDDS complex to the pathogenesis of Parkinson's disease and neurodegenerative processes. In line with this finding Guo *et al*. reported that *NUS1* loss could reduce the number

of dopaminergic neurons with apoptosis in the fly brain.[26]

Overall, the prominent neurological phenotype of our cohort highlights the importance of dolichol metabolism in neuronal subcellular membrane systems and further broaden the clinical and biochemical characterization of *DHDDS*-related conditions, placing them in a spectrum that only partially overlaps with CDG type I. In line with this, serum glycoprotein hypoglycosylation was not observed in these patients and, in contrast to the RP phenotype[20] and other CDGs involving dolichol metabolism,[4,32] the urinary dolichol D18/D19 ratio determined by LC-MS/MS was normal.

Instead, electron microscopy of skin biopsy revealed abundant lipid-like material storage in myelinated fibers and Schwann cells, and secondary lysosomes in stromal fibroblasts suggesting a dysfunction of lysosomal enzymatic digestion machinery.

Consistently with this finding lysosomal cholesterol accumulation has been recently reported in fibroblasts of patients with *de novo NUS1* and *DHDDS* pathogenic variants.[42,43] Given that many lysosomal enzymes are highly N-glycosylated, hypo-glycosylation caused by impaired dolichol synthesis may be contributing factor to the neurological symptoms observed in the patients with defective DHDDS or NUS1[43].

The aberrant functioning of the ER-endolysosomal pathway and the association between cortical myoclonus, severe MD including parkinsonism, neurological deterioration, cerebral atrophy, and storage material in myelinated fibers and fibroblasts, supports the proximity of *DHDDS* related disorders to other inherited storage diseases such as NCLs and other lysosomal disorders.

Additional functional studies with future generation of knockout/knockin mice and iPSC derived neuronal cell models could provide a full understanding of the consequences of these and other *DHDDS* mutations on cellular organelle dynamics, dolichol metabolism, mammalian brain development and neurodegeneration pathways. Many aspects of dolichol dependent glycosylation including N-glycosylation, several steps of synthesis, recycling and regulation of dolichol availability are still largely unknown. This information would be critical not only to understand the biochemical underpinning of this disease, but also to consider the potential for specific disease-targeted therapeutic interventions.

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Competing interests

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DS consulted for Upsher-Smith, Biomarin, Neurogene Marinus and Ovid Therapeutics on unrelated subject matter. He also serves on the advisory board for the non-profit foundations SLC6A1 Connect and FamilieSCN2A.