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Novel *NDUFA12* variants are associated with isolated complex I defect and variable clinical manifestation

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ABSTRACT

Isolated biochemical deficiency of mitochondrial complex I is the most frequent signature amongst mitochondrial diseases and is associated with a wide variety of clinical symptoms. Leigh syndrome represents the most frequent neuroradiological finding in patients with complex I defect and >80 monogenic causes have been involved in the disease. In this report, we describe 7 patients from four unrelated families harbouring

novel *NDUFA12* variants, 6 of them presenting with Leigh syndrome. Molecular genetic characterization was performed using next generation sequencing combined with the Sanger method. Biochemical and protein studies were achieved by enzymatic activities, blue native gel electrophoresis and Western blotting. All patients displayed novel homozygous mutations in the *NDUFA12* gene leading to the virtual absence of the corresponding protein. Surprisingly, despite in none of the analyzed patients NDUFA12 protein was detected, they present a different onset and clinical course of the disease. Our report expands the array of genetic alterations in *NDUFA12* and underlines phenotype variability associated with *NDUFA12* defect.

Key words: mitochondrial disease, Leigh syndrome, NADH ubiquinone oxidoreductase, *NDUFA12*

INTRODUCTION

Mitochondrial complex I (CI; NADH-ubiquinone oxidoreductase) is the largest enzyme of the respiratory chain. It is composed of 45 subunits, 14 of which are highly conserved from the bacteria to humans and represent the enzymatic core of the enzyme whereas the others are considered as accessory or supernumerary subunits (Bridges et al, 2020). Amongst the core subunits, 7 are hydrophilic and are encoded by the nuclear DNA (NDUFS1, NDUFS2, NDUFS3, NDUFS7, NDUFS8, NDUFV1 and NDUFV2) whereas the remaining are hydrophobic and are encoded by the mitochondrial DNA (ND1, ND2, ND3, ND4, ND4L, ND5, ND6). The core subunits house the catalytic activity of the enzyme due to the presence of one flavin mononucleotide (FMN) and eight iron-sulfur [Fe-S] clusters, which ensure the electron transfer from the NADH to the ubiquinone. The role of the accessory subunits is still controversial but one of the hypotheses is that they are essential for the structure and stability of CI.

NDUFA12 is a small hydrophobic accessory subunit of the transmembrane region of CI, likely not involved in CI catalytic activity (Rak and Rustin, 2014). A mutation in *NDUFA12* has been associated with Leigh syndrome (LS; MIM# 256000) and CI defect (CI; MIM# 252010) in only one patient (Ostergaard et al., 2011). Mitochondrial CI defects represent the most common inborn error of mitochondrial metabolism in childhood, accounting for the 20-30% of all mitochondrial diseases (Fassone & Rahman, 2012; Mayr et al.,2015). The spectrum of clinical phenotype is wide and includes severe encephalopathy, fatal infantile lactic acidosis, cardiomyopathy, myopathy and hepatopathy. The most frequent clinical presentation linked to CI deficiency is LS, a progressive neurodegenerative disorder, which includes a heterogeneous group of clinical conditions whose peculiar neuroradiological hallmark is the bilateral symmetrical lesions in the brainstem and basal ganglia. First symptoms usually appear soon after birth, typically after a normal developmental period, and neurological signs can include dystonia, failure to thrive, spasticity, ataxia, optic atrophy, nystagmus, hypotonia ultimately leading to death (Leigh, Al-Sarraj, & DiMauro, 2015; Lake et al., 2016). However, long survival cases as well as adult-onset presentation can occur rarely (Bannwarth et al., 2013; La Piana et al., 2017). The identification of the genetic cause in LS is extremely complicated since more than 80 genes have been implicated in this and all forms of inheritance are possible syndrome (Rahman & Thorburn, 2020). Nevertheless, the introduction of the next generation sequencing (NGS) for the molecular analysis of patients with suspect of mitochondrial disease has been proven to be crucial for the molecular diagnosis of many undiagnosed cases (Pronicka et al., 2016).

In the present work, we report the findings of two unrelated singleton patients and three and two siblings from two additional unrelated families, all carrying novel mutations in *NDUFA12* gene. To the best of our knowledge, only one case with a homozygous

NDUFA12 mutation has been reported so far; therefore, with this report we confirm the causative link between *NDUFA12* mutations and LS, expanding the series of patients and genotypes associated with this gene.

METHODS

Standard protocol approvals, registrations, and patient consent

The study was approved by the Ethical Committees of the Bambino Gesù Children's Hospital, Rome (Italy), the ''C. Besta'' Neurological Institute, Milan (Italy), the Necker Hospital, Paris (France) and the UCL Queen Square Institute of Neurology, London (UK), in agreement with the Declaration of Helsinki. Informed consent for molecular genetic analysis was obtained from the patient's parents.

Next Generation Sequencing, Variant Prioritization and Sanger Sequencing

Total DNA from patients and parents has been isolated using standard procedures. Patients were screened by different NGS approaches. For Pt1 the Trusight One panel (clinical exome, Illumina, San Diego, CA, USA), comprehensive of greater than 4.800 clinically relevant genes, was run on a Miseq system (Illumina, San Diego, CA, USA). For Pt2, in-solution targeted enrichment of exonic sequences was performed by using the 50 Mb SureSelect Human All Exon kit (Agilent). The library was run on the GAIIx system (Illumina, San Diego, CA, USA).

Prioritization of the variants was achieved by selecting variants having allele frequency <0,01 according to public database (Exome Variant Server, http://evs.gs.washington.edu/EVS; gnomAD, http://gnomad-old.broadinstitute.org; ExAC, http://exac.broadinstitute.org) or in house database, quality scores (e.g. coverage >20), amino acid impact (High or Moderate according to *in-silico* prediction tools),

possible clinical significance and a recessive inheritance pattern (Legati et al., 2016;

Saoura et al., 2019). Sanger sequencing analysis, using BigDye chemistry 3.1 and run on an ABI 3130XL automatic sequencer (Applied Biosystems, Life Technologies), was performed to confirm the presence of the identified variants in the probands and to follow the segregation analysis within the family members.

For Pt3, whole exome sequencing and subsequent data analysis were carried out in collaboration with Commissariat à l'Energie Atomique (CEA)/Institut de Génomique (IG)/Centre National de Génotypage (CNG) – INSERM and were described previously (Gardeitchik et al., 2018). For Pt6 and Pt7 WES and segregation analysis using Sanger sequencing was performed by Centogene (Bauer et al., 2019).

Quantitative Real-Time PCR

For *NDUFA12* transcript expression, total RNA was isolated from skin fibroblasts using Total RNA Purification plus Kit (Norgen Biotech corp, Canada) according to the manufacturer instructions. 1 μg of RNA was reverse transcribed using EuroScript M-MLV RT kit (Euroclone S.p.A., Pero (Mi)) and 0,5 μl of cDNA were either PCR amplified or subjected to quantitative real-time PCR and run in a ABI PRISM 7500 Sequence Detection System (Life Technologies) using Power SYBR® Green I dye chemistry; expression of the glucuronidase beta (*GUSB*) transcript was used for normalization.

Biochemical and protein studies

Respiratory chain complexes and citrate synthase activity was assayed in muscle homogenate of Pt1, Pt2 and Pt3, according to previously reported methods (Bugiani et al., 2004). Oxygen consumption rate (OCR) in Pt1 and Pt2 was measured in fibroblasts

with an Extracellular Flux Analyzer (Seahorse XF96 Bioscience, Agilent, Santa Clara, CA), as described (Invernizzi et al., 2012). Subsequent functional studies were performed on available biological samples.

For protein expression analysis, muscle and skin fibroblasts were processed with common procedures and subjected to SDS-PAGE. Respiratory chain complexes assembly state was analyzed by BNGE (Díaz, Barrientos, & Fontanesi, 2009) and analysed either by immunoblot or with *in-gel* activity assay (Zerbetto et al., 1997; Torraco et al., 2017).

The following antibodies were used for immunodetecion: complex I-NDUFA9; complex II-SDHA, complex III-UQCRC2, complex IV-COXIV(Abcam), MTCOI (Thermo Fisher scientific); NDUFA12 (Proteintech, Manchester, UK); VDAC (Proteintech, Manchester, UK) was used as loading control. Reactive bands were detected using Lite Ablot Extend Long Lasting Chemiluminescent Substrate (Euroclone, Pero (Mi), Italy). Densitometry analysis was performed using Quantity One software (BioRad, Hercules, CA, USA). For protein experiments samples concentration was determined by BCA assay (Thermo Fisher scientific).

Statistical analysis

For functional studies on human samples, data are presented as mean ± SD. The Student's t-test was used for the analysis of statistical significance.

RESULTS

Patients description and brain MRI findings

Clinical and MRI data of patients described are summarized in table 1.

Patient 1 (Pt1) is the second child of consanguineous parents of Italian origin. He was born preterm after an uneventful pregnancy at 35 weeks of gestation by cesarean section. From birth, poor sucking and hypotonia were noted. He presented delayed motor skills acquisition and started to walk at 2 years (yrs) of age. At 4 yrs of age the patient presented a sudden onset of convergent strabismus with ptosis in the left eye, with consequent involvement of the right eye as well, which improved during the follow-up. Brain MRI performed at 5 yrs showed bilateral and symmetric T2 hyperintense lesions in the brainstem (red nuclei and tegmental tract) (Figure 1A-B); the MRS was normal. Evoked visual potential (VEP) disclosed an increased latency in both eyes (left>right) and electroretinogram (ERG) was normal. At 5 yrs of age the neurologic examination revealed exclusively a convergent strabismus with minimal ptosis in the left eye, minimal horizontal nystagmus with partial limitations of horizontal ocular movements. He then started treatment with coenzyme Q_{10} (CoQ_{10}), idebenone and riboflavin. At 7 yrs neurological examination showed only minimal horizontal nystagmus, no diplopia nor ophthalmoparesis. Motor function was normal. Brain MRI surprisingly disclosed no signal alteration in the brainstem (Figure 1 C-D). ECG was normal and heart ultrasound revealed a patent foramen ovale with no significant shunt. VEP and ERG were unmodified from the previous evaluations. Brainstem auditory evoked potentials (BAEPs) were normal. At the last examination, at 9 yrs of age, neurological examination showed the same clinical pattern. He repeated a brain MRI that again showed no lesions (not shown). Blood lactic acid was always in the normal range with the exception of one test that was found slightly elevated (2.85 mmol/L; nv 0.5-2.20 mmol/L); plasma aminoacids and urine organic acids were always normal.

Patient 2 (Pt2) is the only child of Italian consanguineous parents. She was born preterm after an uneventful pregnancy at 32 weeks of gestation by urgent cesarean section for

mother eclampsia. At birth, she presented with respiratory distress that required oro-tracheal intubation. Psychomotor development was referred to be normal until the age of 6 yrs when she presented with dystonia of the right arm. The clinical picture rapidly deteriorated until the baby girl lost the autonomous ambulation at 9 yrs of age. Neurological examination at that time showed a spastic-dystonic syndrome together with oromandibular dystonia. Brain MRI showed a typical LS pattern. She then started treatment with CoQ_{10} and riboflavin. Her condition worsened over time and at 13 yrs of age neurological examination showed a severe spastic-dystonic tetraparesis (the patient was unable to maintain the sitting position) and scoliosis; brain MRI disclosed bilateral lesions in the basal ganglia (putamen) (Figure 1 E-F), partial agenesis of septum pellucidum and mild enhancement of left optic nerve after gadolinium; MRS was normal. VEP was abnormal in amplitude in both eyes. At last clinical evaluation, at 15 yrs of age, the neurological picture was quite stable, VEP was worsened compared to the previous ones. Cardiological evaluation was normal. Lactic acid was elevated in blood (3.62 mmol/L; nv 0.5-2.20 mmol/L) and urine (>400 mmol/mol creatinine; nv <200 mmol/mol creatinine); plasma amino acids showed an increase of alanine (852 mmol/L; nv 150 – 400 mmol/L).

Patient 3 (Pt3) is the second girl of four siblings born from consanguineous parents of Moroccan origin. She was born at term after a normal pregnancy. At birth, she had respiratory distress with APGAR 3 and 8 at 1 and 5 minutes of life respectively. After a normal development, at 4 yrs of age she suddenly presented nystagmus and right hemiparesis. Brain MRI performed at that time showed T2 hypersignal of lenticular nuclei and brainstem, and spectroscopy detected a lactate peak (not shown). Metabolic profile showed moderate lactate increase in blood (lactate 2.4 mmol/L; nv 0.50-1.95 mmol/L) and CSF (lactate 2.8 mmol/L; nv 1-1.90 mmol/L). She progressively lost the

autonomous ambulation and language and neurological examination at 5 yrs of age revealed trunk hypotonia and extrapyramidal syndrome. Nerve conduction study disclosed peripheral neuropathy. She was given biotin and thiamin supplementation that improved her condition (she started holding the head and sitting alone). Her dystonia progressively worsened with completely loss of walk and poor language. She is now 13 yrs old.

Her 2 yrs younger sister (Pt4) had a completely normal psychomotor development until aged 9 and she suddenly developed visual impairment. Clinical examination revealed extrapyramidal syndrome and brain MRI showed lenticular nuclei involvement. She also had hyperlactatorachia (lactate 2.8 mmol/L; nv 1-1.90 mmol/L) but normal blood lactate.

Their younger sister (Pt5) developed at 7 yrs of age visual impairment due to isolated optic atrophy. She is now 7 yrs and 5 months old and does not present any other symptom. Brain MRI was normal. She has hyperlactatorachia (2.4 mmol/L; control values 1-1.90 mmol/L).

Patient 6 (Pt6) is the second child from a healthy first cousin parents of Syrian origin. Psychomotor development was normal until the age of 5, then he started to get abnormal gait as elevated heels, recurrent falling with abnormal hand movements as dystonic especially at the left side. Episodes of vomiting were present lasting for 2 days and controlled without hospitalization. No history of epilepsy was ever recorded. On last examination at the age of 11 yrs, the boy was very reactive to surrounding, and did not show cognitive or language impairment. The upper and lower limbs (left >right) showed increased tone, rigidity and abnormal dystonic posture of the hands and feet worsened by action. Nevertheless, the course was stationary as the child is still ambulant and maintained all his routine activities. Fundus examination, VEP, ERG and BAEPs were normal. The serum lactate was mildly elevated (2.6 mmol/L; nv 0.5-2.20 mmol/L).

Acylcarnitine profile and urinary organic acids were in the normal range. Brain MRI, performed at 6 yrs, (Figure 1G-H) showed bilateral high signal intensity at T2W and T2-FLAIR in lentiform nucleus, however, it involved the whole lentiform nucleus in right side while only the putamen in left side. There was also high signal included the red nucleus in brain stem. MRS was normal.

Patient 7 (Pt7) is the younger sister of Pt6 with earlier onset and more severe progression than her eldest brother. The pregnancy and delivery histories were unremarkable again. Psychomotor development was normal till the age of 3 yrs and a half when she gradually started to lose motor skills; no history of vomiting or seizures were recorded. On examination, at the age of 4 yrs and 8 months, the girl could walk or stand only with support, with rigid gait, tip-toe walking and dystonic movement. Similarly, the left side was more severely affected than the right. Oromandibular dystonia was also present and pronunciation of words was unclear. Neurological assessment revealed increased rigidity of the limbs (lower limbs > upper limbs and left > right), dystonia on initiation of movement. Serum lactate was mildly elevated (2.2 mmol/L; nv 0.5-2.20 mmol/L); acylcarnitine profile and urinary organic acids were in the normal range. Brain MRI have been not performed yet.

Biochemical studies

Respiratory chain complex activity measured in muscle homogenate of Pt1, Pt2 and Pt3 showed an isolated defect of CI in all patients tested (Figure 2A). Moreover, in fibroblasts from Pt1 and Pt2 respiratory capacity, assessed by micro-oxygraphy, was decreased in galactose-medium after 12 days, thus indirectly confirming an impairment of the mitochondrial respiratory chain (Figure 2B).

NGS analysis

Bioinformatics analysis carried out on NGS data of Pt1, Pt2, Pt3, and Pt6 led to the identification of four novel homozygous variants in *NDUFA12* (NM_018838.5; NP_061326.1), a gene encoding for a subunit of mitochondrial CI. No other potential pathogenic variants in genes known or predicted to be associated with mitochondrial disease were found in none of the patients. In particular, we identified the *NDUFA12* nucleotide changes c.86G>A (p.Arg29Lys) in Pt1, c.224G>A (p.Trp75*) in Pt3, c.253G>T (p.Glu85*) in Pt6, and the c.395delA (p.Lys132Argfs*50) in Pt2. All mutations segregated within the family members with the parents being heterozygous carriers of the respective mutations. Pt4 and Pt5 were homozygous for the c.224G>A, and Pt7 was homozygous for the c.253G>T mutation (Figure 3A-B). With the exception of the p.Arg29Lys that was considered a VoUS, all the other were considered pathogenic or likely pathogenic according to the ACMG criteria (Table 1) (Richards et al., 2015). In addition, the c.86G>A transition altered the canonical splicing donor site of exon 1, and possibly affected the splicing process, according to *in silico* prediction by HSF.

Real-Time PCR

To assess whether the c.86G>A variant could affect the canonical splicing, we PCR amplified the full-length transcript of *NDUFA12*, both in Pt1 and control cDNA. In the control subject we obtained a band of the expected size (288 bp) whereas in Pt1 we observed a fragment of about 800 bp compatible with retention of intronic regions (Figure 4A). Sequencing analysis revealed the formation of a cryptic exon by the retention of the first 472 bp of intron 1. The aberrant transcript has an altered reading frame which led to the formation of a premature stop codon and the synthesis of an aberrant, truncated protein (p.Arg29Lysfs*2) (Figure 4B). Expression analysis of *NDUFA12* transcript performed in Pt1 and Pt2 by real-time PCR revealed a dramatic

reduction of *NDUFA12* mRNA, in both patients (Figure 4C). In particular, we found an almost undetectable expression of *NDUFA12* in Pt1 and a 90% reduction in Pt2, suggesting mRNA decay associated with c.86G>A and c.395delA variants.

SDS-PAGE and BNGE

In order to evaluate the impact of the c.86G>A/p.Arg29Lysfs*2 and the c.395delA/p.Lys132Argfs*50 variants on NDUFA12 protein stability, we performed western blot analysis on skeletal muscle homogenate (for Pt1) and on digitonized skin fibroblasts (for Pt1 and Pt2). Immunoblotting, using a specific antibody against NDUFA12, did not detect any signal in Pt1 skeletal muscle (Figure 5A**)** and in Pt1 and Pt2 digitonin-permeabilized cells (Figure 5B). In accordance with the specific defect of CI, the expression level of an additional subunit of CI, NDUFA9, was dramatically reduced both in Pt1 skeletal muscle (90%) (Figure 5A) and in Pt1 and Pt2 skin fibroblasts (75% and 60%, respectively) (Figure 5B), whereas the expression level of CII and CIII subunits (SDHA and UQCRC2, respectively) was in the normal range. Surprisingly, in the patients' fibroblasts, but not in Pt1 muscle, we observed a statistical significant reduction of a CIV subunit, COXIV (30% and 50%, respectively) (Figure 5B), probably due to the destabilization of the CI-CIV containing supercomplexes.

To assess the assembly state of CI we carried out BNGE experiments on lauryl maltoside-treated muscle and on digitonin-permeabilized cells of Pt1, Pt2 and control samples and subjected the gels to either immunoblot analysis or in gel activity assay. In muscle specimens, western blot analysis showed a drastic reduction of CI in both patients (Figure 5C). However, we did not detect any subassembly of CI, at least with the antibody we used (NDUFA9). These data are in accordance with previously reported observations.[2] Of note, we noticed a slight reduction of CIV in Pt2 (Figure 5C), in line with the results obtained in Pt2 fibroblasts (Figure 5B). A significant reduction of the

fully assembled CI, without the presence of subassemblies, was observed also in the fibroblasts of Pt1 and Pt2 (more than 90%), when BNGE was subjected to western blot (Figure 5D). This data was supported by the *in-gel* activity assay, which confirmed a CI reduction of about 90% in both patients.

DISCUSSION

Herein, we describe seven patients from four unrelated consanguineous families all carrying novel mutations in *NDUFA12* gene. Experimental studies were performed only in Pt1 and Pt2 in whom we equally found an undetectable NDUFA12 protein signal associated to a drastic reduction of CI amount and activity. All these data are in line with the only patient so far described carrying a nonsense c.178C>T (p.Arg60*) mutation in *NDUFA12* (Ostergaard et al., 2011) and with *in vitro* studies performed in HEK293 cells devoid of NDUFA12 subunit (Rak and Rustin, 2014). Interestingly, despite an almost undetectable CI in our patients, *in gel* activity assay of BNGE showed a partial activity of CI (about 10% residual activity), in accordance with residual activity observed in muscle homogenate by spectrophotometric activity. This could be explained by the fact that even very low amount of CI can retain some activity; moreover low sensibility of the used antibodies against CI could also be considered to interpret this finding. In addition, western blot analysis of SDS PAGE showed a 30 to 50% reduction of a complex IV subunit (COXIV) in digitonin-treated cells of Pt1 and Pt2 but not in the muscle homogenate of the Pt1. Of note, BNGE performed on skeletal muscle of Pt1 and Pt2 confirmed only in the latter patient the reduction of CIV. A combined deficiency of CI with either CIV or CIII has been already reported in other patients affected by LS due to mutations in genes encoding subunits of CI (Saada et al., 2012; Piekutowska-Abramczuk et al., 2018) and the possible explanation is the disassembly of the supercomplexes following the lack of CI.

Due to the unavailable biological samples, we could not perform protein analysis in Pt3, Pt4, Pt5, Pt6 and Pt7; nevertheless, the classification as a class 5 mutations according to ACMG criteria point towards a pathogenic role of the variants.

All patients, except for Pt5, here described shared a neuroradiological picture of LS but presented with different onset and clinical course. Pt1 showed a clinical picture characterized by mild psychomotor delay and early-onset strabismus with brainstem lesions at MRI. Surprisingly, at the follow-up evaluation, the MRI disclosed a complete recovery of brainstem lesion and his clinical picture almost completely recovered. The last neurological examination revealed only reduction in visual acuity and nystagmus. After initial evaluation, patient had began treatment with riboflavin and CoQ10 however we can not state that this treatment contributed to the improvement of MRI and clinical picture. Some cases of lesions' regression/improvement in Leigh or Leigh-like syndrome have already been described (Heidary et al., 2014; Bonifanti et al., 2016). In Pt1 Heidary et al described improvement of brainstem lesions and a disease onset characterized by nystagmus. Improvement of the MRI pattern was also described in other mitochondrial diseases (Melchionda et al., 2014). Lesions at first MRI in our patient (Pt1) could represent an episode of acute metabolic decompensation.

Pt2 and Pt3 presented with a normal early development followed by early-onset dystonia that progressively worsened; their MRI showed lesions in the basal ganglia and brainstem. A milder phenotype was present in Pt4 and Pt5, siblings of Pt3, who exhibit a later onset of vision impairment and extrapyramidal signs (Pt4) or only a visual impairment (Pt5). Pt6 had normal motor neurodevelopment followed by signs of dystonic syndrome in both limbs. Pt7 showed an earlier onset and more severe phenotype compared to her brother. We can speculate that Pt1, Pt4 and Pt5 mild phenotype can be ascribed to the known high phenotypic variability in mitochondrial disorders (Kanungo

et al., 2018) or to disease modifying factors. A great phenotypic variability is described in Leigh syndrome due to mitochondrial nuclear genes associated to CI defect (Simon et al., 2019). A retrospective multicenter study of 130 Leigh syndrome patients shows that predictors of a severe disease course or higher occurrence of exacerbations were the presence of early onset, increased lactate in the CSF, brainstem involvement, cardiac abnormalities. On the other hand, in our cohort Pt1 presented with psychomotor delay and an early onset strabismus, whereas Pt4 and Pt5 had a later onset. All patients except Pt1 had high serum lactate. Noteworthy, Pt1 displayed the same profound CI defect and complete absence of the NDUFA12 compared to Pt2. No specific abnormalities were found in urine organic acids or plasma amino acids. MRS detected a lactate peak only in two (Pt2 and Pt3) of four tested patients.

NDUFA12 associated disease is characterized by a clinical picture with psychomotor delay/regression, dystonia and visual impairment. Patients of our cohort have an early childhood onset of dystonia but do not present cardiac involvement or epileptic seizures as frequently described for other nuclear genes causing LS with complex I defect (Sofou K, et al., 2018). LS with an early onset dystonia but no major organs or systems involvement is described for other mitochondrial nuclear genes with CI defect (Baide-Mairena et al., 2019).

In conclusion, we have expanded the clinical and genotypic spectrum of *NDUFA12* defects, also confirming previous evidences that NDUFA12 inactivation is associated with LS and CI deficiency; moreover, we have further highlighted the importance of this subunit in CI stability and function.

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CONFLICT OF INTEREST

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

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

The data are not publicly available due to privacy or ethical restrictions. All data relevant for the study are included in the article. LOVD links for the identified *NDUFA12* variants: https://databases.lovd.nl/shared/individuals/00310039; https://databases.lovd.nl/shared/individuals/00310608; https://databases.lovd.nl/shared/individuals/00310609; https://databases.lovd.nl/shared/individuals/00310610.

WEB RESOURCES

CADD: https://cadd.gs.washington.edu/

gnomAD: https://gnomad.broadinstitute.org/

Mutation assessor: http://mutationassessor.org/r3/

OMIM: https://www.omim.org/

Polyphen2: http://genetics.bwh.harvard.edu/pph2/

Provean and SIFT: http://provean.jcvi.org/index.php

Clustalw2: https://www.ebi.ac.uk/Tools/msa/clustalw2

HSF: Human Splicing Finder, http://www.umd.be/HSF

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FIGURES

Figure 1

(**A-B**) MRI in Pt1 at onset of disease: Axial T2-weighted images through the brain stem show bilateral and symmetrical lesions in central tegmental tracts (**A**) and in red nuclei (**B**). (**C-D**) MRI in Pt1 after 2 years: Axial T2-weighted images at same level show disappearance of the lesions. (**E-F**) MRI in Pt2: Axial T2 (**E**) and FLAIR (**F**) images of basal ganglia show bilateral symmetric lesions in the putamen. (**G-H**) MRI in Pt5: Axial FLAIR image (**G**) and coronal T2 image (**H**) show respectively hyperintense lesion in the right lentiform nucleus and in the left putamen.

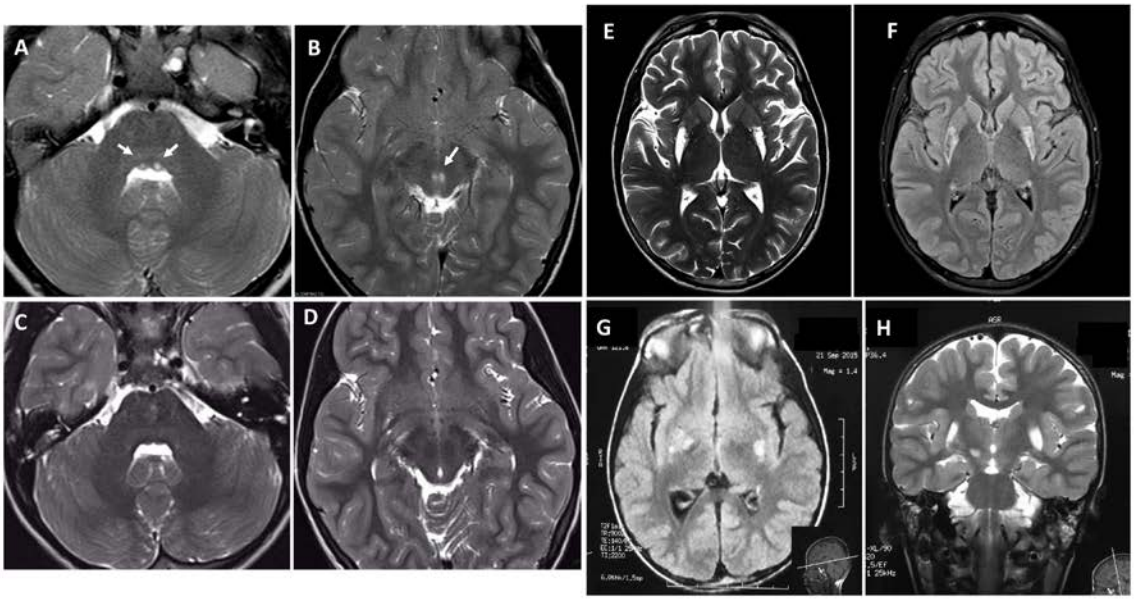


Figure 2

(**A**) Respiratory chain enzymatic activities measure in muscle samples of Pt1, Pt2 and Pt3 showed specific reduction of CI. Values are expressed as percentage of residual activity compared to the lowest values of the control range, after normalization with citrate synthase (CS). (**B**) Maximum respiratory rate (MRR) was measured in fibroblasts of Pt1 and Pt2 after 5 and 12 days (5d and 12d respectively) of galactose supplementation. Data are presented as percentage compared with the controls.

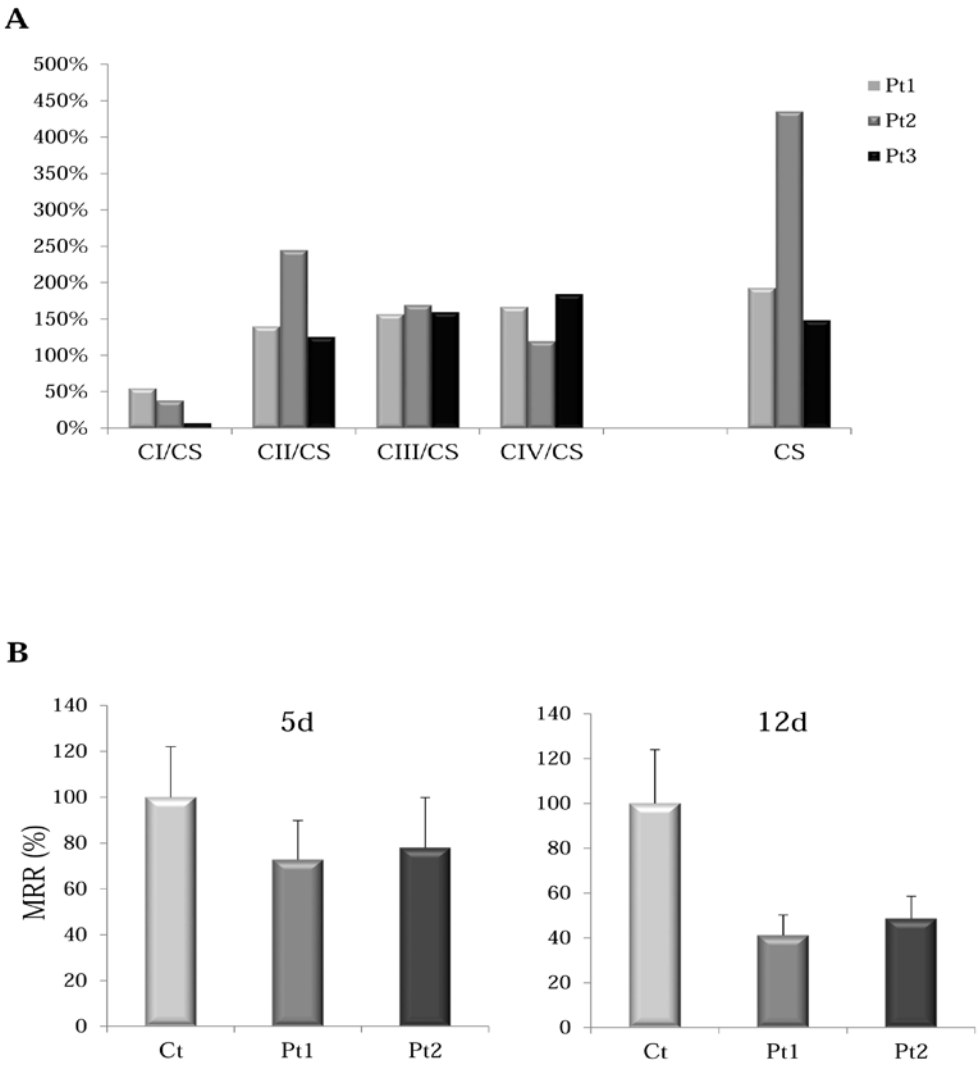


Figure 3

(**A**) Family pedigrees of the seven patients showing *NDUFA12* genotypes and corresponding electropherograms of the *NDUFA12* regions containing the variants in index patients and parents. Black symbols indicate the affected siblings. (**B**) Cartoon for the *NDUFA12* gene showing the positions of the variants reported in this manuscript (black and bold) and the one previously described (red).

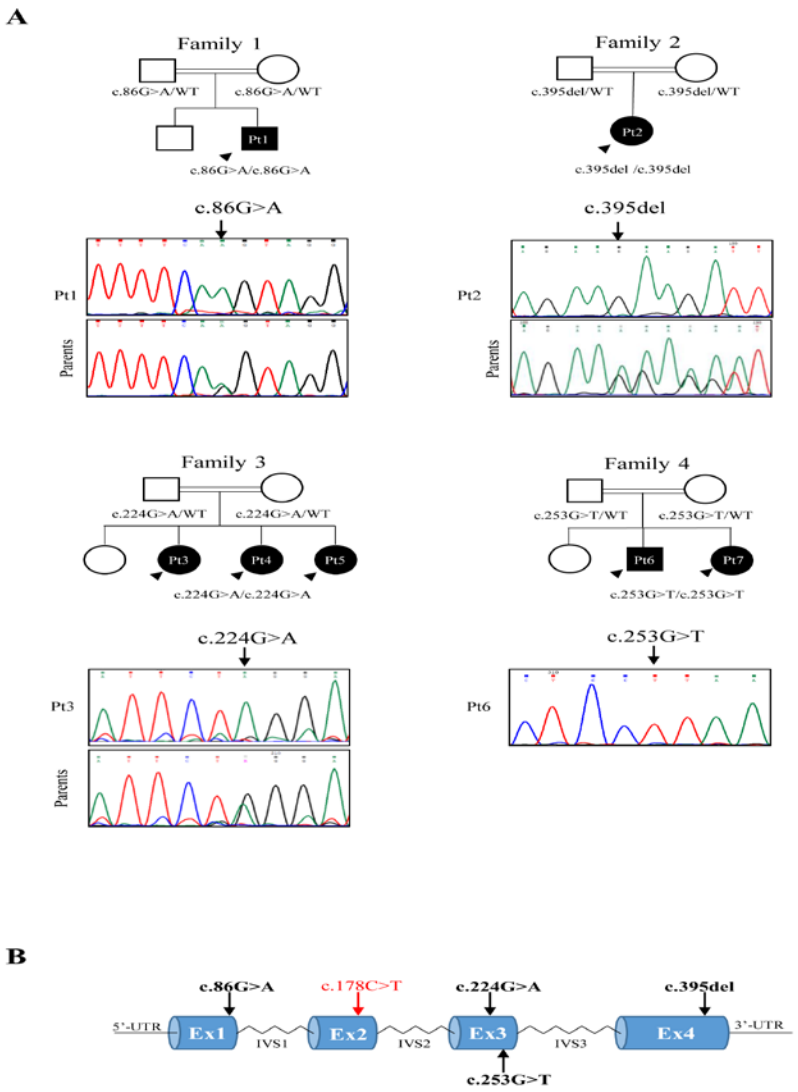


Figure 4

(**A**) PCR amplification of the full length *NDUFA12* transcript in Pt1 and control (C) showing the abnormal migration of the fragments. (**B**) Sequence analysis of the Pt1 and control amplicon displaying the insertion of a portion of IVS1. (**C**) Real time PCR showing the reduction of *NDUFA12* transcript in both patients (Pt1 and Pt2). Ctrls: n=6. Data are presented as a mean±SD of at least three independent experiments; *p<0.05; **p<0.001; ***p<0.0001.

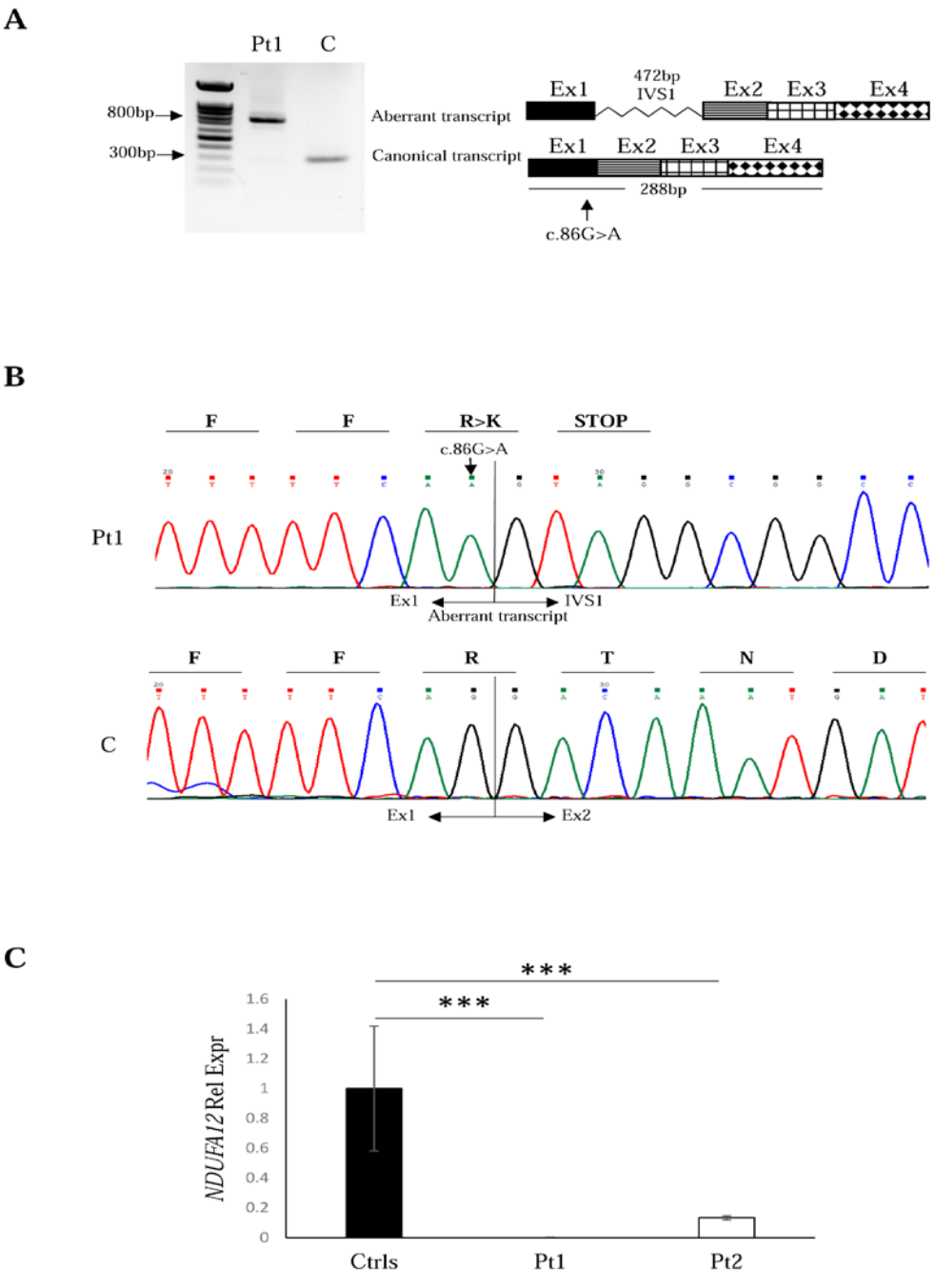
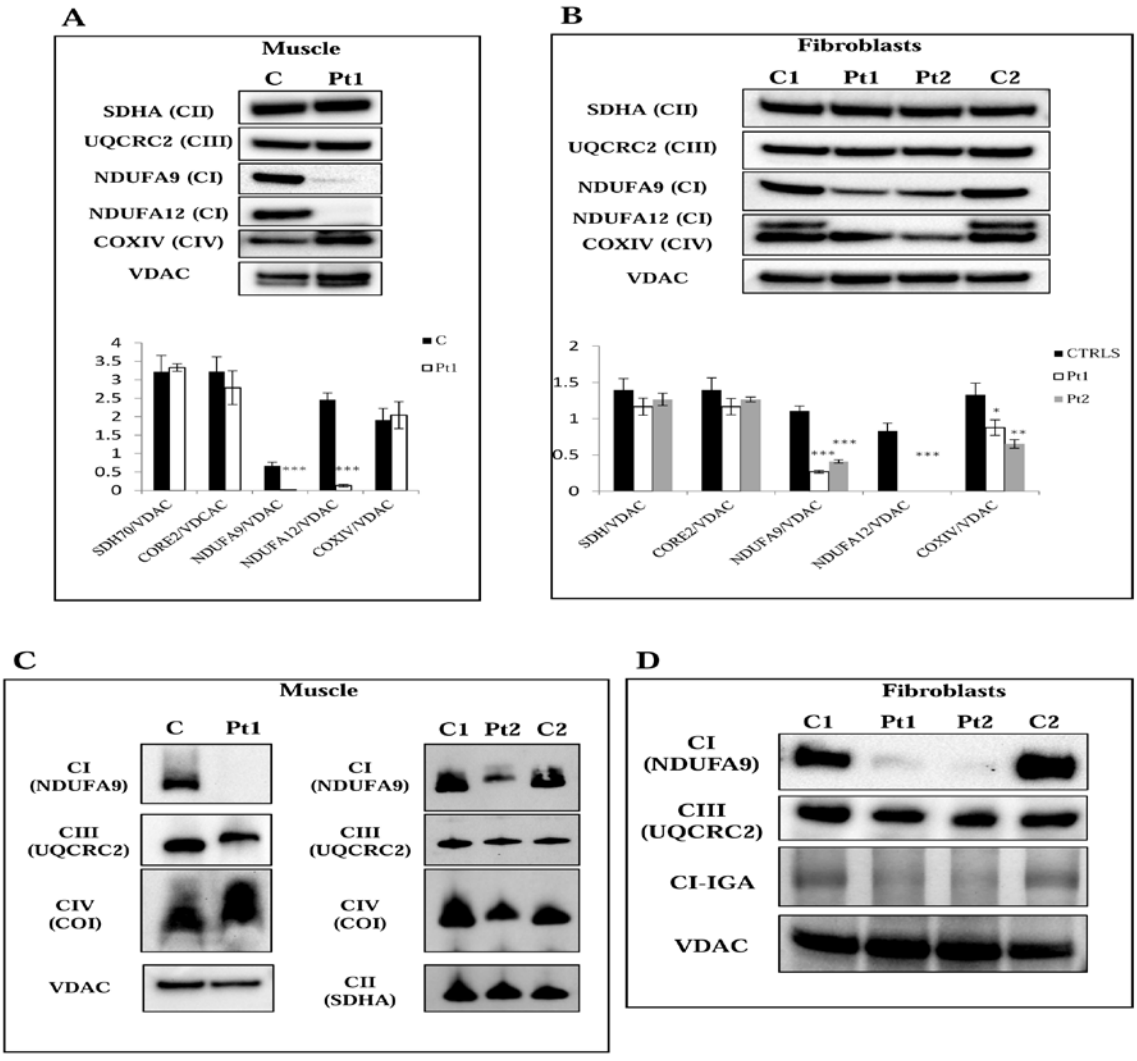


Figure 5

(**A**) SDS-PAGE/western blot analysis using specific antibodies against the respiratory chain complexes subunits was performed on muscle homogenate of control (C) and Pt1 and (**B**) on digitonin-permeabilized fibroblasts of two control subjects (C1 and C2), Pt1 and Pt2. VDAC signal was used as control of equal loading. (**C**) BNGE/western blot performed on muscle sample of Pt1 with a relative control (C) and on Pt2 with two additional controls (C1 and C2). (**D**) BNGE performed on digitonin-permeabilized fibroblasts of controls (C1, C2), Pt1 and Pt2 and probed either with CI (NDUFA9) and CIII (UQCRC2) specific antibodies or subjected to in-gel complex I activity (CI-IGA). Data are presented as a mean±SD of at least three independent experiments; *p<0.05; **p<0.001; ***p<0.0001.



Table

Table 1 Clinical, genetic and MRI features of *NDUFA12* patients in our cohort

Family	**Patient**	**Age**	**Birth**	**Psychomotor development**	**Symptoms at onset**	**Neurological picture**	**Clinical course**	**Brain MRI**	**Mutation** **ACMG classification**
#1	Pt1	9y	Poor sucking, hypotonia	Mild psychomotor delay	Convergent strabismus (4 yrs of age)	Strabismus, nystagmus, minimal ptosis in the left eye	Almost complete regression	-First: Bilateral/symmetric T2 hyperintense lesions in the brainstem -Second: normal	c.86G>A p.Arg29Lys **(VoUS)**
#2	Pt2	15 y	Respiratory distress	Normal	Dystonia of the right arm	Generalized dystonia	Progression/Worsening	Bilateral lesions in the basal ganglia (putamina)	c.395delA p.Lys132Argfs*50 **(Likely pathogenic)**
#3	Pt3	13 y	Respiratory distress	Normal	Nystagmus, right hemiparesis	Generalized dystonia	Progression/Worsening	T2 hypersignal of lenticular nuclei and brainstem	c.224G>A p.Trp75* **(Pathogenic)**
	Pt4	9y	NA	Normal	NA	Extrapyramidal syndrome	NA	Lenticular nuclei involvement	
	Pt5	7y	Normal	Normal	Visual impairment (optic atrophy)	Visual impairment (optic atrophy)	Stable	Normal	
#4	Pt6	11 y	Normal	Normal	Dystonia of the limbs (left>right)	Dystonia of the limbs (left>right)	Stable	T2/FLAIR high signal intensity in lentiform nuclei, red nucleus, brainstem	c.253G>T p.Glu85* **(Pathogenic)**

	Pt7	4y	Normal	Normal	Dystonia of the limbs (distal; left>right) (3 yrs)	Dystonia of the limbs (left>right)	Progression/Worsening	ND	
Reported by Ostergaard E, J Med Genet. 2011 Nov;48(11):737-40. c.178C/T p.Arg60*		10 y	NA	Psychomotor delay	Dystonia of the limbs (right>left)	Generalized dystonia	Stable	Bilateral hyperintense signals in the globus pallidus	

Abbreviations: y: years; NA: information not available; ND: not done