https://doi.org/10.1093/brain/awae057 BRAIN 2024: 147; 1967–1974 | 1967

BRAIN REPORT

Digenic Leigh syndrome on the background of the m.11778G>A Leber hereditary optic neuropathy variant

Beryll Blickhäuser,[1,2,†] Sarah L. Stenton,[3,4,†] Christiane M. Neuhofer,[1,5] Elisa Floride,[6] Victoria Nesbitt,[7] Carl Fratter,[7] Johannes Koch,[8,9] Birgit Kauffmann,[10] Claudia Catarino,[2] Lea Dewi Schlieben,[1,5] Robert Kopajtich,[1,5] Valerio Carelli,[11,12] Alfredo A. Sadun,[13,14] Robert McFarland,[15,16] Fang Fang,[17] Chiara La Morgia,[11,12] Stéphanie Paquay,[18] Marie Cécile Nassogne,[18] Daniele Ghezzi,[19,20] Costanza Lamperti,[19] Saskia Wortmann,[8,9] Jo Poulton,[21] Thomas Klopstock[2,22,23] and Holger Prokisch[1,5]

[†]These authors contributed equally to this work.

Leigh syndrome spectrum (LSS) is a primary mitochondrial disorder defined neuropathologically by a subacute necrotizing encephalomyelopathy and characterized by bilateral basal ganglia and/or brainstem lesions. LSS is associated with variants in several mitochondrial DNA genes and more than 100 nuclear genes, most often related to mitochondrial complex I (CI) dysfunction. Rarely, LSS has been reported in association with primary Leber hereditary optic neuropathy (LHON) variants of the mitochondrial DNA, coding for CI subunits (m.3460G>A in MT-ND1, m.11778G>A in MT-ND4 and m.14484T>C in MT-ND6). The underlying mechanism by which these variants manifest as LSS, a severe neurodegenerative disease, as opposed to the LHON phenotype of isolated optic neuropathy, remains an open question. Here, we analyse the exome sequencing of six probands with LSS carrying primary LHON variants, and report digenic co-occurrence of the m.11778G > A variant with damaging heterozygous variants in nuclear disease genes encoding CI subunits as a plausible explanation.

Our findings suggest a digenic mechanism of disease for m.11778G>A-associated LSS, consistent with recent reports of digenic disease in individuals manifesting with LSS due to biallelic variants in the recessive LHON-associated disease gene *DNAJC30* in combination with heterozygous variants in CI subunits.

1 Institute of Neurogenomics, Computational Health Center, Helmholtz Zentrum München, 85764 Neuherberg, Germany
2 Friedrich-Baur-Institute, Department of Neurology, LMU University Hospital, Ludwig-Maximilians-Universität München, 80336 Munich, Germany
3 Division of Genetics and Genomics, Boston Children's Hospital, Boston, MA 02115, USA
4 Program in Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA
5 Institute of Human Genetics, School of Medicine, Technical University of Munich, 81675 Munich, Germany
6 Institute for Human Genetics, Paracelsus Medical University (PMU), 5020 Salzburg, Austria
7 NHS Highly Specialised Services for Rare Mitochondrial Disorders, Oxford University Hospitals NHS Foundation Trust, Oxford, OX3 7HE, UK
8 University Children's Hospital, Department of Neuropediatrics, Paracelsus Medical University (PMU), 5020 Salzburg, Austria

Received August 09, 2023. Revised December 01, 2023. Accepted January 28, 2024. Advance access publication March 13, 2024

9 Radboud Center for Mitochondrial Medicine, Department of Pediatrics, Amalia Children's Hospital, Radboudumc, 6525 Nijmegen, The Netherlands
10 Klinikum Bremen Mitte, Department of Pediatrics, Neuropediatrics, 28205 Bremen, Germany
11 IRCCS Istituto delle Scienze Neurologiche di Bologna, Programma di Neurogenetica, 40139 Bologna, Italy
12 Department of Biomedical and Neuromotor Sciences, University of Bologna, 40127 Bologna, Italy
13 Doheny Eye Institute, Pasadena, CA 91105, USA
14 Department of Ophthalmology, David Geffen School of Medicine, UCLA, Los Angeles, CA 10833, USA
15 Wellcome Centre for Mitochondrial Research, Translational and Clinical Research Institute, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK
16 NHS Highly Specialised Service for Rare Mitochondrial Disorders, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, NE2 4HH, UK
17 Department of Pediatric Neurology, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health, 100005 Beijing, China
18 Department of Neuropediatrics, University Hospital St Luc, UCLouvain, 1200 Bruxelles, Belgium
19 Unit of Medical Genetics and Neurogenetics, Fondazione IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) Istituto Neurologico Carlo Besta, 20133 Milan, Italy
20 Department of Pathophysiology and Transplantation, University of Milan, 20122 Milan, Italy
21 Nuffield Department of Women's and Reproductive Health University of Oxford, The Women's Centre, Oxford, OX3 9DU, UK
22 German Center for Neurodegenerative Diseases (DZNE), 81377 Munich, Germany
23 Munich Cluster for Systems Neurology (SyNergy), 81377 Munich, Germany

Correspondence to: Holger Prokisch
Institut für Humangenetik, Klinikum rechts der Isar der TUM
Trogerstraße 22, 81675 Munich, Germany
E-mail: holger.prokisch@helmholtz-munich.de

Keywords: Leigh syndrome spectrum (LSS); Leber hereditary optic neuropathy (LHON); digenic inheritance; mitochondrial complex I (CI)

Introduction

Leigh syndrome spectrum (LSS) is a progressive neurodegenerative syndrome and is the most frequent paediatric manifestation of a primary mitochondrial disease.[1] Less frequently, LSS is reported in adults. LSS is characterized by bilateral lesions in the brainstem and/or basal ganglia, often in combination with changes in the thalamus, cerebellum, subcortical white matter or spinal cord. Patients manifest with neurological symptoms, encompassing neurodevelopmental delay or regression, muscle weakness and hypotonia, extrapyramidal movement disorders, spasticity, ataxia, seizures and neuropsychiatric symptoms. The underlying defect in mitochondrial metabolism is evidenced by elevated lactate levels in plasma or CSF, an elevated lactate peak on magnetic resonance spectroscopy (MRS), or measurement of deficient respiratory chain (RC) enzyme activity in affected tissues, among others.[2]

To date, >100 monogenic causes of LSS have been reported, with damaging variants in genes of both the mitochondrial (mtDNA) and nuclear genome. Among mtDNA-encoded causes of LSS are the primary Leber hereditary optic neuropathy (LHON) variants m.3460G>A in MT-ND1, m.11778G>A in MT-ND4 and m.14484T>C in MT-ND6.[3-9] While these three variants account for the majority of reported LHON cases,[10] they are only rarely reported in LSS patients, with or without LHON features.

LHON is a remarkable mitochondrial disease, characterized by subacute (often sequential), bilateral, painless deterioration of central vision, resulting from selective retinal ganglion cell dysfunction and degeneration. Extraocular features are rare. Biochemically, mitochondrial complex I (CI) defects underlie most cases. The mechanism by which primary LHON variants present clinically with LSS or a LHON/LSS overlap syndrome is poorly understood, with speculative hypotheses spanning the role for modifying factors in the nuclear or mitochondrial DNA acting synergistically with the primary LHON variant to cause more severe, often multi-systemic disease (such as additional damaging variants and mitochondrial haplogroup)[4,8] and environmental factors (such as prematurity and oxygen therapy).[7]

Recently, we characterized a recessive form of LHON due to biallelic variants in the nuclear encoded gene *DNAJC30*, responsible for CI repair. Reflecting reports of primary LHON variants, we described three patients manifesting with LSS or a LHON/LSS overlap syndrome.[11,12] For each of these patients, in addition to the biallelic pathogenic *DNAJC30* variants, a rare heterozygous damaging variant was detected in a nuclear-encoded CI subunit, specifically in *NDUFS2* or *NDUFS8*.[11] Since our initial report, this finding has been recapitulated in two unrelated LSS probands, with biallelic *DNAJC30* variants plus a damaging heterozygous variant in *NDUFS8* and *NDUFA9*, respectively.[13,14] Given the role of DNAJC30 in CI repair and the location of the heterozygous second digenic hits in CI subunits, our findings suggested a digenic mechanism of disease for the *DNAJC30*-associated LSS phenotype. We therefore sought to determine if a similar mechanism may be responsible for the LSS manifestation in patients with primary LHON variants.

Herein, we characterize six probands with LSS harbouring LHON variants. For all five probands carrying the m.11778G>A variant, we report evidence supporting a digenic mechanism of disease consistent with reports for *DNAJC30*-associated LSS, providing a plausible explanation for the more severe disease manifestation in these individuals.

Materials and methods

Study participants

Patients with a clinical manifestation of LSS were recruited from six European academic hospitals. All patients with a primary LHON

variant (m.3460G>A in MT-ND1, m.11778G>A in MT-ND4 or m.14484T>C in MT-ND6) were further investigated by whole exome sequencing (WES) analysis. Patients with rarer LHON-associated mtDNA disease causing variants, whose extended phenotype could already be explained by the respective variant, were not included in the study. All study participants met the clinical diagnostic criteria for LSS as recently defined by the ClinGen Mitochondrial Disease Nuclear and Mitochondrial Variant Curation Expert Panel,[2] as follows: (i) brain imaging findings typical of LSS [bilateral T_2-weighted hyperintensities on MRI or hypodensities on CT in the brainstem and/or basal ganglia (symmetric), thalamus, cerebellum, subcortical white matter and/or spinal cord]; (ii) neurological symptoms (developmental regression, developmental delay and/or psychiatric symptoms); and (iii) biochemical findings (mtDNA variant known to cause CI deficiency, elevated plasma and/or CSF lactate, lactate peak on MRS, OXPHOS enzyme activity deficiency).

Molecular genetic investigation

Genomic DNA was extracted from blood using standard techniques followed by WES. No reported (likely) pathogenic biallelic variants in LSS disease genes were identified.[15] In a second step, all genes encoding CI subunits or assembly factors were screened for predicted loss of function (pLoF) and predicted deleterious functional (missense and in-frame indel) variants. A population allele frequency (af) filter of ≤1% in gnomAD v2 was applied.[16] Patient haplogroups were classified using Haplogrep 3.[17]

Structural modelling and analysis

The Cryo-EM (electron microscopy) structure of murine CI structure (6G2J) was obtained from the Protein Data Bank (PDB).[20] Visualization was performed using PyMOL.[21]

Statistical analysis

Comparison of observed and expected values for carriage of a damaging CI subunit variant. pLoF (frameshift, splice acceptor, splice donor and stop gained) variants plus missense variants were downloaded from the gnomAD v2 browser (https://gnomad.broadinstitute.org/) for all 37 nuclear-encoded CI subunits. All missense variants were annotated with REVEL[18] and only missense variants with a REVEL score of ≥0.773 were retained, in line with ClinGen recommendations for 'moderate' evidence of deleteriousness.[19] Variants with a global allele frequency >1% were removed. From the summed total of variant allele frequencies (total allele frequency), the expected number of individuals carrying a damaging variant in a CI subunit was calculated, with N being the total number of LSS probands included in the analysis (total af $\times 2 \times N$). To determine if the observed count of individuals carrying heterozygous damaging variants adjusts to the expected count by random chance, a goodness of fit test following a Chi-squared (χ^2) distribution with one degrees of freedom was applied.

Results

Primary LHON variants manifest as Leigh syndrome spectrum in six probands

Across participating centres, we collected a total of six patients clinically presenting with LSS that carried a homoplasmic primary LHON variant affecting CI, five with the m.11778G>A MT-ND4 variant (Probands 1–5) and one with the m.3460G>A MT-ND1 variant (Proband 6). A summary of the clinical features, brain MRI, ophthalmological and biochemical findings is displayed in Table 1, Fig. 1 and Supplementary Table 1.

Proband 1

Proband 1 presented with absence seizures, amblyopia and muscular coordination problems involving fine and gross motor skills. In subsequent years, he developed strabismus, trunk and gait ataxia, and behavioural problems, which appeared during a period of acute deterioration triggered by ethosuximide treatment, and subsequently by zonisamide treatment. He was treated with carnitine, idebenone, biotin, riboflavin and thiamine. On this therapy, there appears to have been an improvement in both neurological and ophthalmological symptoms. The seizures were successfully treated with sodium valproate.

Proband 2

Proband 2 presented with severe visual impairment. During his early years, he exhibited global developmental delay, ataxia and right hemiplegia. Initially, the clinical symptoms were attributed to prematurity; however, they progressively worsened over time, and in childhood he developed acute neurological deterioration. A clinical description of this case has already been reported in the literature, in a study focused on the potential influence of environmental factors, such as premature birth and oxygen therapy, on the progression of LHON.[7]

Proband 3

Proband 3 presented with gait disturbance and bilateral spastic-dystonic movement disorder. Apart from temporary strabismus at 2.5 years of age, that spontaneously resolved after 6 months, she had normal developmental milestones. At 7 years of age, she developed increasing gait abnormalities. Following genetic diagnosis, idebenone was started (900 mg/day). From 8 years of age, she developed a progressive bilateral spastic-dystonic movement disorder with severe scoliosis losing the ability to walk within 2 years. Currently, aged 12 years, she is virtually immobile with unaffected cognition and vision. She was intellectually bright but needed full motor support for tasks such as writing.

Proband 4

Proband 4 initially presented with bilateral internuclear ophthalmoplegia with lesions in the posterior dorsal midbrain and superior colliculus. A few months later, he developed bilateral sequential acute visual loss. In the next years he also developed ataxia, spastic quadriparesis, hypertrophic cardiomyopathy, respiratory failure, further impairments of vision and ophthalmoplegia.

Proband 5

Proband 5 presented with dystonia, coordination difficulties and dysarthria, with onset at 7 years of age. The brain MRI (images not available) showed atrophy and bilateral T_2 signal hyperintensities in the putamen and caudate.

Proband 6

Proband 6 presented with symptoms of progressive dystonia, choreoathetoid movement disorder, regression of intellectual and motor skills, and short stature. No visual symptoms have been reported.

Table 1 Clinical findings in primary LHON variant carriers manifesting with LSS

Proband ID	Primary LHON variant	Sex, ethnicity	Age at onset of neurological symptoms (years)	Age at last follow-up in years (current age)	Brain imaging findings	Neurologic symptoms	Biochemical findings
Proband 1	m.11778G>A (ND4)	M Caucasian European	7	8 (11)	Bilateral hyperintensities in thalamus and brainstem (Fig. 1A)	Developmental regression (motor), absence seizures, ataxia	Normal RC enzyme activity, elevated CSF lactate (2.4 mmol/l)
Proband 2	m.11778G>A (ND4)	M Caucasian European	11	21 (23)	Bilateral hyperintensities in mesencephalon, pons and medulla oblongata (Fig. 1B)	Developmental delay, ataxia, hemiplegia	Normal RC enzyme activity in liver and muscle, elevated CSF lactate
Proband 3	m.11778G>A (ND4)	F Caucasian European	7	12 (12)	Bilateral hyperintensities in putamen (Fig. 1C)	Developmental regression (motor), gait disturbance, spastic-dystonic movement disorder	CI defect in muscle (0.12 mUnit/mUnit CS), normal serum lactate (1.8 mmol/l)
Proband 4	m.11778G>A (ND4)	M Caucasian European	16	25 (25)	Hyperintensities in mesencephalon, pons and medulla oblongata (Fig. 1D)	Ataxia, spastic quadriparesis	RC enzyme activity not investigated, normal serum lactate (0.5 mmol/l and 1.8 mmol/l)
Proband 5	m.11778G > A (ND4)	F Caucasian European	7	15 (18)	Bilateral hyperintensities in putamen	Developmental regression (motor and intellectual), dystonia, dysarthria	RC enzyme activity not investigated, elevated serum lactate (3.2 mmol/l)
Proband 6	m.3460G > A (ND1)	F Caucasian European	7	13 (18)	Basal ganglia changes (symmetrical changes in caudate, putamen and lentiform) (Fig. 1E)	Developmental regression (motor and intellectual), dystonia, choreoathetoid movement disorder	CI defect, normal CSF and serum lactate

All mitochondrial variants were found at homoplasmy in the proband. All probands self-identified as Caucasian European. CSF lactate reference range: 1.1–2.1 mmol/l. Serum lactate reference range: <2 mmol/l. CI enzyme activity reference range: 0.14–0.35 mUnit/mUnit CS. CI = complex I; CS = citrate synthase; F = female; LHON = Leber hereditary optic neuropathy; LSS = Leigh syndrome spectrum; M = male; NA = not available; RC = respiratory chain.

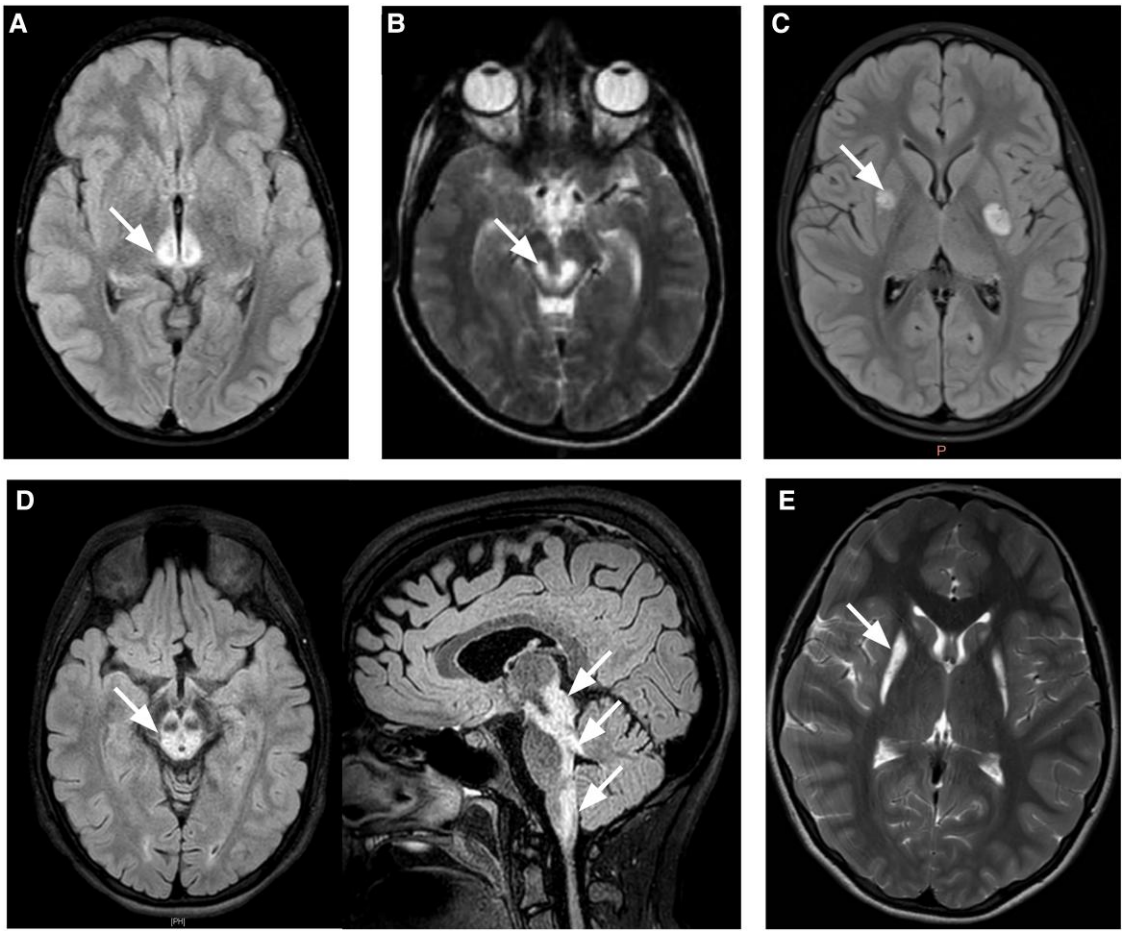

Figure 1 Brain MRI results from LSS probands. (**A**) Axial T^2 FLAIR-weighted MRI of Proband 1 (at 9 years of age), showing bilateral hyperintensities in thalamus (arrow). (**B**) Axial T_2-weighted MRI of Proband 2 showing bilateral increased signal in paramedian regions of mesencephalon (arrow). (**C**) Axial T_2 FLAIR-weighted MRI of Proband 3 (at 2.3 years of age) demonstrating bilateral hyperintensities in the putamen (arrow). (**D**) Axial (*left*) and sagittal (*right*) T_2 FLAIR-weighted MRI of Proband 4 demonstrating widespread confluent hyperintensities in mesencephalon (arrow on *left*), pons and medulla oblongata (arrows on *right*). (**E**) Axial T_2-weighted MRI of Proband 6 demonstrating bilateral hyperintensities of the putamen (arrow). LSS = Leigh syndrome spectrum.

Co-occurrence of the primary m.11778G>A LHON variant with complex I subunit variants in Leigh syndrome spectrum probands

We looked for an explanation for the difference in phenotype between LSS and LHON in patients with the primary LHON variants first by checking the haplogroups of the probands, finding four different top level haplogroups to be represented (haplogroups T, J, K and H) (Table 2). Next, we screened the WES of all six LSS probands for additional rare variants in CI subunits. This analysis revealed all five m.11778G>A probands to carry a rare heterozygous pLoF or predicted deleterious missense variant. In three probands, we detected variants in *NDUFS2*: a pLoF nonsense variant in Proband 1 [c.899G>A (p.Trp300Ter, absent from gnomAD, paternally inherited)], a missense variant in Proband 2 [c.412C>T (p.Arg138Trp, absent from gnomAD, REVEL 0.959, maternally inherited)] and a missense variant in Proband 3 [c.881G>A (p.Arg294Gln, gnomAD af 3.19×10^{-5}, REVEL 0.968)]. In Proband 4 we detected a rare missense variant in *NDUFS7* [c.364G>A (p.Val122Met, gnomAD af 5.69×10^{-5}, REVEL 0.835)] and in Proband 5, a rare pLoF frameshift variant in NDUFS8 [c.513dup (p.Glu172Ter, gnomAD af 7.99×10^{-6})] (Table 2).

NDUFS2, *NDUFS7* and *NDUFS8* are all reported recessive monogenic causes of Leigh syndrome[2] and are in the CI Q-module. To exclude monogenic cause in our probands, we closely examined each CI subunit for a second, potentially biallelic hit. In all five probands, we did not find any additional rare predicted damaging variants to constitute a recessive diagnosis, thereby, to the best of our knowledge, excluding these genes as the primary molecular genetic cause of LSS. Our finding of a second digenic hit in a CI subunit, and specifically in the Q module, is consistent with recent reports proposing a digenic mechanism for *DNAJC30*-associated LSS, where second hits were reported in the recessive Leigh syndrome-associated Q module subunit encoding genes *NDUFA9*, *NDUFS2* and *NDUFS8* (Table 2).

Next, we sought to determine if the m.11778G>A probands carried damaging variants in CI subunits at a significantly higher rate than expected by random chance. Based on the gnomAD v2 reference population ($n = 125\,748$ exomes), carriage of a rare pLoF or damaging missense variant in one of the 37 nuclear-encoded CI subunits is expected to occur only in 8.7 per 100 individuals. If we limit this analysis to the six Q module subunits in which all our proband's CI variants fall, the expected rate drops to 1.3 per 100 individuals. Our observation in five of five m.11778G>A probands is

Table 2 Genetic findings in LSS patients with primary LHON mtDNA variants or DNAJC30 variants

Reference	Proband ID, sex, phenotype	LHON-associated gene	LHON-associated variant[a]	CI subunit	CI variant[b]
LSS patients with primary LHON variants					
This study	Proband 1, Male, LSS	MT-ND4	m.11778G>A, p.Arg340His, homoplasmic, T2b	NDUFS2	c.899G>A, p.Trp300Ter, heterozygous, nonsense, pLoF
This study	Proband 2, Male, LSS	MT-ND4	m.11778G>A, p.Arg340His, homoplasmic, J2a2e	NDUFS2	c.412C>T, p.Arg138Trp, heterozygous, missense, REVEL 0.959
This study	Proband 3, Female, LSS	MT-ND4	m.11778G>A, p.Arg340His, homoplasmic, J2a1a1a	NDUFS2	c.881G>A, p.Arg294Gln, heterozygous, missense, REVEL 0.968
This study	Proband 4, Male, LSS + LHON	MT-ND4	m.11778G > A, p.Arg340His, homoplasmic, K1a+195	NDUFS7	c.364G>A, p.Val122Met, heterozygous, missense, REVEL 0.835
This study	Proband 5, Female, LSS	MT-ND4	m.11778G>A, p.Arg340His, homoplasmic, H1a1c	NDUFS8	c.513dup, p.Glu172Ter, heterozygous, frameshift, pLoF
LSS patients with *DNAJC30* variants					
Stenton *et al.*[11]	Patient 1, Female, LSS	*DNAJC30*	c.152A>G, p.Tyr51Cys, homozygous, NA	NDUFS8	c.305G>A, p.Arg102His, heterozygous, missense, REVEL 0.782
Stenton *et al.*[11]	Patient 2, Female, LSS	*DNAJC30*	c.152A>G, p.Tyr51Cys, homozygous, NA	NDUFS8	c.457T>C, p.Cys153Arg, heterozygous, missense, REVEL 0.926
Stenton *et al.*[11]	Patient 3, Male, LSS + LHON	*DNAJC30*	c.152A>G, p.Tyr51Cys, homozygous, NA	NDUFS2	c.980A>G, p.Tyr327Cys, heterozygous, missense, REVEL 0.935
Zawadzka *et al.*[13]	Patients 2 + 3, Male +Female (siblings), LSS	*DNAJC30*	c.152A>G+c.130_131del, p.Tyr51Cys +, p.Ser44ValfsTer8, compound heterozygous, NA	NDUFS8	c.484G>T, p.Val162Leu, heterozygous, missense, REVEL 0.783
Nesti *et al.*[14]	Patient 1, Male, LSS + LHON	*DNAJC30*	c.130_131del, p.Ser44ValfsTer8, homozygous, NA	NDUFA9	c.801-1G>C, heterozygous, splice, pLoF

Transcripts used for variant annotation: *DNAJC30*, ENST00000395176; *NDUFA9*, ENST00000266544; *NDUFS2*, ENST00000367993; *NDUFS7*, ENST00000233627; *NDUFS8*, ENST00000313468. CI = complex I; LSS = Leigh syndrome spectrum; LHON = Leber hereditary optic neuropathy; NA = not available; pLoF = predicted loss-of-function.
[a]Genomic position, amino acid (AA) change, heteroplasmy level/zygosity, haplogroup.
[b]Genomic position, AA change, zygosity, consequence, *in silico* prediction.

significantly above expectation when compared both to the expected rate for all CI subunits (observed = 5, expected = 0.433483, χ^2 statistic = 52.672, df = 1, *P*-value = 3.941×10^{-13}) and Q module subunits only (observed = 5, expected = 0.06591199, χ^2 statistic =374.29, df = 1, *P*-value < 2.2×10^{-16}). In contrast to the m.11778G>A probands, the LSS proband carrying the m.3460G>A variant did not carry any additional rare variants of interest in CI subunit encoding genes to explain the more severe phenotype.

To ensure that this constellation of variants is not found in other LHON patients or asymptomatic carriers of the same primary homoplasmic LHON variants, we screened 10 additional individuals with the m.11778G>A variant and 17 additional individuals with the m.14484T>C variant that had available exome sequencing for analysis across our centres. We found no additional rare, potentially damaging, variants in CI subunits.

Discussion

The primary LHON mtDNA variants are known for variable penetrance and expressivity, spanning from the asymptomatic carrier, to isolated optic nerve involvement and, in rare cases, to more severe multisystem phenotypes, such as those resembling LSS, as reported here. Focusing on variable expressivity, the mechanism responsible for the observed discrepancy in clinical manifestation in symptomatic individuals remains, to date, an open question. A contributory role of secondary genetic modifiers has been suggested,[4,8] but was only reported in one family with two affected siblings presenting with dystonia, motor delay and MRI findings consistent with LSS, with co-occurrence of the m.11778G>A (MT-ND4) primary LHON variant and a novel m.4716C G (MT-ND2) variant at high heteroplasmy level. The novel variant is, however, only moderately evolutionarily conserved and thereby of questionable functional relevance.[8]

Analysing both the mitochondrial and nuclear DNA by WES in our cohort, we consistently observe a second digenic hit in a CI Q-module subunit in all five of the LSS probands carrying the m.11778G>A (MT-ND4) variant at homoplasmy. Each of these variants is nuclear encoded, heterozygous, rare (af <0.0001%) and either nonsense, frameshift or missense in nature, with all missense variants being associated with a moderately deleterious REVEL prediction indicating probable LOF.[22] The carriage of a variant of this nature in a CI subunit is expected by chance in an estimated 8.7 per 100 individuals and 1.3 per 100 for the Q module, which is significantly lower than our observed rate (5 out of 5), and was not detected in 27 homoplasmic LHON variant carriers without LSS. In all cases, the identified digenic hit was monoallelic. The chance that we missed deep intronic functional relevant compound heterozygous variants to constitute recessive diagnoses in all five cases is extremely low. Nevertheless, the unavailability of patient tissues (e.g. fibroblasts) to investigate this further, such as by RNA sequencing, is a recognized limitation of our study. As we did not find additional rare, potentially deleterious variants in the CI subunits in LSS patients with m.3460G>A, and this study does not provide evidence for a similar pathomechanism for this variant.

Our observation of a second digenic hit in a CI subunit (NDUFS2, NDUFS7 and NDUFS8) in ND4 variant carriers with LSS is consistent with recent reports proposing a digenic mechanism for *DNAJC30*-associated LSS, where second digenic predicted damaging heterozygous hits have been reported in *NDUFA9*, *NDUFS2* and *NDUFS8*, in a total of five probands to date (Table 2).[11,13,14] Notably, each of the subunits in which variants have been observed

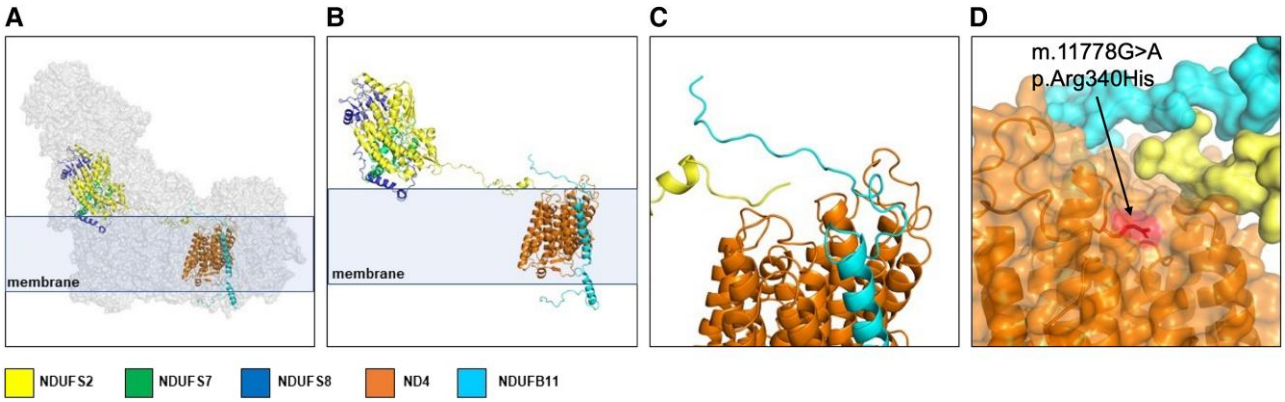

Figure 2 Mitochondrial complex I structure highlighting Q module and ND4 subunits in which deleterious variants were detected. Cryo-EM structure of the murine mitochondrial complex I (CI) (PDB: 6g2j). Single subunits highlighted: NDUFS2 in yellow, NDUFS7 in green, NDUFS8 in blue, ND4 in orange and NDUFB11 in cyan (interaction partner from DNAJC30). (**A**) Overview of the whole CI with inner mitochondrial membrane. (**B**) Location of subunits NDUFS2, 7, 8, ND4 and NDUFB11. (**C**) Detailed view showing proximity between the N-terminus of NDUFS2, ND4 and NDUFB11. (**D**) Potential contact sites between NDUFS2, ND4 and NDUFB11. LHON variant m.11778G>A, p.Arg340His highlighted in red. LHON = Leber hereditary optic neuropathy.

belong to the CI Q-module, spanning *NDUFA9*, *NDUFS2*, *NDUFS7* and *NDUFS8* for *DNAJC30* and m.11778G>A variant carriers, collectively. Moreover, biallelic variants in each of these Q module subunit-encoding genes are associated with recessive LSS.[2] In most recessive patients, the disease progression was significantly more severe than in the cases described here. CI comprises three structurally and functionally defined modules, the N module, that binds and oxidizes NADH; the Q module, responsible for the transfer of electrons to ubiquinone; and the P module, responsible for proton pumping.[23] At the molecular level, the chaperone DNAJC30 is required for the assembly and disassembly of the N module upon the Q module of CI. This is the final step in the assembly of CI. Given that it is a reversible step, DNAJC30 allows replacement of damaged N module subunits. Patients with LOF variants in *DNAJC30* are known to accumulate defective CI.[12] We therefore hypothesize that DNAJC30 acts synergistically with the deleterious variants in NDUFS2, NDUFS7 or NDUFS8 within the Q module to cause the more severe LSS phenotype, likely due to destabilizing the structural integrity and thereby interfering with fully functional assembly of CI. Although the m.11778G>A (MT-ND4) variant is in the P module, far away from the Q module, there is an extension of the N-terminus of the Q module subunit NDUFS2 towards ND4 (Fig. 2). Direct contact between the NDUFS2 and ND4 subunits may explain the observed synergistic effect of the deleterious Q module and ND4 variants. NDUFS2 has the properties of a molecular tether, facilitating membrane integration of CI, and is required to add the final N module to the complex. This interpretation is underscored both by the location of the m.11778G>A variant, p.Arg340His, at the contact point between NDUFS2 and ND4, and by observation that the turnover of the N module is specifically reduced in patients with the m.11778G>A variant, but not in the LHON-associated variant in ND1 (m.3460G>A).[12]

In conclusion, the genetic findings presented here support our hypothesis that the unusually severe clinical manifestation of LSS in patients who harbour the primary m.11778G>A (MT-ND4) LHON variant is caused by a synergistic digenic impairment of CI. The fact that all variants affect the Q module, especially NDUFS2, suggests a mechanistic interaction between the Q module and ND4 and underscores the function of NDUFS2 as a molecular tether for the N, Q and P modules and for the complete functional assembly of CI.

Data availability

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

Acknowledgements

We thank our patients, their families and the clinicians who cared for them. S.W. is a member of the European Reference Network for Rare Hereditary Metabolic Disorders (MetabERN)—Project ID No 739543.

Funding

This study is supported by the German Federal Ministry of Education and Research [Bundesministerium für Bildung und Forschung (BMBF), Bonn, Germany] and Horizon2020 through the European Joint Programme on Rare Diseases 'GENOMIT' (01GM1920A, genomit.eu), as well as a BMBF grant to the 'German Network for Mitochondrial Disorders' (mitoNET, 01GM1906D to H.P. and 01GM1906A to T.K.). S.L.S. is supported by a fellowship from the Manton Center for Orphan Disease Research, Boston Children's Hospital and an Early Career Award from the Thrasher Research Fund. V.N. and J.P. had salary support from the UK NHS Highly Specialised Service for Rare Mitochondrial Disorders of Adults and Children. This study is supported by the ERA PerMed project 'Personalized Mitochondrial Medicine (PerMiM): Optimizing diagnostics and treatment for patients with mitochondrial diseases' [Austrian Science Funds to S.B.W. (4704-B), BMBF to H.P. (01KU2016A)].

Competing interests

T.K., V.C., H.P. and A.S. have received research support, speaker honoraria, consulting fees and travel reimbursement from Santhera Pharmaceuticals, Chiesi GmbH and GenSight Biologics.

Supplementary material

Supplementary material is available at *Brain* online.

References

1. Barca E, Long Y, Cooley V, et al. Mitochondrial diseases in North America: An analysis of the NAMDC registry. *Neurol Genet*. 2020; 6:e402.
2. McCormick EM, Keller K, Taylor JP, et al. Expert panel curation of 113 primary mitochondrial disease genes for the Leigh syndrome spectrum. *Ann Neurol*. 2023;94:696-712.
3. Miyaue N, Yamanishi Y, Tada S, et al. Repetitive brainstem lesions in mitochondrial DNA 11778G>A mutation of Leber hereditary optic neuropathy. *eNeurologicalSci*. 2019;14:74-76.
4. Fruhman G, Landsverk ML, Lotze TE, et al. Atypical presentation of Leigh syndrome associated with a Leber hereditary optic neuropathy primary mitochondrial DNA mutation. *Mol Genet Metab*. 2011;103:153-160.
5. Ji K, Zheng J, Sun B, et al. Novel mitochondrial C15620A variant may modulate the phenotype of mitochondrial G11778A mutation in a Chinese family with Leigh syndrome. *Neuromolecular Med*. 2014;16:119-126.
6. Funalot B, Reynier P, Vighetto A, et al. Leigh-like encephalopathy complicating Leber's hereditary optic neuropathy. *Ann Neurol*. 2002;52:374-377.
7. Paquay S, Benoit V, Wetzburger C, et al. Uncommon Leber "plus" disease associated with mitochondrial mutation m.11778G>A in a premature child. *J Child Neurol*. 2014;29:NP18-NP23.
8. McFarland R, Chinnery PF, Blakely EL, et al. Homoplasmy, heteroplasmy, and mitochondrial dystonia. *Neurology*. 2007;69:911-916.
9. Grazina MM, Diogo LM, Garcia PC, et al. Atypical presentation of Leber's hereditary optic neuropathy associated to mtDNA 11778G>A point mutation–A case report. *Eur J Paediatr Neurol*. 2007;11:115-118.
10. Yu-Wai-Man P, Chinnery PF. *Leber hereditary optic neuropathy*. University of Washington; 2021.
11. Stenton SL, Tesarova M, Sheremet NL, et al. DNAJC30 defect: A frequent cause of recessive Leber hereditary optic neuropathy and Leigh syndrome. *Brain*. 2022;145:1624-1631.
12. Stenton SL, Sheremet NL, Catarino CB, et al. Impaired complex I repair causes recessive Leber's hereditary optic neuropathy. *J Clin Invest*. 2021;131:e138267.
13. Zawadzka M, Krygier M, Pawłowicz M, et al. Expanding the phenotype of DNAJC30-associated Leigh syndrome. *Clin Genet*. 2022;102:438-443.
14. Nesti C, Ticci C, Rubegni A, et al. Additive effect of DNAJC30 and NDUFA9 mutations causing Leigh syndrome. *J Neurol*. 2023;270: 3266-3269.
15. Landrum MJ, Lee JM, Benson M, et al. ClinVar: Public archive of interpretations of clinically relevant variants. *Nucleic Acids Res*. 2016;44(D1):D862-D868.
16. Karczewski KJ, Francioli LC, Tiao G, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*. 2020;581:434-443.
17. Schönherr S, Weissensteiner H, Kronenberg F, Forer L. Haplogrep 3—An interactive haplogroup classification and analysis platform. *Nucleic Acids Res*. 2023;51(W1):W263-W268.
18. Ioannidis NM, Rothstein JH, Pejaver V, et al. REVEL: An ensemble method for predicting the pathogenicity of rare missense variants. *Am J Hum Genet*. 2016;99:877-885.
19. Pejaver V, Byrne AB, Feng BJ, et al. Calibration of computational tools for missense variant pathogenicity classification and ClinGen recommendations for PP3/BP4 criteria. *Am J Hum Genet*. 2022;109:2163-2177.
20. Berman HM, Westbrook J, Feng Z, et al. The protein data bank. *Nucleic Acids Res*. 2000;28:235-242.
21. PyMOL. Accessed 7 July 2023. https://pymol.org/2/
22. Hopkins JJ, Wakeling MN, Johnson MB, Flanagan SE, Laver TW. REVEL is better at predicting pathogenicity of loss-of-function than gain-of-function variants. *medRxiv*. [Preprint] doi:10.1155/2023/8857940
23. Guerrero-Castillo S, Baertling F, Kownatzki D, et al. The assembly pathway of mitochondrial respiratory chain Complex I. *Cell Metab*. 2017;25:128-139.