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Qualification en BIOLOGIE MÉDICALE

Dual occurrence of mtDNA pathogenic variants: an underestimated phenomenon?

About three cases and a literature review

Double occurrence de variants pathogènes de l'ADNmt : un phénomène sous-estimé ?

A propos de 3 cas et revue de littérature

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LISTE DES ABREVIATIONS

ADPKD	Autosomal Dominant Polycystic Kidney Disease
ANCA	Anti-Neutrophil Cytoplasmic Autoantibody
BN-PAGE	Blue Native-Polyacrylamide Gel Electrophoresis
bp	Base Pair
CI	Complex I
CO1	Cytochrome C Oxidase I
CPEO	Chronic Progressive External Ophthalmoplegia
CS	Citrate Synthase
DNA	Deoxyribonucleic Acid
ES	Exome Sequencing
KSS	Kearns-Sayre Syndrome
LHON	Leber's Hereditary Optic Neuropathy
LS	Leigh Syndrome
MELAS	Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes syndrome
MERRF	Myoclonic Epilepsy with Ragged Red Fibers
MIDD	Maternally Inherited Diabetes and Deafness
MIEH	Maternally Inherited Essential Hypertension
MIND	Maternally Inherited Nonsyndromic Deafness
MT-ATP6	Mitochondrially encoded ATP synthase membrane subunit 6
MT-CO1	Mitochondrially encoded Cytochrome C Oxidase I
MT-CO3	Mitochondrially encoded Cytochrome C Oxidase III
MT-CYB	Mitochondrially encoded Cytochrome B
MT-ND4	Mitochondrially encoded NADH:Ubiquinone Oxidoreductase Core Subunit 4
MT-ND4L	Mitochondrially encoded NADH:Ubiquinone Oxidoreductase Core Subunit 4L
MT-ND5	Mitochondrially encoded NADH:Ubiquinone Oxidoreductase Core Subunit 5
MT-ND6	Mitochondrially encoded NADH:Ubiquinone Oxidoreductase Core Subunit 6
MT-TH	Mitochondrially encoded tRNA-His
MT-TK	Mitochondrially encoded tRNA-Lys
MT-TL1	Mitochondrially encoded tRNA-Leu
NAION	Non-Arteritic Ischemic Optic Neuropathy
NGS	Next-Generation Sequencing
OCT	Optical Coherence Tomography
OXPHOS	Oxidative Phosphorylation
PCR	Polymerase Chain Reaction
PEM	Progressive Encephalomyopathy
PKD1	Polycystin 1, transient receptor potential channel interacting
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SMRT	Single Molecule, Real-Time
T2DM	Type 2 Diabetes Mellitus
YARS2	Tyrosyl-tRNA Synthetase 2

CONTEXTE DE LA THESE ET PROBLEMATIQUE :

Les mitochondries sont des organites intracellulaires ubiquitaires chez les eucaryotes (1). Elles sont impliquées dans différentes voies de signalisation, comme l'homéostasie cellulaire ou l'apoptose (2) et ont un rôle central dans la production d'énergie grâce aux réactions d'oxydation phosphorylante qui se déroulent au sein des complexes de la chaîne respiratoire mitochondriale (3). Parmi tous les organites cellulaires, il est le seul à être sous la double influence de l'ADN nucléaire (ADNn) et de son propre génome, l'ADN mitochondrial (ADNmt) (4). L'ADNmt humain est une molécule circulaire double brin de 16569 pb, constituée de 37 gènes codant pour les 13 sous-unités de la chaîne respiratoire mitochondriale, deux ARN ribosomiques (ARNr) et 22 ARN de transfert (ARNt) (5). De plus, la transmission de l'ADNmt n'est pas mendélienne mais maternelle (4). Enfin, chaque mitochondrie contient une à dix copies d'ADNmt (6).

Les maladies mitochondriales primaires font partie des pathologies du métabolisme les plus fréquentes, avec une prévalence estimée à 1/4300 (7). Elles peuvent avoir pour origine une anomalie de l'ADNn, qui affecte la synthèse et l'assemblage des composants de la chaine respiratoire, la maintenance de l'ADNmt (8) ou le processus de fusion et fission des mitochondries (9), pouvant conduire à une réduction de la masse mitochondriale (10). L'atteinte de l'ADNmt, quant à elle, conduit à des dysfonctions des sous-unités de la chaine respiratoire (11) associées à une surproduction d'espèces réactives de l'oxygène (12) et une incapacité à générer suffisamment d'énergie pour répondre aux besoins des différents organes (13). Les maladies mitochondriales sont caractérisées par une pénétrance incomplète et une expressivité clinique hétérogène (14). Les manifestations cliniques peuvent être d'un seul organe comme la neuropathie optique héréditaire de Leber (NOHL, MIM #535000), ou bien systémiques comme le syndrome de Leigh (MIM #256000) ou le syndrome

encéphalomyopathie mitochondriale, acidose lactique, pseudo-épisodes vasculaires cérébraux (MELAS, MIM #540000).

L'ADNmt est très vulnérable, son taux de mutation est 20 à 25 fois supérieur à celui des gènes nucléaires (15). Ceci est dû entre autres aux erreurs lors de la réplication (8), au fait que les systèmes de réparation de l'ADN soient plus frustres (4) et à l'absence d'histones protectrices contre les espèces réactives de l'oxygène (16). Chaque cellule contient des milliers de mitochondries organisées en un réseau (9). Ce réseau mitochondrial est en dynamique continue grâce aux mécanismes de fusion et de fission afin de mieux répondre aux besoins énergétiques cellulaires (17). Ainsi, ces mécanismes entraînent un important renouvellement de l'ADNmt normal et muté. Les variants pathogènes peuvent donc affecter une proportion variable de l'ADNmt dans la cellule, définissant l'hétéroplasmie (4). Lorsque la totalité de l'ADNmt est identique, on parle d'homoplasmie (18). Ce phénomène explique en partie l'expressivité et l'hétérogénéité clinique des maladies mitochondriales (19). De plus, lorsque les cellules se divisent, les mitochondries seraient réparties selon un processus stochastique dans les cellules filles (17). De ce fait, lorsqu'une cellule porteuse d'un variant pathogène hétéroplasmique se divise, les ADNmt mutants sont répartis au hasard. Par conséquent, le pourcentage d'ADNmt mutant peut différer selon les tissus et organes chez un même individu (20).

Dans la littérature, les maladies mitochondriales semblent essentiellement causées par la présence d'un seul variant pathogène de l'ADNmt. Sur la base de données Mitomap (21), près d'une centaine de variants pathogènes avec un statut « confirmé » sont répertoriés. Cependant, dans certains cas, la présence d'un second variant pathogène permettrait expliquer la présentation clinique complexe ou la pénétrance incomplète d'une pathologie. Ce phénomène de co-occurrence de variants pathogènes de l'ADNmt est très peu décrit dans la

littérature et concerne essentiellement la NOHL. Par ailleurs, les bases de données existantes ne permettent pas d'inventorier ce type de co-occurrences.

Historiquement, le diagnostic des maladies mitochondriales reposait sur des approches ciblées réalisées avec différents outils standard de biologie moléculaire, tels que la PCR ou le séquençage Sanger. Cette stratégie, en plus d'être chronophage, restreignait le diagnostic aux variants pathogènes les plus communs et associés à la clinique, mais ne permettaient pas la détection de co-occurrences de variants. Aujourd'hui, le séquençage total de l'ADNmt par séquençage à haut débit est devenu un outil de routine permettant un gain significatif en efficacité et en sensibilité, ainsi que la détection de co-occurrence de variants pathogènes de l'ADNmt, qui pourrait expliquer la pénétrance et l'expressivité phénotypique de certaines maladies mitochondriales.

L'objectif de cette étude était de déterminer la prévalence des co-occurrences de variants pathogènes de l'ADNmt au laboratoire de cytogénomique au CHU d'Angers et l'impact de ces doubles diagnostics sur la prise en charge des patients. Les résultats obtenus sont présentés sous la forme d'un article scientifique, en vue d'une publication.

Dual occurrence of mtDNA pathogenic variants: an underestimated phenomenon?

About three cases and a literature review

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ABSTRACT

The prevalence of primary mitochondrial diseases is 1:4300, with more than 350 pathogenic or likely pathogenic mitochondrial DNA (mtDNA) variations listed in databases. However, despite a mutation rate 20 to 25 times higher than nuclear DNA, the co-occurrence of mtDNA pathogenic variants appears to be rare in the literature, with few cases described.

The objective of this work was to assess the prevalence of co-occurrence of mtDNA pathogenic variants in patients referred to our laboratory for molecular diagnosis of mitochondrial diseases between 2014 and 2022, using whole mtDNA next-generation sequencing (NGS).

The analysis of mtDNA sequencing data from 4102 individuals revealed the co-occurrence of two pathogenic or likely pathogenic variants in about 0.5% of subjects. This includes eight individuals (0.19%) from three families with a "confirmed" status in the Mitomap database, nine individuals (0.22%) carrying a "confirmed" pathogenic variant and a "reported" one, and two individuals with a combination of two "reported" variants. These subjects were not only suffering from Leber's hereditary optic neuropathy (LHON) but also from syndromic mitochondrial diseases. Our study shows that the co-occurrence of mtDNA pathogenic variants is not a rare event as suggested in the literature, and its prevalence is probably underestimated. Furthermore, the long-read sequencing results are in favour of mtDNA homologous recombination events, leading to 4 different mtDNA sequences per patient. Identifying combinations of variants through systematic whole mtDNA sequencing improves mitochondrial disease diagnosis, notably in complex phenotypes, and offers better genetic counselling to families.

INTRODUCTION

Human mitochondrial DNA (mtDNA) is a 16,569 bp circular double-stranded DNA molecule, maternally inherited and encoding for 13 polypeptides involved in oxidative phosphorylation (OXPHOS), two ribosomal RNAs (rRNA), and 22 transfer RNAs (tRNA) (3). In addition to structural and inheritance features, mtDNA is characterised by a high mutation rate, 20 to 25 times higher than nuclear DNA (nDNA), according to the studies (22). Imperfections in the mtDNA maintenance system, replication errors and an incomplete DNA repair system compared to nDNA (23), absence of protective histones (24) and the high exposure to reactive oxygen species (ROS) generated by the mitochondrial respiration chain (25) can explain this high mutation rate. All these reasons make mtDNA more vulnerable to molecular variations than nDNA. Therefore, the prevalence of mitochondrial diseases due to mtDNA pathogenic variants is estimated to be three times higher than that of pathogenic variants in nuclear-encoded mitochondrial genes (7). Pathogenic variants occurring in mtDNA can affect either the protein-coding genes (26), the tRNA (27) or the rRNA genes (28). They could be responsible for a wide range of clinical and biological symptoms from a multisystemic failure of neonatal onset to single organ involvement, like Leber's hereditary optic neuropathy (LHON; MIM #535000) (29). These heterogeneous phenotypes can be explained by the nature of the pathogenic variant and because mtDNA pathogenic variants can affect a variable proportion of the mtDNA in the cell, namely heteroplasmy. When the totality of the mtDNA is affected by a molecular variant, we talk about homoplasmy (18). As the percentage of mutant mtDNA increases, energy production declines to a level below which the minimum necessary for the physiological maintenance of cellular functions is no longer sufficient, resulting in disease (19). However, if mtDNA is more prone to acquire molecular variants, the coexistence of mtDNA pathogenic variants seems to be rare in literature and confined to LHON pathogenic variants(30–34). In this study, we describe three new families in which at least one member

carries a combination of two known mtDNA pathogenic variants. Contrary to the literature, this phenomenon is not only involved in LHON but also responsible for syndromic disorders. It highlights the importance of analysing the whole mtDNA for diagnosing mitochondrial diseases. Indeed, it allows the detection of variant co-occurrence, which can explain, in some cases, the phenotypic variability of mitochondrial diseases.

PATIENTS AND METHODS

Patients

The whole mtDNA from 4102 individuals referred to the cytogenomic laboratory of the University Hospital of Angers for the diagnosis of mitochondrial disease was sequenced. All individuals were included after obtaining written informed consent (Institutional Review Board Committee of the University Hospital of Angers, Authorization number: AC-2012-1507). Demographic data, clinical manifestations and molecular data were collected retrospectively only for individuals with two or more mtDNA pathogenic variants and are reported in Table I and Supplementary Patients and Methods.

mtDNA analysis

Total DNA was extracted from blood and urine as previously described (35). The whole mtDNA was sequenced by next-generation sequencing (NGS) on an Ion Torrent® platform (Supplementary Patients and Methods). Signal processing and base calling were performed by the integrated pre-processing Ion Torrent® pipeline. Alignment against reference (NC_012920.1), variant calling and annotation, and haplogroup determination were performed using a homemade bioinformatics pipeline (Supplementary Patients and Methods). "Confirmed" and "reported" mtDNA pathogenic variants taken from the Mitomap database (18) were screened for each sample (Supplementary Table S1). Disease risk factors variants, susceptibility variants or whose population base frequency was more than 0.2% were excluded from the analysis. A second technique confirmed all putative variants identified by NGS. For the m.3243A>G in *MT-TL1*, m.8344A>G in *MT-TK*, m.11778G>A in *MT-ND4*, m.14459G>A in *MT-ND6*, and 14484T>C in *MT-ND6,* heteroplasmy loads were also quantified by fluorescent PCR-RFLP technique (Supplementary Patients and Methods). Other variants were confirmed by

Sanger sequencing with Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA).

nDNA analysis

Exome sequencing (ES) was performed for all members of family A, II.3, and III.3 from family B and II.2 from family C on Illumina NextSeq 550 platform (Illumina Inc, San Diego, CA, USA) (Supplementary Patients and Methods).

Long-read sequencing

Long-read mtDNA sequencing was performed for individuals III.4 from family B and II.2 from family C. One part of the mtDNA was amplified by long-range PCR using a combination of primers as previously described (36) and in Supplementary Patients and Methods. Long-range PCR amplicons were sequenced in a single read by using Sequel I from PacBio® (Pacific Biosciences, Menlo Park, CA, USA), real-time (SMRT) sequencing technology as described elsewhere (37) and in Supplementary Patients and Methods.

Respiratory chain assay

For individuals II.2 and III.1 from family A, a functional study of respiratory chain complexes was performed on cultured fibroblasts. Activities of OXPHOS complexes were evaluated by spectrophotometry (Supplementary Patients and Methods).

Quantification of mtDNA content

The mtDNA content was quantified by quantitative PCR, as described in Supplementary Patients and Methods, in those members of family A whose blood samples were available (Table I).

RESULTS

Prevalence of co-occurrence of mtDNA pathogenic variants

The co-occurrence of at least two mtDNA pathogenic or likely pathogenic variants was found in 19 individuals (0.46%). Among these, eight (0.19%) had the co-occurrence of two pathogenic variants with a "confirmed" status on the Mitomap database (Table I), and nine (0.22%) carried the association of an actual variant and a "reported" variant (Table II). Lastly, two individuals had two "reported" variants (Supplementary Table S2). However, we did not find the co-occurrence of more than two pathogenic variants in our cohort. Most patients/families belonged to European haplogroups (H, J, T U, W), with only one patient belonging to haplogroup L and one to haplogroup C.

Co-occurrence of confirmed mtDNA pathogenic variants

Herein, we report the cases of eight individuals with two "confirmed" mtDNA pathogenic variants from three families (Figure 1 and Table I). In family A, belonging to haplogroup W1a, the co-occurrence of two of the three main LHON pathogenic variants was identified: m.11778G>A (p.Arg340His) in *MT-ND4* at a heteroplasmic rate and the homoplasmic m.14484T>C (p.Met64Val) in *MT-ND6* (Table I). Proband (III.5) was referred to the laboratory in the context of undiagnosed familial optic atrophy in men (Figure 1B and Supplementary Patients and Methods). His clinical history was marked by a sudden decrease in visual acuity in the right eye at fifteen, a few months after smoking onset. Then, within one month, the second eye was sequentially impaired to 1/20. Less than a year later, after quitting smoking, he recovered vision of the right eye at 8/10 and the left eye at 9/10, but with the persistence of a cæcocentral scotoma. Familial segregation identified the two variants in all tested individuals, regardless of their clinical status, with variable levels of heteroplasmy for m.11778G>A but consistently homoplasmic for m.14484T>C (Table I). Of note, all members

of generation II and subject III.4 had autosomal dominant polycystic kidney disease (ADPKD, MIM #173900), with a heterozygous pathogenic variant in *PKD1* (NM_001009944.3):c.2638_2657dup (p.Trp887Profs*18) (38). As individual II.5 had undergone a kidney transplant, interpreting the quantification of heteroplasmy in their urine was impossible. Exome sequencing (ES) did not reveal any pathogenic variant in mitochondrial nuclear-encoded genes, including mitochondrial maintenance genes. Functional assays performed on cultured fibroblasts of individuals II.2 and III.1 showed a slight decrease of complex I activity related to citrate synthase (CI/CS) for both, i.e., 0.28 for II.2 and 0.27 for III.1, compared to reference values (0.30 – 0.70). However, despite their different clinical presentations, no difference was observed between the two individuals (Supplementary Table S4). Only the energetic activity of complex I in raw value revealed a difference, activity being twice as high in the asymptomatic II.2 (192 nmol/min/mg) than in the symptomatic III.1 (100 nmol/min/mg). MtDNA content appeared roughly the same between family members (Supplementary Table S5), with no statistical difference between affected and unaffected individuals (p=0.41). However, index case (III.5) increased his mtDNA content by about 50% (33.7 *vs* 49.3) after visual recovery.

Table I. Demographic data, clinical and molecular features of patients carrying two mtDNA pathogenic variants. *These patients are affected by Autosomal Dominant Polycystic Kidney Disease (ADPKD). **This patient received a kidney transplant; his urine result cannot be interpreted. †The pathogenic variant was only identified by Sanger sequencing. LHON: Leber's Hereditary Optic Neuropathy; MERRF: Myoclonic Epilepsy with Ragged-Red Fibers; MELAS: Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like episodes; ND: Not Determined.

			Age (Y)			Pathogenic variant heteroplasmy (%)			
Family (Haplogroup)	**Individuals**	**Sex**	**Diagnosis**	**Clinical onset**	**Clinical features**	**Blood**		**Urine sediment**	
A						**m.11778G>A**	**m.14484T>C**	**m.11778G>A**	**m.14484T>C**
(W1a)	II.1*	M	ND	17	LHON	ND	ND	ND	ND
	II.2*	F	62	ND	Asymptomatic	93	100	100	100
	II.5*	F	59	ND	Asymptomatic	19	100	10**	53**
	III.1	M	43	17	LHON	100	100	100	100
	III.2	F	42	ND	Asymptomatic	100	100	ND	ND
	III.3	M	29	4	LHON	ND	ND	ND	ND
	III.4*	M	35	ND	Asymptomatic	22	100	45	100
	III.5	M	24	15	LHON	85	100	88	100
B						**m.13042G>A**	**m.3243A>G**	**m.13042G>A**	**m.3243A>G**
(H)	II.3	F	51	ND	Asymptomatic	0	0	heteroplasmic†	0
	II.4	M	52	34	LHON	heteroplasmic†	0	heteroplasmic†	0
	III.3	F	28	ND	Asymptomatic	0	0	0	0
	III.4	F	25	19	LHON	34	7	34	3
C						**m.8344A>G**	**m.3243A>G**	**m.8344A>G**	**m.3243A>G**
(**T2b25**)	II.1	F	ND	ND	MERRF	ND	ND	ND	ND
	II.2	F	65	60	MELAS	ND	ND	57	20
	II.3	F	71	50	MERRF	heteroplasmic†	0	ND	ND
	III.1	M	45	20	MERRF	55	ND	63	0
	III.2	M	39	34	MERRF	55	0	76	0
	III.3	M	43	ND	MERRF	ND	ND	ND	ND
	III.4	M	38	ND	MERRF	ND	ND	ND	ND
	III.5	M	52	38	Muscular pain, cramps	ND	ND	ND	ND

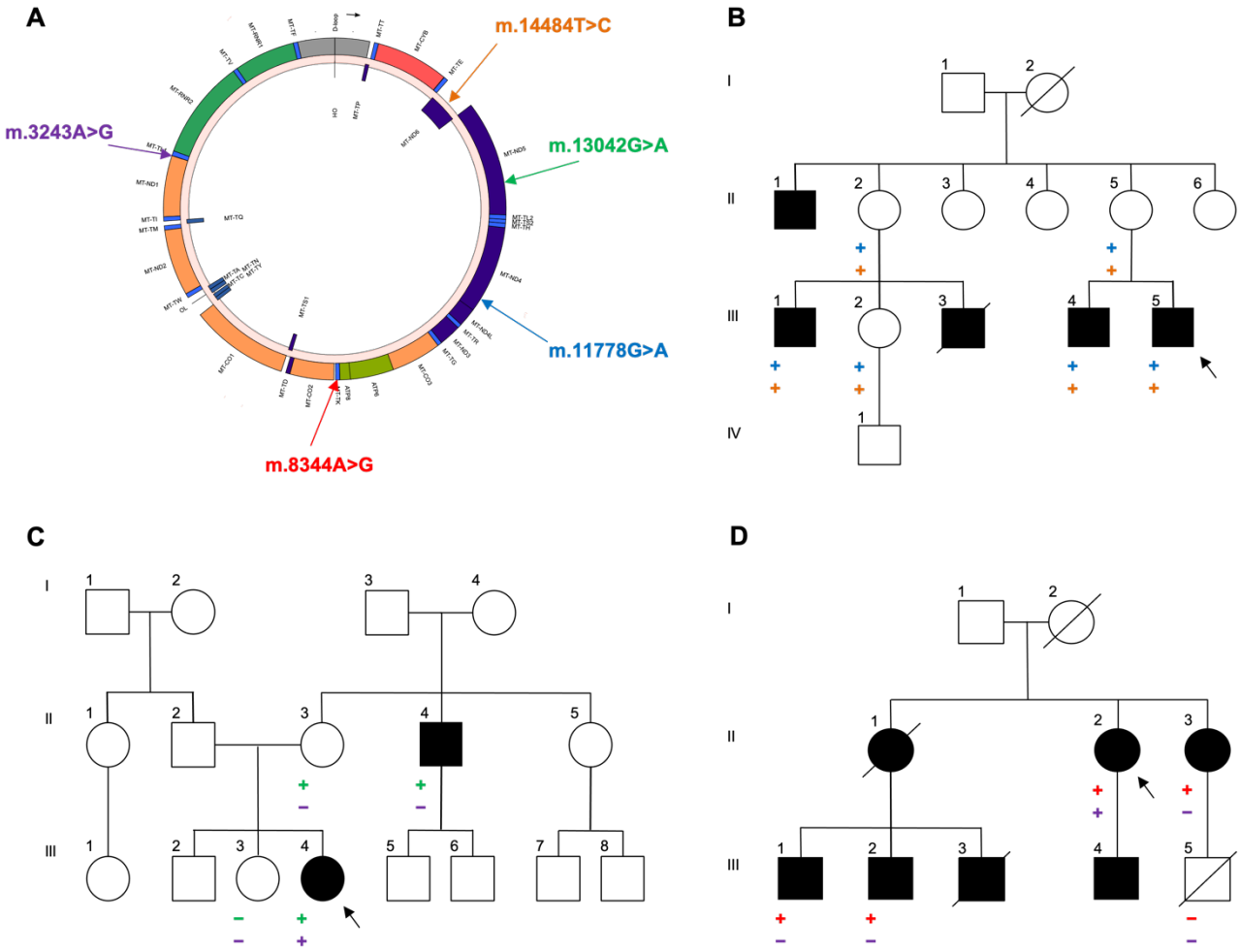

Figure 1. Pedigrees of the families carrying two mtDNA pathogenic variants. A. Schematic representation of the human mitochondrial genome and the localization of the "confirmed" pathogenic variants identified in families A, B and C. **B.** Family A pedigree with m.11778G>A (blue) and m.14484T>C (orange). **C.** Family B pedigree with m.13042G>A (green) and m.3243A>G (purple). **D.** Family C pedigree with m.8344A>G (red) and m.3243A>G (purple). Empty symbols represent unaffected individuals, filled symbols affected individuals, arrow: proband, +: individual positive for the variant, -: individual negative for the variant.

Family B was referred for LHON (Figure 1C). The proband (III.4) was a young woman of twenty-five years old. She had a sudden decline in visual acuity in her right eye at nineteen years old, followed by the other eye two years later. On ophthalmological examination, both optic nerves had a rather pale appearance, visual acuity of 24/100, and Parinaud 6 in both eyes. Ganglion cell-inner plexiform layer was preserved on OCT. Whole mtDNA sequencing, performed in blood and urine, identified the co-occurrence of two heteroplasmic pathogenic variants in haplogroup H background: m.3243A>G in *MT-TL1* at 7% in blood and 3% in urine,

and m.13042G>A (p.Ala236Thr) in *MT-ND5* at 34% in both samples reported in various mitochondrial diseases (39,40) (Table I). The m.3243A>G variant was not detected in all tested individuals within the limits of the fluorescent PCR-RFLP detection threshold (~2%). Conversely, the m.13042G>A was detected in her symptomatic maternal uncle (II.4) and her asymptomatic mother (II.3). Within the limit of Sanger sequencing, the LHON variant was not detected in her asymptomatic sister (III.3). ES did not reveal any pathogenic or likely pathogenic variant in mitochondrial nuclear-encoded genes. Long-read sequencing revealed the presence of four types of mtDNA molecules in variable proportions (Figure 2A): 60.6% of wild-type molecules, 37.8% carrying an isolated pathogenic variant, respectively 34.7% with the m.13042G>A and 3.1% the m.3243A>G, and 1.6% carrying the two pathogenic variants.

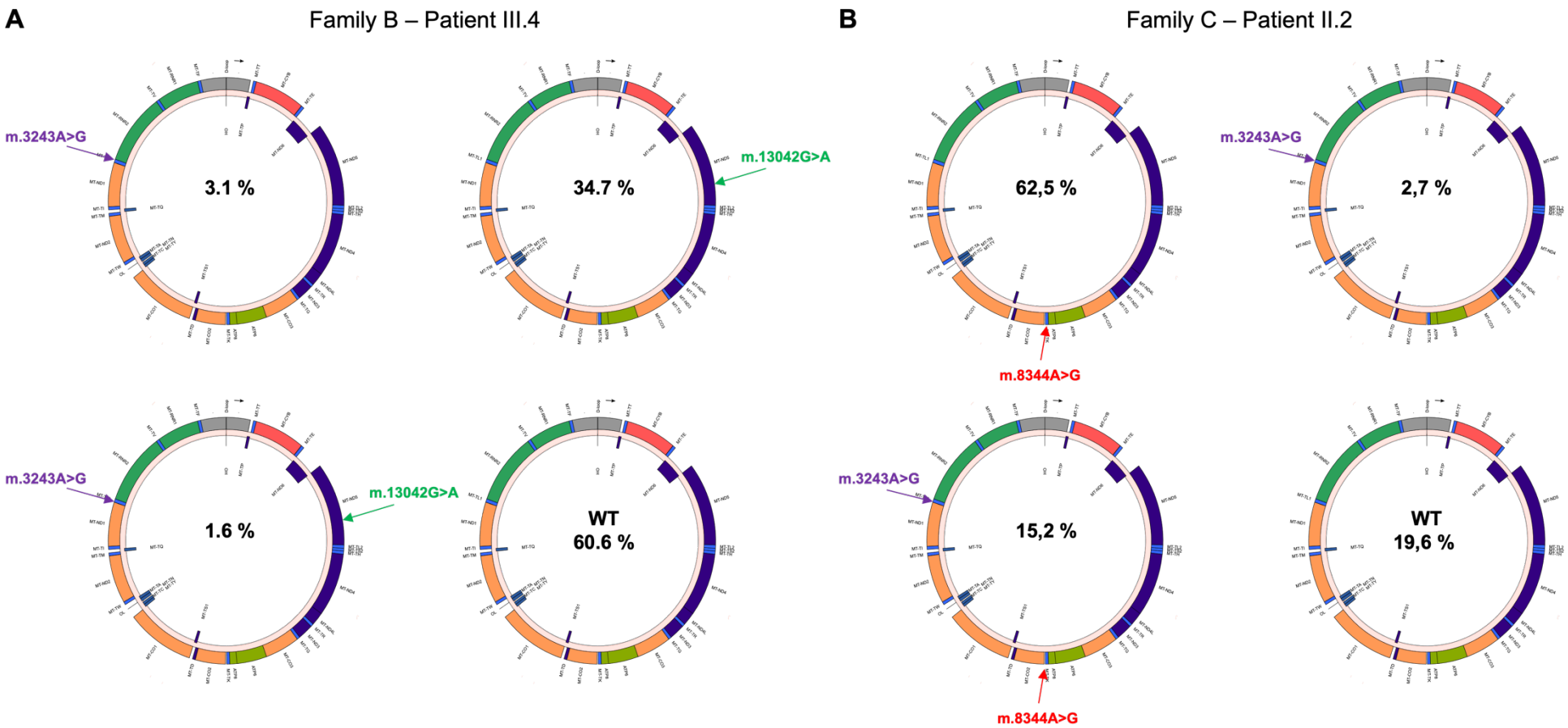

Figure 2. The proportion of the four types of mtDNA molecules identified by long-read sequencing in the two patients harbouring the co-occurrence of two heteroplasmic pathogenic variants. A. Proportion of mtDNA molecules with m.13042G>A variant (green), m.3243A>G variant (purple), both variants and wild-type (WT) of individual III.4 from family B. **B.** Proportion of mtDNA molecules with m.8344A>G variant (red), m.3243A>G variant (purple), both variants and wild-type (WT) of individual II.2 from family C.

The proband (II.2) from family C (Figure 1D) was a sixty-five-year-old female followed since 2016 for a neuromuscular disorder, which would have appeared at the age of sixty. The electroneuromyogram (ENMG) objectified a sensory-motor axonal neuropathy with a length-dependent pattern. She also suffered from bilateral sensorineural deafness from age fifty, arterial hypertension and glucose intolerance. Familial history was marked by a sister (II.3) (Supplementary Patients and Methods) followed for more than twenty years at the University Hospital of Angers for Myoclonic Epilepsy with Ragged Red Fibers syndrome (MERRF, MIM #545000) due to the m.8344A>G pathogenic variant in *MT-TK*. First, targeted Sanger sequencing and PCR-RFLP identified the heteroplasmic m.8344A>G in patient II.2 urine sample. Then, an additional pathogenic variant was identified by NGS: m.3243A>G in *MT-TL1* at 20% (Table I) in mitochondrial haplogroup T2b25 background. Long-read sequencing showed the presence of four types of mtDNA molecules (Figure 2B): 19.6% of wild-type molecules, 65.2% carrying an isolated pathogenic variant, respectively 62.5% with the m.8344A>G and 2.7% the m.3243A>G, and 15.2% carrying the two pathogenic variants. Familial segregation identified the m.8344A>G pathogenic variant in two symptomatic nephews (III.1 and III.2) but not in her son (III.5), suffering from systemic scleroderma and ANCA vasculitis (Supplementary Patients and Methods). None of those three individuals carried the m.3243A>G within the limits of the fluorescent PCR-RFLP. ES did not reveal any pathogenic or likely pathogenic variant in mitochondrial nuclear-encoded genes.

Co-occurrence of mtDNA likely pathogenic variants

Among the 4102 mtDNA samples analysed in our laboratory, nine individuals had the combination of a "confirmed" pathogenic variant and a likely pathogenic variant with a "reported" status in the Mitomap database (Table II) or two likely pathogenic variants (Supplementary Table S2). Subjects P1, P2, and P3 belonged to the same family (Table II -

Family D). They were, respectively, the proband affected by Leigh syndrome, his asymptomatic mother and maternal aunt. All three carried the m.14459G>A (p.Ala72Val) in *MT-ND6* at homoplasmic state, a known pathogenic variant and responsible for Leigh syndrome, among others (41–43), and the m.15773G>A (p.Val343Met) homoplasmic variant of *MT-CYB* in the proband but heteroplasmic in his relatives. The m.15773G>A variant was reported responsible for a severe form of LHON with myoclonus (44). ES did not reveal any pathogenic or likely pathogenic variant in mitochondrial nuclear-encoded genes.

Patient P4 suffered from epilepsy complicated by left hemianopia. After an exhaustive exploration, mtDNA analysis objectified the presence of heteroplasmic variant m.3243A>G associated with the homoplasmic variant m.8668T>C (p.Trp48Arg) in *MT-ATP6*, previously reported in LHON (45).

Patient P5 was referred to the laboratory for dystonia. MtDNA analysis showed the co-occurrence of homoplasmic variants: the LHON pathogenic variant m.14484T>C and m.10680G>A (p.Ala71Thr) in *MT-ND4L*, reported as increasing penetrance of m.14484T>C LHON disease (46–48). Patients P6 to P9 suffered from LHON. For each one, a known pathogenic variant involved in optic atrophy was identified: m.11778G>A (P6 and P7), m.14484T>C (P8), and 13042G>A (P9) at variable heteroplasmy rates. They also had the co-occurrence of missense variants reported in other pathologies, which could modulate LHON penetrance: m.9101T>C (p.Ile192Thr) in *MT-ATP6* reported in LHON in individual P6 (49–53), m.3421G>A (p.Val39Ile) in *MT-ND1* described in Maternally Inherited Diabetes and Deafness (MIDD, MIM #520000) in individual P7 (54), m.9957T>C (p.Phe251Leu) in *MT-CO3* related among others in Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke-like episodes (MELAS, MIM #540000) in individual P8 (55) or m.15453T>C (p.Leu236Pro) in *MT-CYB* described in isolated complex III deficiency in individual P9 (56).

In addition, in our cohort, we found two individuals (P10 and P11) suffering from optic neuropathy and carrying two "reported" variants each (Supplementary Table S2). P10 had the m.3388C>A (p.Leu28Met) homoplasmic variant in *MT-ND1*, reported in Maternally Inherited Nonsyndromic Deafness (MIND, MIM #500008) (57) and m.14465G>A (p.Thr70Ile) heteroplasmic variant in *MT-ND6*, reported in LHON (58,59). Patient P11 carried the homoplasmic m.6489C>A (p.Leu196Ile) in *MT-CO1*, reported in CO1 deficiency (60), and the heteroplasmic m.13376T>C (p.Ile347Thr) in *MT-ND5* reported in MELAS with cerebral atrophy (61,62).

Table II. Demographic data, clinical and molecular features of patients carrying one "confirmed" pathogenic and one "reported" as pathogenic mtDNA variant. *These patients had only PCR-RFLP in blood to identify the "confirmed" pathogenic variant. B: Blood; U: Urine sediment; LHON: Leber's Hereditary Optic Neuropathy; MIDD: Maternally Inherited Diabetes and Deafness; MELAS: Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like episodes; LS: Leigh Syndrome; PEM: Progressive Encephalomyopathy; NAION: Non-Arteritic Ischemic Optic Neuropathy; ON: Optic Neuropathy; CPEO: Chronic Progressive External Ophthalmoplegia.

Patient	Haplogroup	Age	Sex	Clinical features	"Confirmed" pathogenic variant		"Reported" pathogenic variant						
					Nucleotide position (Heteroplasmy %)	Disease	Nucleotide position (Heteroplasmy %)	Locus	Mitomap frequency in 54594 FL seqs	gnomAD 3.1 frequency in 56417 seqs	Conservation	Disease	References
P1*	J1c3h	9	M	Leigh syndrome	m.14459G>A (B: 100%, U: 100%)	LS	m.15773G>A (U: 100%)	*MT-CYB*	0.116%	0.144%	100%	LHON with myoclonus	(44)
P2		37	F	Asymptomatic	m.14459G>A (B: 100%, U: 100%)		m.15773G>A (B: 72%, U: 72%)						
P3*		32	F	Asymptomatic	m.14459G>A (B: 100%, U: 100%)		m.15773G>A (U: 72%)						
P4*	L1c3a1b	17	M	Epilepsy, left hemianopia	m.3243A>G (B: 49%, U: 95%)	MELAS, LS	m.8668T>C (U: 100%)	*MT-ATP6*	0.060%	0.17%	91%	LHON	(45)
P5	H5s	58	F	Dystonia	m.14484T>C (B: 100%)	LHON	m.10680G>A (B: 100%)	*MT-ND4L*	0.031%	0.021	93%	LHON	(46–48)
P6	C4a1b	27	M	LHON	m.11778G>A (B: 82%)	LHON	m.9101T>C (B: 100%)	*MT-ATP6*	0.088%	0.1%	13%	LHON	(49,52,63–65)
P7	U5a2c1	38	M	LHON	m.11778G>A (B: 100%)	LHON	m.3421G>A (B: 100%)	*MT-ND1*	0.135%	0.223%	29%	MIDD	(54)
P8	J1c3e2	45	F	LHON	m.14484T>C (B: 65%)	LHON	m.9957T>C (B: 100%)	*MT-CO3*	0.082%	0.03%	98%	MELAS, PEM, NAION, CPEO	(55) (66–68)
P9	T2c1-146	40	F	ON	m.13042G>A (B: 19%, U: 66%)	ON, LS	m.15453T>C (B: 100%, U: 100%)	*MT-CYB*	0.020%	0.003%	42%	Isolated complex III deficiency	(56)

DISCUSSION

Prevalence of mtDNA pathogenic/likely pathogenic variants

The coexistence of two or more mtDNA pathogenic variants remains a rare event, with only a few reports in the literature (Table III). In our study, sequencing the whole mtDNA allowed us to identify the co-occurrences of two pathogenic variants in nearly 0.2% of the cohort. Recently, Wei *et al.* (69) estimated the prevalence of two or more disease-causing mutations at less than 1%, using GenBank sequences. They suggested this prevalence was influenced by mtDNA background, the proportion of double mutations being less important in European mtDNA. In contrast to these data, in our cohort, 80% (8/10) of families identified with two mtDNA pathogenic variants belonged to European haplogroups. Yang *et al.* (70) studied more than 26,000 whole mtDNA searching for the co-occurrence of three primary LHON variants and pathogenic or candidate variants of hearing loss. Among their cohort, this kind of association was found in 200 individuals (0.77%). Interestingly, most of the previously reported cases concern the co-occurrence of homoplasmic variants related to LHON, deafness or pathogenic variants characterised by incomplete penetrance (Table III)(31–34,71–75). However, there are few descriptions of co-occurrence of variants known to be involved in syndromic diseases, with the description in two subjects of a large-scale deletion and the heteroplasmic m.3243A>G variant (72,73) and four individuals from the same family carrying the m.3243A>G and the m.8356T>C in *MT-TK* (74) (Table III). Our results showed a greater diversity of mtDNA pathogenic variant combinations, responsible for LHON but also for syndromic diseases such as MIDD/MELAS or MERRF syndromes, with at least one variant found at a heteroplasmic state.

Table III. Literature review of patients/families with co-occurrence of two or more mtDNA pathogenic variants. LHON: Leber's Hereditary Optic Neuropathy; MELAS: Mitochondrial Encephalomyopathy Lactic Acidosis and Stroke-like episodes; KSS: Kearns-Sayre Syndrome; MERRF: Myoclonic Epilepsy with Ragged-Red Fibers; CI: Complex I.

References	mtDNA variation	Patients	Diseases	Country of origin	Functional studies	Consequences of the co-occurrence
Riordan-Eva et al. (1995)	m.11778G>A m.14484T>C	1	LHON	UK	No	Synergistic
Ohno et al. (1996)	m. 3243A>G Large-scale deletion (2532 bp)	1	MELAS/KSS overlap syndrome Myopathy Autoimmune polyendocrinopathy	US	No	Synergistic
Brown et al. (2001)	m.11778G>A m.14484T>C	1	LHON	UK	Biochemical assay of CI-linked respiration and CI activity	Biochemical deleterious effect Mitochondrial dysfunction No clinical influence but early age of disease onset
Howell et al. (2002)	m.11778G>A m.14484T>C	5 (2 families)	LHON Fatal infantile encephalopathy	Australia, US	No	No clinical influence
Zhao et al. (2004)	m.1494C>T m.1555A>G	28 (1 family)	Hearing loss	China	Respiration assay	Synergistic Mitochondrial dysfunction
Wei et al. (2007)	m.14484T>C m.1555A>G	4 (1 family)	LHON Hearing loss	China	No	Synergistic
Zhang et al. (2008)	m.11778G>A m.1555A>G	12 (1 family)	LHON	China	No	Increase penetrance
Tonska et al. (2008)	m.11778G>A m.3460G>A	5 (1 family)	LHON	Poland	No	No clinical influence
Nakamura et al. (2010)	m.8356T>C m.3243A>G	4 (1 family)	MERRF/MELAS overlap syndrome	Japan	No	Synergistic
Khan et al. (2013)	m.11778G>A m.1555A>G	4 (2 families)	LHON	India	No	Increase penetrance
Yu et al. (2016)	m. 3243A>G Large-scale deletion (4977 bp)	1	MELAS/KSS overlap syndrome	China	No	Increase clinical severity
Cruz-Bermúdez et al. (2016)	m.11778G>A m.14484T>C m.11253T>C	1	LHON	Spain	Biochemical assay in cybrid cell lines	No biochemical influence Mitochondrial dysfunction
Catarino et al. (2017)	m.11778G>A m.14484T>C	4 (1 family)	LHON	Germany	Biochemical assay of CI-linked respiration in fibroblasts	Synergistic Mitochondrial dysfunction

If we also consider likely pathogenic variants and variants reported to increase penetrance or expressivity, the prevalence of co-occurrences rises, with nearly 0.4% of additional individuals (Table II and Supplementary Table S2). In the literature, the description of this type of co-occurrences is a little more abundant. Most of them concern LHON patients (Supplementary Table S3) and attribute to the second variant a modulator function, increasing the penetrance and expressivity of the disease (82–85) or playing a protective role (86,87). Recent studies have demonstrated that the co-occurrence of rare mtDNA polymorphisms, as the combination of m.14258G>A (p.Pro139Leu) in the *MT-ND6* gene, m.10680G>A in *MT-ND4L*, and m.12033A>G (p.Asn425Ser) in *MT-ND4* (47), or the association of m.4136A>G (p.Tyr277Cys) in *MT-ND1*, m.9139G>A (p.Ala205Thr) in *MT-ATP6*, and m.15773G>A (p.Val434Met) in *MT-CYB* (44), may lead to a reduction in oxygen consumption, OXPHOS deficiency, and may be expressed by clinical impairments as LHON. Thus, identifying these co-occurrences of pathogenic variants or rare polymorphisms should slightly improve the performance of mtDNA analysis, which was previously estimated at around 10-15%, depending on the cohorts (28)(88).

Identification of co-occurrences of variants for better genotype-phenotype correlation

The influence of variant co-occurrence on the clinical phenotype remains debated in the literature. In some cases, each pathogenic variant could be responsible for one part of the patient's phenotype. For example, Mimaki *et al*. (89) described a patient with LHON and cardiomyopathy carrying the m.11778G>A variant responsible for LHON and m.12192G>A in the *MT-TH* gene already reported in cardiomyopathy (90). In our cohort, the co-occurrence of pathogenic variants may explain the clinical expressivity and some complex phenotypes, as described in our family C (Figure 1D and Table I). Because of her family history of MERRF, patient II.2's symptoms had been initially attributed to the m.8344A>G variant identified by

Sanger sequencing. However, later, whole mtDNA sequencing revealed the m.3243A>G pathogenic variant at a rate of 20%. This variant could explain the different clinical expression compared to its relatives carrying only the m.8344A>G. Indeed, only individual II.2 suffered from glucose dysregulation and hearing loss. The m.3243A>G is known to be the most diabetogenic mtDNA pathogenic variant, with intermediate levels of heteroplasmy (91,92), whereas diabetes in the m.8344A>G variant was rarer (93). It is typically observed in adulthood and before the age of 70 (94), often in association with bilateral deafness (95,96). We can also hypothesise that the m.3243A>G potentiates the severity of proband's neurological symptoms (97,98) or deafness (93). This example shows, despite clinical evidence of a syndrome and the presence of a familial mutation, the importance of systematically performing whole mtDNA sequencing to reveal pathogenic variant co-occurrences and thus explain the phenotypic variability of mitochondrial pathologies other than by simple heteroplasmy variations.

As in clinical reports, *in vitro* functional assays, only realised on LHON mtDNA pathogenic variants, led to conflicting results on the synergistic effect of those co-occurrences. Howell *et al.* have concluded that the association of m.11778G>A and m.14484T>C led to a deleterious synergistic impact in their patients (33), which could be consistent with a more severe phenotype than those carrying a single LHON pathogenic variant. Catarino *et al.* also suggested a synergistic effect of the combination of pathogenic variants (34). However, Cruz-Bermudez *et al.* described a case of a patient harbouring three variants implied in LHON: two "confirmed", m.11778G>A and m.14484T>C, and a "reported" m.11253T>C variants (81). They did not find any synergistic effect in cybrid cells carrying these three pathogenic variants and those carrying a single variant responsible for LHON. Some authors have also suggested that the co-occurrence of LHON pathogenic variants did not influence severity but could lead to an earlier age of pathology onset (30). In our study, functional studies have shown a slight decrease in

complex I activity for both tested individuals (Supplementary Table S4) but a lower mitochondrial mass for the symptomatic subject (III.1) than for his asymptomatic aunt (II.2) (Supplementary Table S5). Moreover, for the index (III.5), the mitochondrial mass increased by 50% after vision recovery (Supplementary Table S5), supporting the hypothesis that the wild-type copy number is correlated with symptoms (99,100).

Heteroplasmy is well known as one of the leading causes of phenotypic variability of mtDNA pathogenic variants (19). In family D (Table II, P1 to P3), it could be hypothesised that the homoplasmic m.15773G>A increases the penetrance of the m.14459G>A variant in the proband which suffered from Leigh syndrome, unlike his mother and maternal aunt who were asymptomatic and had the variant in a heteroplasmic state. Conversely, heteroplasmy levels did not explain the LHON penetrance in family A. While they all carried the co-occurrence of m.11778G>A and m.14484T>C variants, only males developed LHON. This observation was consistent with literature where males are more often affected than females in a ratio of approximately 2.5:1 for m.11778G>A and 5.7:1 for m.14484T>C (31). Fifty per cent of male carriers of those variants develop symptoms, but only ten per cent of female carriers (31)(101). However, this penetrance variability is not fully understood even if some factors are already known: mtDNA modulator variants (80)(102), nuclear genetic determinants (103,104), and environmental factors may modulate the susceptibility of LHON pathogenic variants carriers to develop symptoms (105,106). For example, Jiang *et al.* (103) reported that the combination of a mutation in the nuclear gene *YARS2* and the mitochondrial variant m.11778G>A enhanced mitochondrial damage, leading to a severe phenotype of LHON. Among environmental factors, a significant decrease in mtDNA copy number has been shown in smoking subjects with LHON (107). Still, mtDNA content was similar within the family regardless of their symptoms or tobacco consumption. As only women were unharmed, the

protective role of oestrogens can also be mentioned, as these hormones ameliorate mitochondrial dysfunction in LHON through the oestrogen receptors (108).

Identification of co-occurrences of variants for better genetic counselling

Despite the development of mtDNA NGS, targeted sequencing of a few pathogenic variants, according to patient phenotype, remains adopted by some teams. However, analysing the whole mtDNA by NGS can reveal the co-occurrence of pathogenic variants at low levels, which, although they probably do not influence the phenotype of patients, will modify their genetic counselling. For instance, in patient III.4 from family B (Figure 1C and Table I), the m.13042G>A at 34% is responsible for her optic neuropathy (39). In addition, the m.3243A>G variant at about 7% was identified. This pathogenic variant was reported in a wide range of phenotypes according to heteroplasmy rates (109) as well as other surrounding factors (110), including MELAS (111) or Leigh syndrome (112) for high heteroplasmy rates and MIDD for intermediate ones (113). However, it has been shown that for it, there is a relatively high phenotypic threshold of heteroplasmy (>60%) (19) which explains the expressivity of the disease. We can therefore assume that this variant at a rate of 7% will not be responsible for a specific phenotypic presentation but may act as a phenotype modifier (27). In addition, one or both mtDNA pathogenic variants could be transmitted to offspring at higher rates of heteroplasmy due to bottleneck theory, leading to a clinical expression (114).

In some other cases, only one pathogenic variant is responsible for the patient's phenotype, as reported by Khan *et al.* (2013) (80), with two families also harbouring m.14484T>C and m.1555A>G. Still, they had optic atrophy with an absence of hearing impairment. Identifying the second variant nevertheless allows for improved patient management and appropriate genetic counselling. Indeed, known pathogenic variants associated with hearing loss should be regarded as "actionable" since they may modify patient management by avoiding specific drugs

such as aminoglycosides, audiometric follow-up and hearing protection. Thus, identifying the co-occurrence of mtDNA pathogenic variants with low heteroplasmy rate will improve but complicate genetic counselling, especially for carriers.

Unknown origin of co-occurrence of pathogenic variants

In family A (Figure 1B), both LHON pathogenic variants were present in all individuals, making the timing of mutational events impossible. However, the m.14484T>C variant was homoplasmic and m.11778G>A heteroplasmic, which could mean that the latter appeared after, but no proof of this has been obtained to date. There is no further data in the literature regarding these co-occurrences of variants. In the two other families, it is possible to specify the chronology of mutational events, as one of the variants was observed in only one individual and supposed to be *de novo* within the limits of sensitivity of the techniques used (~2%) and the tissues analysed. As mtDNA is more prone to accumulate mutations than the nuclear genome (22), *de novo* mtDNA variants are not uncommon (115). Among them, the m.3243A>G pathogenic variant was frequently reported as *de novo* (116–119), especially in twins (120), with moderate rates of heteroplasmy (20%) and without clinical expression. Several reports have suggested that oxidative damage and environmental factors such as smoking may accelerate the process of this variant's apparition and accumulation (65)(121). In the two individuals carrying the *de novo* m.3243A>G, this second mutational event could not be explained by variants in nuclear-encoded mitochondrial genes involved in mitochondrial maintenance. Interestingly, in these families, the long-read sequencing revealed four different mtDNA molecules (Figure 2) with a mixture of molecules without any pathogenic variant, molecules with only one of the two variants and molecules with both. Therefore, wild-type mtDNA molecule content, which allows the proper function of the mitochondria, depends on whether pathogenic variants are combined. This rate could partly explain the biochemical

defect of the mitochondrial respiratory chain and the clinical heterogeneity of mitochondrial diseases (99)(122). On the other hand, the coexistence of four molecules of different sequences in both patients supports the possibility of recombination in human mtDNA. Already known in yeast, plants and animals (123–126), this mechanism of homologous recombination, which allows the repair of DNA double-strand breaks and the exchange of genetic material (127,128), remains debated for years in humans (129–135). However, it seems more likely that the occurrence of two mutations and then recombination mechanisms are at the origin of the four types of molecules observed rather than the occurrence of four distinct mutational events. This probable recombination makes it challenging to determine whether the second event occurred on wild-type or mutant mtDNA molecule. It also increases the complexity of genetic counselling, as patients risk transmitting only one of the two mutations or their combination to their offspring at random.

Conclusion

According to our results, the coexistence of two pathogenic variants is not as rare as previously suggested in the literature. Moreover, this phenomenon does not only concern LHON pathogenic variants but also variants reported in syndromic disorders. We can assume that combinations of mitochondrial variants may be an additional element to explain the phenotypic variability of mitochondrial diseases, not only for pathogenic variant combinations but also for other rare variant associations. Thus, detecting the co-occurrence of mtDNA variants is essential for an accurate diagnosis and appropriate genetic counselling, and it highlights the need to develop specific tools and interoperable databases to index them.

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ANNEXES

Supplementary Patients and Methods

Analysis of patients with mtDNA deletions

Family A (Figure 1B and Table I):

Individual II.1 developed bilateral blindness at the age of seventeen with the recovery of visual acuity six months later, without sequelae. He also reported having started smoking one year before the first symptoms. We could not obtain a sample for DNA analysis because of the patient's refusal.

The proband's aunt (II.2) was asymptomatic and had been smoking since she was twenty-eight years old. Her ophthalmological examination revealed a decrease in the vision of her right eye to 7/10 with temporal atrophy of this eye on OCT, stable with time (Supplementary figures 1A and 1B).

The proband's mother (II.5) was also asymptomatic. She had been smoking for thirty-five years, about twenty cigarettes per day.

The proband's cousin (III.1) presented with a sudden decrease in vision in both eyes when he was seventeen. He had a period of tobacco intoxication one year before the onset of symptoms until he was thirty-three years old. Suddenly, he presented bilateral complete optic atrophy with diffuse visual field deficit in both eyes.

Individual III.3 developed a sudden drop in vision in both eyes very early in childhood, at the age of four. He never recovered his vision, and the last ophthalmic examination showed visual acuity of 1/10 in the right eye and 2/10 in the left one year before his death at thirty. Therefore, we did not have any mtDNA samples to analyse for him.

Family C (Figure 1D and Table I):

The proband (II.2) from family C was a sixty-five-year-old female followed since 2016 for a neuromuscular disorder, which would have appeared at the age of sixty. Two years later, she suffered from balance disorders with weakness of the two lower limbs and nocturnal cramps. Clinical examination showed a proximal weakness of the legs and static cerebellar syndrome. Biological tests revealed an elevation of muscle enzymes. Her symptoms had worsened since 2020, with increased falls and balance problems leading to difficulties in getting up and a tendency to hyperglycaemia, which did not require medication.

The proband sister's (II.3) symptomatology started more than 30 years ago with a painful fatigability. She has been developing balance disorders at fifty years old with progressive evolution. The clinical examination revealed a mild kinetic cerebellar syndrome of the lower limbs and a diffuse osteotendinous areflexia but no oculomotor impairment or myoclonus. Brain MRI showed discrete cerebellar atrophy. However, in the last 5-6 years, she had an apparent increase in her disorders, leading to repeated falls resulting in fractures.

The proband's nephew (III.1) was previously asymptomatic. For the last 3-4 years, he had been suffering from involuntary movements of limbs compatible with myoclonus, gait disorders, and fatigue on exertion. Clinical examination was poor, brain imaging did not reveal any abnormalities, but an electroneuromyogram (ENMG) showed a predominantly sensory neuropathy. The rest of his assessment was regular.

The other proband's nephew (III.2) was hospitalised at the University Hospital of Angers to explore persistent pain in the upper and lower limbs with aches not relieved by level I analgesics. Biological tests showed an increase of serum creatine phosphokinase to twice the standard value but no muscle abnormality on ENMG. The mother (II.1) and brother (III.3) of these two patients would have had the same diagnosis of MERRF and died early.

Individual III.5 suffered from cramps for several years associated with diffuse muscular pain, mainly after exertion with hands and face oedema. An exhaustive workup concluded the diagnosis of systemic scleroderma and ANCA vasculitis.

Analysis of patients in Table II and Supplementary Table S2

The proband (P1) was a nine-year-old boy with a suspected mitochondrial disease based on the presence of progressive motor signs of dystonia. There was no ophthalmological involvement on the first workup performed two years earlier. He was the only affected case in the family, and his parents had no other children at that moment. His mother (P2) and his maternal aunt (P3) were asymptomatic.

Individual P4 presented with headaches associated with left hemianopia. Brain MRI performed on this subject showed pseudostrokes, which may point to a mitochondrial disease.

Individual P5 is a fifty-eight-year-old female who had isolated dystonia.

Individuals P6 to P9 suffered from optic neuropathy.

Individual P10 was affected by LHON.

Individual P11 suffered from optic neuropathy.

DNA extraction

Total DNA was extracted from blood and urine sediment according to the manufacturer's protocols:

- EZ1 DNA Tissue kit (Qiagen, Venlo, Netherlands) on BioRobot EZ1 for urine sediment.
- NucleoMag Blood 200µl Kit (Macherey-Nagel, Hoerdt, France) on Microlab STARlet M (Hamilton Company, Reno, NV, USA) and Thermofisher Starlet Presto (Thermo Fisher Scientific, Waltham, MA, USA). DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

Heteroplasmy quantification

Fluorescent PCR – Restriction

Heteroplasmy quantifications were performed in a two-step process: PCR with one of the primers tagged with the fluorescent dye FAM and enzymatic restriction of the PCR products. General conditions are detailed in the following paragraph, and specificities for each mutation are in the table.

PCR reaction included: 25 µL of GoldStar® Mix (Eurogentec, Seraing, Belgium), 1µL of each primer (10µM), 1 µl of MgCl2, 17 µL of RNAse-free water, and 30 ng of patient DNA.

The amplification conditions were 94°C for 10 minutes, the n cycles of 1 minute at 94°C, 1 minute at Tm temperature, 1 minute at 72°C, and the last step at 72°C for 5 minutes.

Fluorescent PCR-Restriction conditions. *6-FAM Dye

Mutation	PCR				Restriction		
	Forward primer (5'→3')	Reverse primer (5'→3')	Cycle	Tm (°C)	Enzyme	T (°C)	Time (min)
m.3243A>G	*CCCTGTACGAAAGGACAAGAGAAATAACGCC	CGTTCGGTAAGCATTAGGAATGCCATTGC	30	58	HaeIII	37	30
m.8344A>G	GTAGTATTTAGTTGGGGCATTTCACTGTAAAGCCGTGTTGG	*CTACCCCCTCTAGAGCCCAC	28	55	BgII	37	60
m.11778G>A	*GCCCACGGGCTTACATC	AAACCCGGTAATGATGTCGG	30	58	Tsp45I	65	120
m.14459G>A	ATGCCTCAGGATACTCCTCAATAGCCGTC	AGGATCAGGCAGGCGCCAAG	30	58	Tsp45I	65	240
m.14484T>C	CACTCACCAAGACCTCAACC	GGGTTTGTGGGGTTTTCTTC	33	60	BccL	37	60

Fragments analysis

The product of enzymatic digestion was analysed by capillary electrophoresis on ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) using DNA ladder GeneScan™ 600 LIZ™ dye Size Standard or GeneScan™ 350 ROX™ dye Size Standard. Data were analysed using Peak Scanner™ v1.0 software (Applied Biosystems). Heteroplasmy load

is calculated as the ratio of the area under the curve of the mutated peak divided by the sum of the areas under the curves of the mutated and wild-type peaks.

Whole mtDNA next-generation sequencing

Ion torrent sequencing

Deep sequencing was performed on all individuals of family A and D, individual III.4 from family B, and individuals II.2, III.2, III.5 from family C.

The whole mitochondrial genome was amplified in two overlapping fragments of 8009 bp (amplicon A) and 8994 bp (amplicon B). The primer pairs used for both PCRs, tested on DNA extracted from cells devoid of mtDNA-(Rho Zero cells) to avoid pseudogene amplification, were located on the cytochrome b and cytochrome c oxidase one genes: 5′-TACGTTGTAGCCCACTTCCACT-3′ and 5′-GCCCGATGTGTAGGAAGAG-3′ for amplicon A; 5′-AACTTCGGCTCACTCCTTGG-3′ and 5′-AGTAACGTCGGGGCATTCCG-3′ for amplicon B. Long-range PCR was performed with KapaLongRange DNA polymerase according to the manufacturer's recommendations (Kapa Biosystems, Boston, MA, USA). Agarose gel electrophoresis (1%) was used to confirm the presence of PCR products. The concentrations of PCR products were quantified by the Qubit® 2.0 Fluorometer using the Qubit® dsDNA HS Assay Kit (Thermo Fischer technologies, Waltham, MA, USA). For each sample, amplicons A and B were pooled equimolarly. Libraries were compiled from 50 to 200 ng of pooled PCR products with the enzymatic fragmentation method of the Library Builder Automate using the dedicated Ion Xpress™ Plus Fragment Library Kit (Thermo Fischer technologies). All purification procedures were performed using Agencourt AMpure XP beads (Beckman Coulter, Brea, CA, USA).

Processing the sequencing data

Sequencing was performed using Ion Torrent Proton, and the pre-processing embedded pipeline did the signal processing and base calling. Demultiplexed reads were mapped on an mtDNA revised Cambridge Reference Sequence (rCRS; NC_012920) before being analysed with a dedicated in-house pipeline integrating various modules for coverage analysis, variant calling, annotation, and prioritisation. The calling module uses a consensus-based approach and combines the prediction of six callers (Torrent Suite Variant Caller, GATK, VarScan, SNVer, LoFreq, and Platypus). All the variant calling formats (VCFs) generated were normalised and

decomposed before launching the annotation-prioritization module, combining the NCBI Variant Reporter for gene annotation and ANNOVAR for variant prioritisation. ANNOVAR allowed the inclusion of several databases, i.e., Mitomap and MitImpact2, and prioritisation tools, i.e., Polyphen2, SIFT, and MutationTaster. All the results were then included in a private database that may be interrogated via a dedicated graphical user interface for reducing the number of candidate variants by applying prioritisation filters. Putative variants were further filtered using the following criteria: at least three callers predicting the variant, including the Torrent Suite Variant Caller, coverage greater than ×500, quality score >150, and a minimum mtDNA heteroplasmy level of 2%, was validated as a sensitive threshold for the detection of variants. All types of variants were studied.

Long-read sequencing

mtDNA amplification

The mitochondrial genome was amplified in a fragment of 7986 bp for patient III.4 from family B and 9290 bp for patient II.2 from family C. The primer pairs used for both PCRs, tested on DNA extracted from cells devoid of mtDNA-(Rho Zero cells) to avoid pseudogene amplification, were: 5′-CGTTACATGGTCCATCATAGAA-3′ and 5′-TTCGATGTTGAAGCCTGAGAC-3′ for family B; 5′-AACCAAACCCCAAAGACACC-3′ and 5′-GCCAATAATGACGTGAAGTCC-3′ for family C. Long-range PCR was performed with TaKaRa LA Taq® DNA Polymerase according to the manufacturer's recommendations (Takara Bio Inc., Japan). The amplification conditions were 94°C for 5 minutes, 35 cycles of 30 seconds at 94°C, 20 seconds at 62°C, 5 minutes at 68°C, and the last step at 68°C for 3 minutes. Agarose gel electrophoresis (1%) was used to confirm the presence of PCR products.

Single molecule real-time technology (SMRT)

LR-PCR reaction products were purified with 0.6× AMPure PB Beads (Pacific Biosciences, Menlo Park, CA, USA) in conditions recommended by the manufacturer and eluted in 20 µL Elution Buffer (EB, PacBio) before DNA quantitation with the Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, Montigny-le-Bretonneux, France). All other reagents are from PacBio unless indicated. Libraries were prepared by following the Preparing Amplicon Libraries using PacBio Barcoded Adapters for the Multiplex SMRT sequencing procedure. End-repair and ligation reaction for multiplexing was carried out with equimolar ratios of input amplicons with

the SMRTbell Barcoded Adapter Prep Kit and Barcoded Adapter Plate-96. Products were pooled together, and purification was carried out as indicated above. DNA damage repair and exonuclease digestion were successively carried out by using 500 ng DNA with the SMRTbell DNA Damage Repair Kit before two successive rounds of purification and then quantitation of SMRTbell Templates in conditions described above. Final libraries (III.4 from family B and II.2 from family C), consisting of the circular templates, polymerase, and Sequencing Primer v3 or v4, were prepared with the Sequel Binding Kit 2.0 or 3.0, loaded onto SMRT cells 1M v2 or v3 LR at a 4 pM final concentration by diffusion, and sequenced with the Sequel Sequencing Kit 2.1 or 3.0 in the Sequel System, respectively. Sequencing data were collected from either 10-hour or 20-hour movie time.

Data quality control and analysis

The primary output data analysis was done with SMRTLink v5.1.0 (PacBio). Briefly, barcode demultiplexing was carried out automatically at the end of the runs. Data were analysed by the Long Amplicon Analysis (LAA) module, which is dedicated to phasing information by generating long haploid reads (136), with a minimum predicted accuracy of 0.999 (LAA parameters: minimum read length = 2,500 bp; maximum read length = 3,200 bp; minimum allele/haplotype read fraction = 0.2; maximum phasing reads = 500). Fastq files were exported to SeqOne (Montpellier, France) for processing. Fastq files were aligned on reference genome GRCh38/hg38 with Vulcan (137). The sequence of each read was retrieved using the Python library "Pysam" (version 0.16.0.1). The number of reads that support each haplotype were counted for positions 3243 and 13042 (Family B) or 3243 and 8344 (Family C). The counts were finally reported in tabular form for each family with each row corresponding to a combination of genotypes at the two positions of interest.

Exome sequencing

Illumina sequencing

Library preparation was done by capture using the Twist Human Core Exome Plus Kit (Twist BioSciences, San Francisco, CA, USA). The prepared DNA libraries were sequenced in a NextSeq550 sequencer (Illumina Inc, San Diego, CA, USA) using the NextSeq™ 500/550 High Output v2 kit.

Bioinformatics analysis

For bioinformatics analysis, the raw data obtained (bcl) are converted to sequence format (fastq) using the GenerateFastq module (Illumina®). Quality control of these sequences is then performed using the Fastqc software (v 0.11.9). The sequences produced are aligned and compared to the human reference genome (GRCh38/hg38). The sequencing depth of coding exons and splice sites (source: RefSeq) was calculated with a quality threshold of a percentage of bases covered by at least 20 reads greater than 98% and an average read depth greater than 50x.

Functional analysis

Respiratory chain assay

The respiratory chain assay was performed according to the protocol described elsewhere (136). Primary skin fibroblasts from individuals II.2 and III.1 of family A, one patient control with CI deficiency, and three healthy controls were cultured. Mitochondrial respiration rate was measured on a high-resolution oxygraphy, and the activities of the mitochondrial OXPHOS complexes were evaluated by spectrophotometry (136).

Quantification of mtDNA content

Quantification of mtDNA copy numbers for individuals II.2, II.5, III.1, III.2, III.4, and III.5 from family A were performed using a previously described (137). It was conducted before and after visual recovery for the latter individual (III.5). Real-time quantitative PCR (Q-PCR) was performed using the Chromo4 System (Biorad®, Hercules, CA, USA) in a 20 µl reaction volume containing ×1 IQ SYBR Green Supermix (Biorad®) and a final concentration of 0.5 µM of each gene-specific primer and 3 µl of the template. The pairs of primers selected were F1R1 (forward 1 and reverse 1; nucleotide positions 8342–8359 and 8546–8559) to quantify the mtDNA in blood samples, and NCOA3 F/R (nuclear receptor coactivator three forward: 5′-AAAGTTAATGGAGTTTCCTG-3′ and reverse: 5′-AGGACTGGCGTTTATGTCTT-3′) to quantify the nuclear DNA in blood samples too. The reactions were performed as described elsewhere (138).

S1 Table

Details of 93 "confirmed" pathogenic variants in Mitomap.

Locus	Allele	Amino Acid or RNA	Plasmy Reports (Homo/Hetero)	Disease	Mitomap Status	GenBank Frequency
MT-TF	m.583G>A	tRNA Phe	-/+	MELAS / MM & EXIT	Confirmed	0.000%
MT-TF	m.616T>C	tRNA Phe	+/+	Maternally inherited epilepsy / mito tubulointerstitial kidney disease (MITKD) / Gitelman-like syndrome	Confirmed [LP]	0.002%
MT-RNR1	m.1494C>T	12S rRNA	+/-	DEAF	Confirmed [LP]	0.007%
MT-RNR1	m.1555A>G	12S rRNA	+/+	DEAF; autism spectrum intellectual disability; possibly antiatherosclerotic	Confirmed	0.141%
MT-TV	m.1606G>A	tRNA Val	-/+	AMDF	Confirmed [VUS]	0.000%
MT-TV	m.1630A>G	tRNA Val	-/+	MNGIE-like disease / MELAS	Confirmed	0.000%
MT-TV	m.1644G>A	tRNA Val	-/+	Leigh Syndrome / HCM / MELAS	Confirmed [LP]	0.000%
MT-TL1	m.3243A>G	tRNA Leu (UUR)	-/+	MELAS / Leigh Syndrome / DMDF / MIDD / SNHL / CPEO / MM / FSGS / ASD / Cardiac + multi-organ dysfunction	Confirmed	0.018%
MT-TL1	m.3243A>T	tRNA Leu (UUR)	-/+	MM / MELAS / SNHL / CPEO	Confirmed	0.000%
MT-TL1	m.3256C>T	tRNA Leu (UUR)	-/+	MELAS; possible atherosclerosis risk	Confirmed [P]	0.000%
MT-TL1	m.3258T>C	tRNA Leu (UUR)	-/+	MELAS / Myopathy	Confirmed [LP]	0.002%
MT-TL1	m.3260A>G	tRNA Leu (UUR)	-/+	MMC / MELAS	Confirmed	0.000%
MT-TL1	m.3271T>C	tRNA Leu (UUR)	-/+	MELAS / DM	Confirmed	0.000%
MT-TL1	m.3273delT	tRNA Leu (UUR)	-/+	PEM / retinal dystrophy in MELAS	Confirmed	0.000%
MT-TL1	m.3280A>G	tRNA Leu (UUR)	-/+	Myopathy	Confirmed [VUS]	0.000%
MT-TL1	m.3291T>C	tRNA Leu (UUR)	-/+	MELAS / Myopathy / Deafness + Cognitive Impairment	Confirmed	0.000%
MT-TL1	m.3302A>G	tRNA Leu (UUR)	-/+	MM	Confirmed	0.000%

MT-TL1	m.3303C>T	tRNA Leu (UUR)	+/+	MMC	Confirmed	0.000%
MT-ND1	m.3376G>A	E24K	+/+	LHON MELAS overlap	Confirmed	0.000%
MT-ND1	m.3460G>A	A52T	+/+	LHON	Confirmed	0.057%
MT-ND1	m.3635G>A	S110N	+/-	LHON	Confirmed	0.016%
MT-ND1	m.3697G>A	G131S	+/+	MELAS / Leigh Syndrome / LDYT / BSN	Confirmed [LP*]	0.000%
MT-ND1	m.3700G>A	A132T	+/-	LHON	Confirmed [VUS*]	0.005%
MT-ND1	m.3733G>A	E143K	+/+	LHON	Confirmed [VUS*]	0.004%
MT-ND1	m.3890G>A	R195Q	-/+	Progressive Encephalomyopathy / Leigh Syndrome / Optic Atrophy	Confirmed	0.002%
MT-ND1	m.3902_3908inv	DLA-GKV	-/+	EXIT + myalgia / severe LA + cardiac / 3-MGA aciduria / nephropathy + deafness + diabetes	Confirmed	0.000%
MT-ND1	m.4171C>A	L289M	+/+	LHON / Leigh-like phenotype	Confirmed	0.004%
MT-TI	m.4298G>A	tRNA Ile	-/+	CPEO / MS	Confirmed	0.000%
MT-TI	m.4300A>G	tRNA Ile	+/+	MICM	Confirmed	0.000%
MT-TI	m.4308G>A	tRNA Ile	-/+	CPEO	Confirmed	0.000%
MT-TQ	m.4332G>A	tRNA Gln	-/+	Encephalopathy / MELAS	Confirmed	0.000%
MT-TM	m.4450G>A	tRNA Met	-/+	Myopathy / MELAS / Leigh Syndrome	Confirmed	0.000%
MT-TW	m.5521G>A	tRNA Trp	-/+	Mitochondrial myopathy	Confirmed	0.000%
MT-TW	m.5537_5539insAT	tRNA Trp	-/+	Leigh Syndrome	Confirmed	0.000%
MT-TA	m.5650G>A	tRNA Ala	-/+	Myopathy	Confirmed	0.002%
MT-TN	m.5690A>G	tRNA Asn	-/+	CPEO + ptosis + proximal myopathy	Confirmed	0.000%
MT-TN	m.5703G>A	tRNA Asn	-/+	CPEO / MM	Confirmed	0.002%
MT-TN	m.5728T>C	tRNA Asn	-/+	Multiorgan failure/myopathy	Confirmed	0.002%
MT-CO1	m.7445A>G	term514term	+/+	SNHL	Confirmed	0.002%
MT-TS1 precursor	m.7445A>G	tRNA Ser (UCN) precursor	+/+	SNHL	Confirmed [P]	0.002%
MT-TS1	m.7471_7472insC	tRNA Ser (UCN)	+/+	PEM / AMDF / Motor neuron disease-like	Confirmed	0.012%
MT-TS1	m.7497G>A	tRNA Ser (UCN)	+/+	MM / EXIT	Confirmed	0.002%

MT-TS1	m.7510T>C	tRNA Ser (UCN)	-/+	SNHL	Confirmed	0.002%
MT-TS1	m.7511T>C	tRNA Ser (UCN)	+/+	SNHL/Deafness	Confirmed	0.004%
MT-TK	m.8306T>C	tRNA Lys	-/+	Severe adult-onset multisymptom myopathy / Myoclonic epilepsy	Confirmed	0.000%
MT-TK	m.8313G>A	tRNA Lys	-/+	MNGIE / Progressive mito-cytopathy	Confirmed	0.002%
MT-TK	m.8340G>A	tRNA Lys	-/+	Myopathy / Exercise Intolerance / Eye disease+SNHL	Confirmed	0.000%
MT-TK	m.8344A>G	tRNA Lys	-/+	MERRF; Other - LD / Depressive mood disorder / leukoencephalopathy / HICM	Confirmed [P]	0.007%
MT-TK	m.8356T>C	tRNA Lys	-/+	MERRF	Confirmed	0.000%
MT-TK	m.8363G>A	tRNA Lys	-/+	MICM+DEAF / MERRF / Autism / Leigh Syndrome / Ataxia+Lipomas	Confirmed	0.000%
MT-ATP6	m.8851T>C	W109R	+/+	BSN / Leigh syndrome	Confirmed [VUS*]	0.005%
MT-ATP6	m.8969G>A	S148N	-/+	Mitochondrial myopathy, lactic acidosis and sideroblastic anaemia (MLASA) / IgG nephropathy	Confirmed	0.002%
MT-ATP6	m.8993T>C	L156P	-/+	NARP / Leigh Disease / MILS / other	Confirmed [P*]	0.004%
MT-ATP6	m.8993T>G	L156R	+/+	NARP / Leigh Disease / MILS / other	Confirmed [P*]	0.011%
MT-ATP6	m.9035T>C	L170P	+/+	Ataxia syndromes	Confirmed	0.000%
MT-ATP6	m.9155A>G	Q210R	-/+	MIDD, renal insufficiency	Confirmed	0.000%
MT-ATP6	m.9176T>C	L217P	+/+	FBSN / Leigh Disease / Spinocerebellar Ataxia	Confirmed [LP*]	0.005%
MT-ATP6	m.9176T>G	L217R	+/+	Leigh Disease / Spastic Paraplegia / Spinocerebellar Ataxia	Confirmed	0.002%
MT-ATP6	m.9185T>C	L220P	+/+	Leigh Disease / Ataxia syndromes / NARP-like disease / Episodic weakness and Charcot-Marie-Tooth	Confirmed	0.005%
MT-ATP6	m.9205_9206del	Ter-M	+/-	Encephalopathy / Seizures / Lacticacidemia	Confirmed	0.000%
MT-TG	m.10010T>C	tRNA Gly	-/+	PEM	Confirmed	0.000%
MT-ND3	m.10158T>C	S34P	+/+	Leigh Disease / MELAS	Confirmed [P*]	0.000%
MT-ND3	m.10191T>C	S45P	-/+	Leigh Disease / Leigh-like Disease / ESOC	Confirmed	0.000%
MT-ND3	m.10197G>A	A47T	+/+	Leigh Disease / Dystonia / Stroke / LDYT	Confirmed	0.007%
MT-ND4L	m.10663T>C	V65A	+/-	LHON	Confirmed	0.004%
MT-ND4	m.11777C>A	R340S	-/+	Leigh Disease	Confirmed	0.000%
MT-TH	m.12147G>A	tRNA His	-/+	MERRF-MELAS / Encephalopathy	Confirmed	0.000%
MT-TH	m.12201T>C	tRNA His	-/+	MIND	Confirmed	0.002%

MT-TS2	m.12258C>A	tRNA Ser (AGY)	-/+	DMDF / RP+SNHL	Confirmed	0.002%
MT-TL2	m.12276G>A	tRNA Leu (CUN)	-/+	CPEO	Confirmed	0.002%
MT-TL2	m.12294G>A	tRNA Leu (CUN)	-/+	CPEO / EXIT+Ophthalmoplegia	Confirmed	0.000%
MT-TL2	m.12315G>A	tRNA Leu (CUN)	-/+	CPEO / KSS / possible carotid atherosclerosis risk, the trend toward myocardial infarction risk	Confirmed	0.000%
MT-TL2	m.12316G>A	tRNA Leu (CUN)	-/+	CPEO	Confirmed	0.000%
MT-ND5	m.12706T>C	F124L	-/+	Leigh Disease	Confirmed	0.000%
MT-ND5	m.13042G>A	A236T	-/+	Optic neuropathy/ retinopathy/ LD	Confirmed	0.002%
MT-ND5	m.13051G>A	G239S	+/-	LHON	Confirmed	0.000%
MT-ND5	m.13094T>C	V253A	+/+	Ataxia+PEO / MELAS, LD, LHON, myoclonus, fatigue	Confirmed	0.002%
MT-ND5	m.13379A>G	H348R	+/-	LHON	Confirmed	0.000%
MT-ND5	m.13513G>A	D393N	-/+	Leigh Disease / MELAS / LHON-MELAS Overlap Syndrome	Confirmed [P*]	0.002%
MT-ND5	m.13514A>G	D393G	-/+	Leigh Disease / MELAS / Ca2+ downregulation	Confirmed	0.000%
MT-ND6	m.14453G>A	A74V	-/+	MELAS / Leigh Disease	Confirmed	0.000%
MT-ND6	m.14459G>A	A72V	+/+	LDYT / Leigh Disease / dystonia / carotid atherosclerosis risk	Confirmed [P*]	0.005%
MT-ND6	m.14482C>A	M64I	+/+	LHON	Confirmed	0.004%
MT-ND6	m.14482C>G	M64I	+/+	LHON	Confirmed	0.000%
MT-ND6	m.14484T>C	M64V	+/+	LHON	Confirmed	0.117%
MT-ND6	m.14487T>C	M63V	-/+	Dystonia / Leigh Disease / ataxia / ptosis / epilepsy	Confirmed	0.000%
MT-ND6	m.14495A>G	L60S	-/+	LHON	Confirmed	0.004%
MT-ND6	m.14568C>T	G36S	+/-	LHON	Confirmed	0.011%
MT-TE	m.14674T>C	tRNA Glu	+/-	Reversible COX deficiency myopathy	Confirmed	0.018%
MT-TE	m.14709T>C	tRNA Glu	+/+	MM+DMDF / Encephalomyopathy / Dementia+diabetes+ophthalmoplegia	Confirmed	0.002%
MT-TE	m.14710G>A	tRNA Glu	-/+	Encephalomyopathy + Retinopathy	Confirmed	0.000%
MT-CYB	m.15579A>G	Y278C	-/+	Multisystem Disorder, EXIT	Confirmed	0.000%
MT-TP	m.15990C>T	tRNA Pro	-/+	MM / PEO	Confirmed	0.000%

S1 Table

Details of 264 "reported" pathogenic variants in Mitomap.

Locus	Allele	Amino Acid Change or RNA	Plasmy Reports (Homo/Hetero)	Diseases	Mitomap Status	Genbank Frequency
MT-CR	m.576A>G	noncoding MT-TF precursor	NR/NR	Hearing loss patient	Reported	0.005%
MT-TF	m.606T>G	tRNA Phe	+/+	Maternally inherited epilepsy	Reported	0.002%
MT-RNR1	m.1420T>G	12S rRNA	+/+	DEAF	Reported	0.000%
MT-TV	m.1607T>G	tRNA Val	+/+	Suspected mito disease	Reported	0.018%
MT-TV	m.1640A>G	tRNA Val	+/+	MELAS	Reported	0.004%
MT-TV	m.1643A>G	tRNA Val	+/+	Late infantile onset fatal mito disease	Reported	0.002%
MT-RNR2	m.3196G>A	16S rRNA	+/+	ADPD	Reported	0.026%
MT-TL1	m.3242G>A	tRNA Leu (UUR)	+/+	MM / HCM+renal tubular dysfunction	Reported	0.000%
MT-ND1	m.3335T>C	I10T	+/-	LHON	Reported	0.103%
MT-ND1	m.3340C>T	P12S	+/-	Encephaloneuromyopathy	Reported	0.005%
MT-ND1	m.3380G>A	R25Q	-/+	MELAS	Reported	0.005%
MT-ND1	m.3388C>A	L28M	NR/NR	Maternally Inherited Nonsyndromic Deafness	Reported	0.046%
MT-ND1	m.3391G>A	G29S	+/-	LHON	Reported	0.097%
MT-ND1	m.3395A>G	Y30C	+/+	LHON / HCM with hearing loss	Reported	0.048%
MT-ND1	m.3421G>A	V39I	+/-	MIDD	Reported	0.141%
MT-ND1	m.3461C>T	A52V	NR/NR	LHON	Reported	0.000%
MT-ND1	m.3472T>C	F56L	+/+	LHON	Reported	0.009%
MT-ND1	m.3481G>A	E59K	-/+	MELAS / Progressive Encephalomyopathy	Reported	0.000%
MT-ND1	m.3488T>C	L61P	+/-	LHON	Reported	0.002%
MT-ND1	m.3496G>T	A64S	+/-	LHON	Reported	0.018%
MT-ND1	m.3502T>C	S66P	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.000%
MT-CR	m.351A>G	noncoding	NR/NR	Patient with CPEO	Reported	0.000%
MT-ND1	m.3551C>T	A82V	+/-	LHON	Reported	0.000%

MT-ND1	m.3571del	frameshift	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.000%
MT-ND1	m.3632C>T	S109F	+/-	LHON	Reported	0.000%
MT-ND1	m.3634A>G	S110G	+/-	LHON	Reported	0.000%
MT-ND1	m.3685T>C	Y127H	-/+	Leigh Syndrome	Reported	0.000%
MT-ND1	m.3688G>A	A128T	+/-	Leigh Syndrome	Reported	0.000%
MT-ND1	m.3713T>C	V136A	+/-	LHON	Reported	0.000%
MT-ND1	m.3733G>C	E143Q	-/+	LHON	Reported	0.000%
MT-ND1	m.3736G>A	V144I	NR/NR	LHON	Reported	0.159%
MT-ND1	m.3745G>A	A147T	+/+	LHON / high altitude variant	Reported	0.194%
MT-ND1	m.3761C>A	S152term	-/+	Deafness with relapsing/remitting neurological symptoms	Reported	0.000%
MT-ND1	m.3769C>G	L155V	+/-	LHON	Reported	0.000%
MT-ND1	m.3781T>C	S159P	+/-	LHON	Reported	0.000%
MT-ND1	m.3919T>C	S205P	+/-	LHON	Reported	0.000%
MT-ND1	m.3945C>A	I213M	NR/NR	Leigh-like phenotype	Reported	0.000%
MT-ND1	m.3946G>A	E214K	+/+	MELAS	Reported	0.002%
MT-ND1	m.3949T>C	Y215H	-/+	MELAS	Reported	0.002%
MT-ND1	m.3958G>A	G218S	+/-	LHON	Reported	0.000%
MT-ND1	m.3959G>A	G218D	NR/NR	MELAS	Reported	0.000%
MT-ND1	m.3995A>G	N230S	NR/NR	MELAS	Reported	0.033%
MT-ND1	m.4081T>C	F259L	+/-	LHON	Reported	0.002%
MT-ND1	m.4123A>T	I273F	+/-	LHON	Reported	0.000%
MT-ND1	m.4136A>G	Y277C	+/-	LHON	Reported - possibly synergistic	0.126%
MT-ND1	m.4142G>A	R279Q	-/+	Developmental delay, seizure, hypotonia	Reported	0.000%
MT-ND1	m.4142G>T	R279L	-/+	Leigh Syndrome	Reported	0.000%
MT-ND1	m.4160T>C	L285P	+/-	LHON / LHON plus	Reported	0.002%
MT-ND1	m.4163T>C	M286T	+/-	LHON	Reported	0.002%
MT-TI	m.4290T>C	tRNA Ile	+/+	Progressive Encephalopathy / PEO, myopathy	Reported	0.000%

MT-TI	m.4295A>G	tRNA Ile	+/+	MHCM / Maternally inherited hypertension / Maternally inherited deafness	Reported	0.188%
MT-TI	m.4316A>G	tRNA Ile	+/+	HCM with hearing loss / poss. hypertension factor	Reported	0.039%
MT-ND2	m.4611del	M-Term	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.000%
MT-ND2	m.4633C>G	A55G	+/-	LHON candidate	Reported	0.000%
MT-ND2	m.4659G>A	A64T	+/-	possible PD risk factor / LHON	Reported	0.178%
MT-ND2	m.4681T>C	L71P	-/+	Leigh Syndrome	Reported	0.002%
MT-ND2	m.4852T>A	L128Q	+/-	LHON	Reported	0.000%
MT-ND2	m.5001_5002insA	frameshift	-/+	Developmental delay, seizure, cardiomyopathy, lactic acidosis	Reported	0.000%
MT-ND2	m.5095T>C	I209T	NR/NR	Proximal muscle weakness and atrophy	Reported	0.033%
MT-ND2	m.5133_5134del	frameshift	NR/NR	Exercise intolerance (EXIT)	Reported	0.000%
MT-ND2	m.5244G>A	G259S	-/+	LHON	Reported	0.000%
MT-ND2	m.5367_5385del	frameshift	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.000%
MT-ND2	m.5452C>T	T328M	+/-	Progressive Encephalomyopathy	Reported	0.037%
MT-TA	m.5587T>C	tRNA Ala	+/+	LHON / possible DEAF modifier / dilated cardiomyopathy / hypertension / tic disorder	Reported	0.062%
MT-CO1	m.6020_6024del	AELGQ-AGPATerm	-/+	Motor Neuron Disease	Reported	0.000%
MT-CO1	m.6145G>A	W81term	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.000%
MT-CO1	m.6328C>T	S142F	+/-	EXIT (Exercise Intolerance)	Reported	0.000%
MT-CO1	m.6474A>G	T191A	+/-	Maternally inherited childhood epilepsy and ataxia	Reported	0.000%
MT-CO1	m.6489C>A	L196I	-/+	CO1 deficiency with epilepsia partialis continua	Reported	0.158%
MT-CO1	m.6526T>C	M208T	NR/NR	Developmental delay, hypotonia, myopathy, failure to thrive	Reported	0.000%
MT-CO1	m.6547T>C	L215P	-/+	Leigh Syndrome	Reported	0.007%
MT-CO1	m.6579G>A	G226term	-/+	Leigh Syndrome	Reported	0.000%
MT-CO1	m.6597C>A	Q232K	-/+	MELAS-like syndrome	Reported	0.000%
MT-CO1	m.6698del	K-K_frameshift	-/+	Myopathy	Reported	0.000%

MT-CO1	m.6708G>A	G269term	-/+	MM & Rhabdomyolysis	Reported	0.000%
MT-CO1	m.6721T>C	M273T	-/+	Acquired Idiopathic Sideroblastic Anaemia	Reported	0.000%
MT-CO1	m.6742T>C	I280T	-/+	Acquired Idiopathic Sideroblastic Anaemia	Reported	0.000%
MT-CO1	m.6955G>A	G351D	+/+	Mild EXIT and MR	Reported	0.002%
MT-CO1	m.7023G>A	V374M	-/+	MELAS-like syndrome	Reported	0.002%
MT-CO1	m.7222A>G	Y440C	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.000%
MT-CO1	m.7299A>G	M466V	+/-	LHON	Reported	0.148%
MT-CO1	m.7402del	frameshift	-/+	Isolated complex IV deficiency	Reported	0.000%
MT-CO1	m.7443A>G	term514G	+/-	DEAF	Reported	0.002%
MT-CO1	m.7445A>C	term514S	+/-	DEAF	Reported	0.027%
MT-TS1	m.7472A>C	tRNA Ser (UCN)	+/+	MM / DMDF modulator	Reported	0.018%
MT-TS1	m.7512T>C	tRNA Ser (UCN)	+/+	PEM / MERME / MELAS	Reported	0.000%
MT-CO2	m.7587T>C	M1T	-/+	Mitochondrial Encephalomyopathy	Reported	0.000%
MT-CO2	m.7623C>T	T13I	+/-	LHON	Reported	0.000%
MT-CO2	m.7630del	frameshift	-/+	MELAS	Reported	0.000%
MT-CO2	m.7671T>A	M29K	-/+	MM	Reported	0.000%
MT-CO2	m.7695T>C	L37P	-/+	Cerebellar and pyramidal syndrome with cognitive impairment	Reported	0.000%
MT-CO2	m.7706G>A	A41T	+/+	Alpers-Huttenlocher-like	Reported	0.020%
MT-CO2	m.7868C>T	L95F	+/-	LHON	Reported - possibly synergistic	0.026%
MT-CO2	m.7887G>A	G101D	-/+	Cerebellar ataxia + neuropathy + exercise intolerance	Reported	0.000%
MT-CO2	m.7970G>T	E129term	-/+	Encephalopathy	Reported	0.000%
MT-CO2	m.7989T>C	L135P	-/+	Rhabdomyolysis	Reported	0.000%
MT-CO2	m.8010T>C	V142A	-/+	Developmental delay, ataxia, seizure, hypotonia, lactic acidosis	Reported	0.004%
MT-CO2	m.8042_8043del	frameshift	-/+	Lactic Acidosis	Reported	0.000%
MT-CO2	m.8078G>A	V165I	+/-	DEAF	Reported	0.048%
MT-CO2	m.8088del	frameshift	-/+	Mitochondrial myopathy with complex IV deficiency	Reported	0.000%

MT-CO2	m.8108A>G	I175V	+/-	SNHL	Reported	0.132%
MT-CO2	m.8156del	frameshift	-/+	Multi-system mitochondrial disorder	Reported	0.000%
MT-CO2	m.8249G>A	G222term	+/-	Mitochondrial myopathy	Reported	0.002%
MT-TK	m.8296A>G	tRNA Lys	+/+	DMDF / MERRF / HCM / epilepsy / hearing loss	Reported	0.069%
MT-ATP8	m.8381A>G	T6A	+/-	MIDD / LVNC cardiomyopathy-assoc.	Reported	0.024%
MT-ATP8	m.8403T>C	I13T	+/-	Episodic weakness and progressive neuropathy	Reported	0.005%
MT-ATP8	m.8411A>G	M16V	+/-	Severe mitochondrial disorder	Reported	0.004%
MT-ATP8	m.8418T>C	L18P	+/-	Mitochondrial Respiratory Chain Disorder	Reported	0.002%
MT-ATP8/6	m.8561C>G	ATP8:P66A ATP6:P12R	+/+	Ataxia w neuropathy, DM, SNHL, and hypogonadism	Reported	0.000%
MT-ATP8/6	m.8561C>T	ATP8:P66S ATP6:P12L	-/+	Ataxia w psychomotor delay	Reported	0.000%
MT-ATP6	m.8578C>T	P18S	+/-	Spinocerebellar ataxia	Reported	0.055%
MT-ATP6	m.8597T>C	I24T	-/+	Leigh Syndrome	Reported	0.031%
MT-ATP6	m.8608C>T	P28S	+/-	Patient with suspected mitochondrial disease	Reported	0.002%
MT-ATP6	m.8611_8612insC	frameshift	-/+	Ataxia, microcephaly, developmental delay, intellectual disability	Reported	0.000%
MT-ATP6	m.8612T>C	L29P	+/-	Patient with suspected mitochondrial disease	Reported	0.000%
MT-ATP6	m.8618_8619insT	frameshift	-/+	NARP/cognitive decline + abnormal brain MRI + impaired kidney function	Reported	0.000%
MT-ATP6	m.8639T>C	I38T	+/-	Possible LHON modulator	Reported	0.027%
MT-ATP6	m.8668T>C	W48R	+/-	LHON	Reported	0.060%
MT-ATP6	m.8691A>G	K55K	NR/NR	Infantile mito disease w subclinical hypothyroidism	Reported	0.013%
MT-ATP6	m.8719G>A	G65term	-/+	Suspected mito disease	Reported	0.000%
MT-ATP6	m.8723G>T	R66L	+/-	Patient with suspected mitochondrial disease	Reported	0.000%
MT-ATP6	m.8779C>T	L85F	+/-	Possible LHON modulator	Reported	0.000%
MT-ATP6	m.8782G>A	G86term	-/+	Cerebellar ataxia+diabetes+kidney disease / ataxia+myoclonic epilepsy	Reported	0.000%

MT-ATP6	m.8783G>A	G86E	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.002%
MT-ATP6	m.8812A>G	T96A	-/+	Spinocerebellar ataxia	Reported	0.114%
MT-ATP6	m.8821T>G	S99A	NR/NR	Possible LHON helper variant	Reported	0.000%
MT-ATP6	m.8839G>C	A105P	-/+	NARP syndrome	Reported	0.000%
MT-ATP6	m.8881T>C	S119P	NR/NR	Patient with suspected mitochondrial disease	Reported	0.002%
MT-ATP6	m.8909T>C	F128S	+/-	Recurrent severe kidney disease and multiple systemic dysfunctions	Reported	0.000%
MT-ATP6	m.8921G>A	G132D	+/-	Patient with suspected mitochondrial disease	Reported	0.013%
MT-ATP6	m.8936T>A	L137H	-/+	Atypical Leigh syndrome	Reported	0.000%
MT-ATP6	m.8938A>G	I138V	+/-	Patient with suspected mitochondrial disease	Reported	0.082%
MT-ATP6	m.8950G>A	V142I	+/-	LDYT / Spinocerebellar Ataxia	Reported	0.148%
MT-ATP6	m.8951T>C	V142A	NR/NR	Patient with ataxia	Reported	0.016%
MT-ATP6	m.8959G>A	E145K	+/+	Developmental delay, intellectual disability, low citrulline	Reported	0.007%
MT-ATP6	m.8989G>C	A155P	-/+	NARP syndrome	Reported	0.000%
MT-ATP6	m.8993TG>CA	L156?	+/+	Developmental delay & myopathy	Reported	0.000%
MT-ATP6	m.8999T>C	V158A	+/-	Patient with suspected mitochondrial disease	Reported	0.013%
MT-ATP6	m.9010G>A	A162T	-/+	Unspecified neurological disorder	Reported	0.049%
MT-ATP6	m.9016A>G	I164V	-/+	LHON	Reported	0.024%
MT-ATP6	m.9017T>C	I164T	-/+	Unspecified neurological disorder	Reported	0.024%
MT-ATP6	m.9025G>A	G167S	+/-	Motor neuropathy, Leigh-like, colon cancer	Reported	0.062%
MT-ATP6	m.9026G>A	G167D	-/+	Spinocerebellar ataxia/patient with suspected mitochondrial disease	Reported [VUS*]	0.005%
MT-ATP6	m.9029A>G	H168R	+/+	LHON-like	Reported	0.002%
MT-ATP6	m.9032T>C	L169P	-/+	NARP / Complex phenotype with microcephaly, ataxia, hearing loss, lactic acidosis	Reported [VUS*]	0.000%
MT-ATP6	m.9041A>G	H172R	-/+	Patient with suspected mitochondrial disease	Reported	0.095%
MT-ATP6	m.9049G>A	G175term	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.000%

MT-ATP6	m.9071C>T	S182L	+/-	Potentially functional variant co-segregating with LHON3635A	Reported	0.027%
MT-ATP6	m.9101T>C	I192T	+/-	LHON	Reported	0.088%
MT-ATP6	m.9115A>G	I197V	+/-	Patient with suspected mitochondrial disease	Reported	0.048%
MT-ATP6	m.9127_9128del	IL-Pterm	-/+	NARP	Reported	0.000%
MT-ATP6	m.9133G>A	E203K	+/-	Patient with suspected mitochondrial disease	Reported	0.007%
MT-ATP6	m.9134A>G	E203G	NR/NR	Hypotonia, lactic acidosis, HCM, IUGR	Reported	0.000%
MT-ATP6	m.9139G>A	A205T	+/-	LHON	Reported - possibly synergistic	0.084%
MT-ATP6	m.9152T>C	I209T	-/+	Patient with suspected mitochondrial disease	Reported	0.027%
MT-ATP6	m.9155A>T	Q210L	+/+	Developmental delay, intellectual disability, low citrulline	Reported	0.000%
MT-ATP6	m.9166T>C	F214L	+/+	EXIT+more / bilateral optic neuropathy	Reported	0.000%
MT-ATP6	m.9191T>C	L222P	-/+	Leigh Disease	Reported [LP*]	0.000%
MT-CO3	m.9237G>A	V11M	NA/NA	Mitochondrial Respiratory Chain Disorder	Reported	0.000%
MT-CO3	m.9267G>C	A21P	-/+	MIDD	Reported	0.000%
MT-CO3	m.9331T>C	L42P	+/-	Failure to thrive with metabolic acidosis, cognitive impairment, optic atrophy	Reported	0.000%
MT-CO3	m.9379G>A	W58term	-/+	MM w lactic acidosis	Reported	0.000%
MT-CO3	m.9399A>G	S65G	NR/NR	Patient with epilepsy, myopathy, hypoacusis, psychiatric disorders	Reported	0.002%
MT-CO3	m.9478T>C	V91A	-/+	Leigh Disease	Reported	0.038%
MT-CO3	m.9537_9538insC	frameshift	+/-	Leigh Disease	Reported	0.000%
MT-CO3	m.9544G>A	G113E	NR/NR	Sporadic bilateral optic neuropathy	Reported	0.000%
MT-CO3	m.9559del	frameshift	-/+	Rhabdomyolysis	Reported	0.000%
MT-CO3	m.9660A>C	M152L	+/-	LHON	Reported	0.000%
MT-CO3	m.9738G>T	A178S	+/-	LHON	Reported	0.000%
MT-CO3	m.9789T>C	S195P	-/+	Myopathy	Reported	0.000%
MT-CO3	m.9952G>A	W249term	-/+	Mitochondrial Encephalopathy	Reported	0.000%
MT-CO3	m.9957T>C	F251L	-/+	PEM / MELAS / NAION / HCM / gout	Reported	0.082%

MT-CO3	m.9984G>A	G260term	NR/NR	Suspected mito disease	Reported	0.000%
MT-ND3	m.10134C>A	Q26K	-/+	Leigh Disease	Reported	0.000%
MT-ND3	m.10237T>C	I60T	+/-	LHON	Reported	0.172%
MT-ND3	m.10254G>A	D66N	-/+	Leigh Disease	Reported	0.000%
MT-ND3	m.10350C>A	L98M	+/-	LHON	Reported	0.000%
MT-ND4L	m.10543A>G	H25R	-/+	LHON	Reported	0.000%
MT-ND4L	m.10591T>G	F41C	-/+	LHON	Reported	0.000%
MT-ND4L	m.10680G>A	A71T	+/-	LHON / synergistic combo 10680A + 12033G + 14258A	Reported / possibly synergistic	0.031%
MT-ND4	m.11232T>C	L158P	-/+	CPEO	Reported	0.000%
MT-ND4	m.11240C>T	L161F	-/+	Leigh Syndrome	Reported	0.000%
MT-ND4	m.11406T>A	L216H	-/+	MELAS	Reported	0.000%
MT-ND4	m.11470A>C	K237N	-/+	MELAS	Reported	0.000%
MT-ND4	m.11519A>C	T254P	+/-	ND4 mutation set found in a Multiple Sclerosis patient	Reported	0.000%
MT-ND4	m.11523A>C	K255T	+/-	ND4 mutation set found in a Multiple Sclerosis patient	Reported	0.000%
MT-ND4	m.11527C>T	H256H	+/-	ND4 mutation set found in a Multiple Sclerosis patient	Reported	0.040%
MT-ND4	m.11621_11622del	frameshift	-/+	CPEO, exercise intolerance	Reported	0.000%
MT-ND4	m.11874C>A	T372N	+/-	LHON	Reported	0.000%
MT-ND4	m.11984T>C	Y409H	+/-	Leigh Syndrome	Reported	0.112%
MT-ND4	m.12015T>C	L419P	-/+	Atypical MELAS	Reported	0.004%
MT-ND4	m.12033A>G	N425S	+/-	LHON synergistic combo 10680A + 12033G + 14258A	Reported: individually neutral variants causing LHON in combination	0.038%
MT-TH	m.12146A>G	tRNA His	+/+	MELAS	Reported	0.000%
MT-TS2	m.12264C>T	tRNA Ser (AGY)	+/+	Multisystem Disease with Cataracts / Myopathy+epilepsy+DEAF+atypical autism	Reported	0.000%

MT-TL2	m.12297T>C	tRNA Leu (CUN)	+/+	Dilated Cardiomyopathy / Leigh Syndrome / Failure to Thrive & LA	Reported	0.083%
MT-TL2	m.12311T>C	tRNA Leu (CUN)	+/+	CPEO	Reported	0.100%
MT-ND5	m.12414del	frameshift	NR/NR	EXIT	Reported	0.000%
MT-ND5	m.12425del	frameshift	-/+	Mitochondrial myopathy & renal failure	Reported	0.005%
MT-ND5	m.12770A>G	E145G	-/+	MELAS	Reported	0.002%
MT-ND5	m.12782T>G	I149S	-/+	LHON	Reported	0.000%
MT-ND5	m.12848C>T	A171V	-/+	LHON	Reported	0.000%
MT-ND5	m.12858C>A	Y174term	NR/NR	Unspecified suspected mitochondrial disorder	Reported	0.000%
MT-ND5	m.12955A>G	N207D	-/+	EXIT and developmental delay	Reported	0.000%
MT-ND5	m.13045A>C	M237L	-/+	MELAS / LHON / Leigh overlap syndrome	Reported	0.000%
MT-ND5	m.13046T>C	M237T	-/+	LHON/MELAS overlap syndrome	Reported	0.000%
MT-ND5	m.13063G>A	V243I	-/+	Adult-onset Encephalopathy / Ataxia	Reported	0.004%
MT-ND5	m.13084A>T	S250C	-/+	MELAS / Leigh Disease	Reported	0.000%
MT-ND5	m.13091T>C	M252T	-/+	MELAS + Migraine	Reported	0.000%
MT-ND5	m.13271T>C	L312P	-/+	Exercise intolerance (EXIT)	Reported	0.002%
MT-ND5	m.13340T>C	F335S	+/-	LHON	Reported	0.000%
MT-ND5	m.13345G>A	A337T	+/-	LHON	Reported	0.000%
MT-ND5	m.13376T>C	I347T	+/-	MELAS w medial temporal lobe atrophy	Reported	0.002%
MT-ND5	m.13379A>C	H348P	+/-	LHON	Reported	0.000%
MT-ND5	m.13511A>T	K392M	-/+	Leigh-like syndrome	Reported	0.000%
MT-ND5	m.13528A>G	T398A	+/-	LHON-like, LHON, MELAS	Reported	0.104%
MT-ND5	m.13730G>A	G465E	-/+	LHON	Reported	0.000%
MT-ND5	m.13849A>C	N505H	+/-	MELAS	Reported - possibly secondary	0.002%
MT-ND5	m.14063T>C	I576T	+/-	Potentially functional variant co-segregating with LHON3635A	Reported	0.044%
MT-ND5	m.14091A>T	K585N	-/+	Developmental delay, seizure, hearing loss, diabetes	Reported	0.000%

MT-ND6	m.14258G>A	P139L	+/-	LHON synergistic combo 10680A + 12033G + 14258A also combo 14258A + 14582G	Reported: individually neutral variants causing LHON in combination	0.053%
MT-ND6	m.14279G>A	S132L	+/-	LHON	Reported	0.011%
MT-ND6	m.14325T>C	N117D	+/-	LHON	Reported	0.095%
MT-ND6	m.14340C>T	V112M	+/-	SNHL	Reported	0.037%
MT-ND6	m.14430A>G	W82R	+/-	Thyroid Cancer / Leigh Syndrome	Reported	0.000%
MT-ND6	m.14439G>A	P79S	+/-	Mitochondrial Respiratory Chain Disorder	Reported	0.000%
MT-ND6	m.14441T>C	Y78C	NR/NR	Leigh-like phenotype	Reported	0.000%
MT-ND6	m.14465G>A	T70I	-/+	LHON / various suspected mitochondrial disease	Reported	0.000%
MT-ND6	m.14498T>C	Y59C	+/+	LHON	Reported	0.000%
MT-ND6	m.14512_14513del	frameshift	-/+	EXIT + mild myopathy & hyperkalaemia	Reported	0.000%
MT-ND6	m.14535_14536insC	frameshift	NR/NR	DMDF	Reported	0.000%
MT-ND6	m.14538A>G	F46L	+/-	LHON	Reported	0.000%
MT-ND6	m.14596A>T	I26M	+/-	LHON	Reported	0.000%
MT-ND6	m.14597A>G	I26T	-/+	LHON / Leigh Syndrome	Reported	0.000%
MT-ND6	m.14600G>A	P25L	+/+	Leigh Disease w/optic atrophy / ASD mouse model	Reported	0.000%
MT-CYB	m.14787_14790del	frameshift	-/+	PD / MELAS	Reported	0.000%
MT-CYB	m.14864T>C	C40R	-/+	MELAS	Reported	0.004%
MT-CYB	m.14894T>C	F50L	NR/NR	LHON	Reported	0.015%
MT-CYB	m.14970A>G	Y75C	NR/NR	LHON	Reported	0.013%
MT-CYB	m.15059G>A	G105term	-/+	MM / carotid atherosclerosis risk / essential hypertension	Reported	0.000%
MT-CYB	m.15060G>A	G105E	+/-	Mitochondrial Respiratory Chain Disorder	Reported	0.000%
MT-CYB	m.15084G>A	W113term	-/+	EXIT	Reported	0.000%
MT-CYB	m.15092G>A	G116S	-/+	MELAS	Reported	0.000%
MT-CYB	m.15150G>A	W135term	-/+	EXIT	Reported	0.000%
MT-CYB	m.15153G>A	G136D	-/+	Suspected mito disease	Reported	0.011%

MT-CYB	m.15158A>G	M138V	-/+	Suspected mito disease	Reported	0.000%
MT-CYB	m.15168G>A	W141term	-/+	EXIT	Reported	0.000%
MT-CYB	m.15170G>A	G142term	-/+	EXIT	Reported	0.000%
MT-CYB	m.15197T>C	S151P	-/+	EXIT	Reported	0.000%
MT-CYB	m.15234G>A	W163term	NR/NR	Leigh stroke-like leukodystrophy	Reported	0.000%
MT-CYB	m.15237T>C	I164T	+/-	Potentially functional variant co-segregating with LHON3635A	Reported	0.011%
MT-CYB	m.15242G>A	G166term	-/+	Mitochondrial Encephalomyopathy	Reported	0.000%
MT-CYB	m.15246G>A	G167D	-/+	Mitochondrial Respiratory Chain Disorder	Reported	0.000%
MT-CYB	m.15453T>C	L236P	+/-	Isolated complex III deficiency	Reported	0.020%
MT-CYB	m.15498_15521del	GDPDNYTL-del	-/+	EXIT	Reported	0.000%
MT-CYB	m.15615G>A	G290D	-/+	EXIT / Antimycin resistance	Reported	0.000%
MT-CYB	m.15620C>A	L292I	-/+	Leigh Syndrome helper mutation	Reported	0.000%
MT-CYB	m.15649_15666del	ILAMIP-del	-/+	Multisystem Disorder, EXIT	Reported	0.000%
MT-CYB	m.15699G>C	R318P	-/+	Muscle Weakness SNHL and Migraine	Reported	0.000%
MT-CYB	m.15723G>A	W326term	-/+	EXIT	Reported	0.000%
MT-CYB	m.15761G>A	G339term	/+	MM	Reported	0.000%
MT-CYB	m.15762G>A	G339E	-/+	MM	Reported	0.000%
MT-CYB	m.15773G>A	V343M	+/-	LHON	Reported - possibly synergistic	0.119%
MT-CYB	m.15800C>T	Q352term	-/+	EXIT / Myopathy	Reported	0.000%
MT-TT	m.15923A>G	tRNA Thr	+/+	LIMM / MERRF / mito disease	Reported	0.000%

S2 Table

Patients with the co-occurrence of two "reported" mtDNA variants. LHON: Leber's Hereditary Optic Neuropathy; MIND: Maternally Inherited Nonsyndromic Deafness; MELAS: Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes; CO1: Cytochrome C Oxidase 1; NR: Not Rated.

Patient	Haplogroup	Age	Sex	Clinical features	Nucleotide position (Blood heteroplasmy %)	Locus	Mitomap frequency in 54594 FL seqs	gnomAD 3.1 frequency in 56417 seqs	Conservation	Disease	Reference(s)
P10	H2a2b1a1	42	M	LHON	m.3388C>A (100%)	*MT-ND1*	0.046%	0.07%	98%	MIND	(57)
					m.14465G>A (18%)	*MT-ND6*	0	NR	89%	LHON	(58,59)
P11	T2f1a1	55	F	ON	m.6489C>A (100%)	*MT-CO1*	0.158%	0.33%	98%	CO1 deficiency	(60)
					m.13376T>C (1%)	*MT-ND5*	0.002%	0.007%	98%	MELAS	(61)(139)

S3 Table

Literature review of patients or families carrying one "confirmed" pathogenic and one "reported" pathogenic variant, or two "reported" pathogenic mtDNA variants. MERRF: Myoclonic Epilepsy with Ragged Red Fibers; LHON: Leber's Hereditary Optic Neuropathy; MELAS: Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes; MIDD: Maternally Inherited Diabetes and Deafness; MIEH: Maternally Inherited Essential Hypertension; CPEO: Chronic Progressive External Ophthalmoplegia; T2DM: Type 2 Diabetes Mellitus.

References	mtDNA variation	Mitomap status	Disease	Patients	Clinical features	Country of origin
Arenas et al. (1999)	m.8363G>A m.8296A>G	Confirmed Reported	MERRF MERRF	5 (1 family)	MERRF syndrome	Spain
Qu et al. (2006)	m.11778G>A m.4435A>G	Confirmed Reported	LHON LHON	8 (1 family)	LHON	China
Li et al. (2006)	m.11778G>A m.15951A>G	Confirmed Reported	LHON LHON	6 (1 family)	LHON	China
Wang et al. (2008)	m.11778G>A m.13708G>A	Confirmed Reported	LHON LHON	20 (2 families)	LHON	China
Mezghani et al. (2013)	m.1555A>G m.3308T>C	Confirmed Reported	Deafness MELAS, LHON	1	MIDD	Tunisia
Jin et al. (2015)	m.3635G>A m.14502T>C	Confirmed Reported	LHON LHON	14 (1 family)	LHON	China
Qiao et al. (2015)	m.11778G>A m.14502T>C	Confirmed Reported	LHON LHON	4 (2 families)	LHON	China
Xie et al. (2017)	m.11778G>A m.11696G>A	Confirmed Reported	LHON LHON, Deafness	37 (8 families)	LHON	China
Zhixin Jiang et al. (2019)	m.3243A>G m.16093T>C	Confirmed Reported	MELAS Cyclic vomiting syndrome	2 (1 family)	MIDD	China
Ding et al. (2020)	m.11778G>A m.5601C>T	Confirmed Reported	LHON MELAS, MIEH	4 (1 family)	LHON	China
De Vries et al. (1996)	m.11696A>G m.14596T>G	Reported Reported	LHON, Deafness LHON	1	LHON Hereditary spastic dystonia	The Netherlands
Guo et al. (2022)	m.15992A>G m.15077G>A	Reported Reported	Deafness LHON, Deafness	9 (1 family)	MIEH	China

X. Yu, Li, et Ding (2022)	m.5601C>T m.12311T>C	Reported Reported	MELAS, MIEH CPEO	3 (1 family)	Hearing loss	China
Jiang et al. (2022)	m.12338T>C m.5587T>C	Reported Reported	LHON, Deafness LHON	4 (1 family)	T2DM	China
Yang et al. (2022)	m.5514A>G m.12337C>T	Reported Undescribed	Neonatal mitochondriopathy Not determined	6 (1 family)	MIDD	China
Mkaouar-Rebai et al. (2021)	m.12058A>C m.3911A>G	Undescribed Undescribed	Not determined Not determined	1	Neurodevelopmental delay, hearing loss, hypotonia	Tunisia

S4 Table

Enzymatic activities of mitochondrial respiratory chain complexes for individuals II.2 and III.1 from family A. CI: Complex I, CII: Complex II, CIII: Complex III, CIV: Complex IV, CV: Complex V, CS: Citrate Synthase.

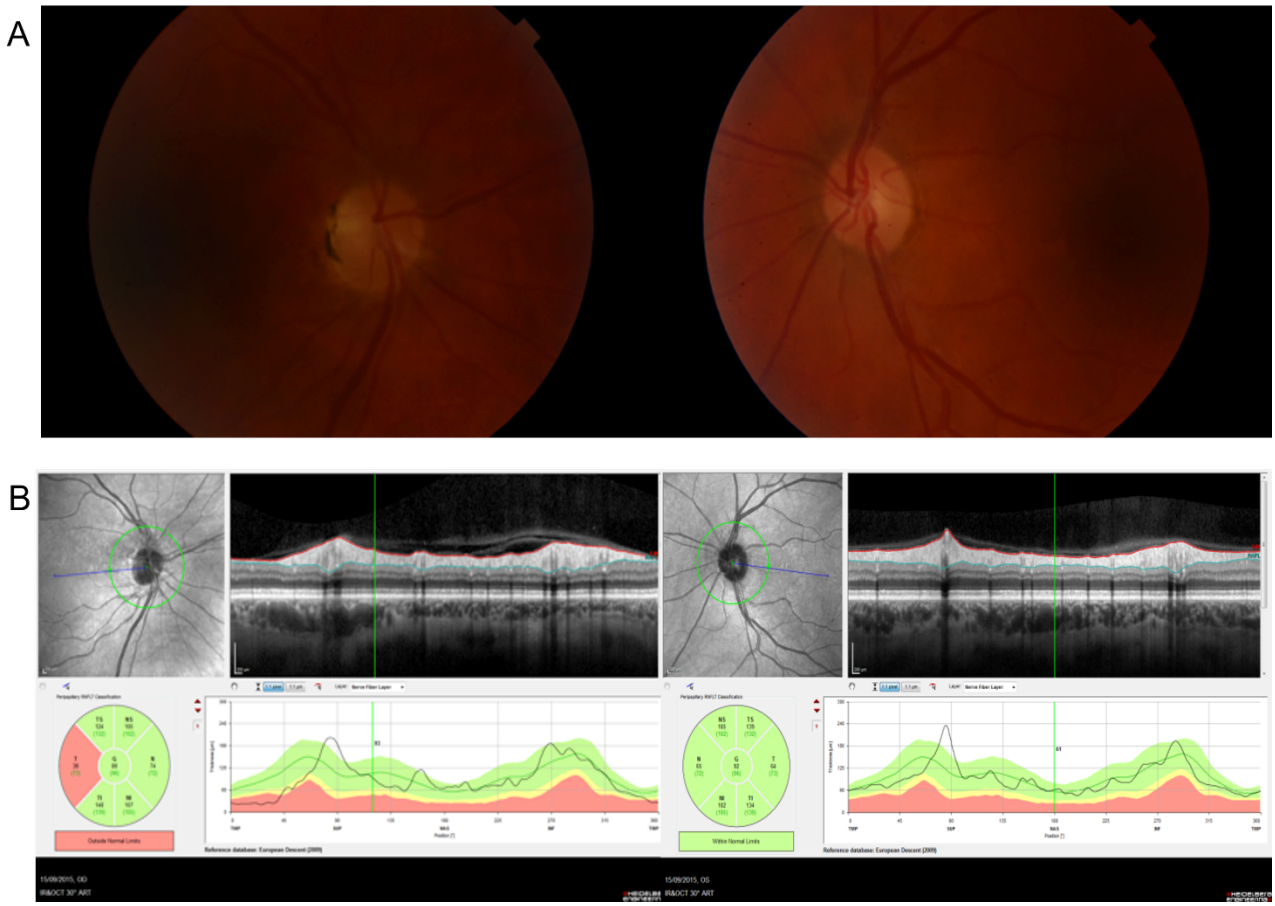
	II.2	III.1	References values
CI (nmol/min/mg)	192	100	100 - 390
CII (nmol/min/mg)	84	58	30 – 100
CIII (nmol/min/mg)	789	480	200 – 750
CIV (nmol/min/mg)	579	344	400 – 1045
CV (nmol/min/mg)	275	145	80 - 190
CS (nmol/min/mg)	570	333	300 - 670
CI/CS	**0.28**	**0.27**	0.30 - 0.70
CII/CS	0.15	0.18	0.09 - 0.25
CIII/CS	1.38	1.44	0.50 - 2.00
CIV/CS	1.02	1.03	0.85 - 2.30
CIV/CV	0.47	0.42	0.10 - 0.35
CV/CS	0.48	0.44	0.16 - 0.38

S5 Table

mtDNA copy number in blood cells for individuals from family A.

		mtDNA quantification
II.2		59,6
II.5		64,9
III.1		45,5
III.2		21,3
III.4		48,3
III.5	at diagnosis	33,7
	after visual recovery	49,3

Supplementary Figure



Ophthalmological investigations for individual II.2 from family A. A. Non-mydriatic retinophotography of the right (left) and left (right) eyes focused on the optic nerves. **B.** Optical coherence tomography (OCT) of the right (left) and left (right) optic nerves.

BENARBIA Randa

Double occurrence de variants pathogènes de l'ADNmt : un phénomène sous-estimé ?

A propos de 3 cas et revue de littérature

RÉSUMÉ

La prévalence des maladies mitochondriales primaires est de 1/4300, avec plus de 350 variations pathogènes ou probablement pathogènes de l'ADN mitochondrial (ADNmt) répertoriées dans les bases de données. Cependant, malgré un taux de mutation 20 à 25 fois supérieur à celui de l'ADN nucléaire, la co-occurrence de variants pathogènes de l'ADNmt semble rare dans la littérature, avec peu de cas décrits.
L'objectif de ce travail était d'évaluer la prévalence de la co-occurrence de variants pathogènes de l'ADNmt chez les patients adressés à notre laboratoire pour un diagnostic moléculaire de maladies mitochondriales entre 2014 et 2022, en utilisant le séquençage de nouvelle génération de la totalité de l'ADNmt.
L'analyse des données de séquençage de l'ADNmt de 4102 individus a révélé la co-occurrence de deux variants pathogènes ou probablement pathogènes chez environ 0,6% des sujets. Il s'agit de huit individus (0,19 %) issus de trois familles présentant deux variants pathogènes dont le statut est "confirmé" dans la base de données Mitomap, de neuf individus (0,22 %) porteurs d'un variant pathogène "confirmé" et d'un autre "rapporté", et de deux individus présentant une combinaison de deux variants "rapportés". Ces sujets étaient atteints non seulement de la neuropathie optique héréditaire de Leber (NOHL) mais aussi de maladies mitochondriales syndromiques. Notre étude montre que la co-occurrence de variants pathogènes de l'ADNmt n'est pas un événement rare comme le suggère la littérature, et que sa prévalence est probablement sous-estimée. L'identification des combinaisons de variants par le séquençage systématique de la totalité de l'ADNmt améliore le diagnostic des maladies mitochondriales, notamment dans les phénotypes complexes, et offre un meilleur conseil génétique aux familles.

Mots-clés : mitochondrie, maladie mitochondriale, ADN mitochondrial, séquençage de nouvelle génération

Dual occurrence of mtDNA pathogenic variants: an underestimated phenomenon?

About three cases and a literature review

ABSTRACT

The prevalence of primary mitochondrial diseases is 1:4300, with more than 350 pathogenic or likely pathogenic mitochondrial DNA (mtDNA) variations listed in databases. However, despite a mutation rate 20 to 25 times higher than nuclear DNA, the co-occurrence of mtDNA pathogenic variants appears to be rare in the literature, with few cases described.
The objective of this work was to assess the prevalence of co-occurrence of mtDNA pathogenic variants in patients referred to our laboratory for molecular diagnosis of mitochondrial diseases between 2014 and 2022, using whole mtDNA next-generation sequencing (NGS).
The analysis of mtDNA sequencing data from 4102 individuals revealed the co-occurrence of two pathogenic or likely pathogenic variants in about 0.6% of subjects. This includes eight individuals (0.19%) from three families carrying two pathogenic variants with a "confirmed" status in the Mitomap database, nine individuals (0.22%) carrying a "confirmed" pathogenic variant and a "reported" one, and two individuals with a combination of two "reported" variants. These subjects were not only suffering from Leber's hereditary optic neuropathy (LHON) but also from syndromic mitochondrial diseases. Our study shows that the co-occurrence of mtDNA pathogenic variants is not a rare event as suggested in the literature, and its prevalence is probably underestimated. Identifying combinations of variants through systematic whole mtDNA sequencing improves mitochondrial disease diagnosis, notably in complex phenotypes, and offers better genetic counselling to families.

Keywords: mitochondria, mitochondrial diseases, mitochondrial DNA, next-generation sequencing

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