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RESEARCH ARTICLE

Primary mitochondrial disorders and mimics: Insights from a large French cohort

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Abstract

Objective: The objective of this study was to evaluate the implementation of NGS within the French mitochondrial network, MitoDiag, from targeted gene panels to whole exome sequencing (WES) or whole genome sequencing (WGS) focusing on mitochondrial nuclear-encoded genes. **Methods**: Over 2000 patients suspected of Primary Mitochondrial Diseases (PMD) were sequenced by either targeted gene panels, WES or WGS within MitoDiag. We described the clinical, biochemical, and molecular data of 397 genetically confirmed patients, comprising 294 children and 103 adults, carrying pathogenic or likely pathogenic variants in nuclear-encoded genes. **Results**: The cohort exhibited a large genetic heterogeneity, with the identification of 172 distinct genes and 253 novel variants. Among children, a notable prevalence of pathogenic variants in genes associated with oxidative phosphorylation (OXPHOS) functions and mitochondrial translation was observed. In adults, pathogenic variants were primarily identified in genes linked to mtDNA maintenance. Additionally, a substantial proportion of patients (54% (42/78) and 48% (13/27) in children and adults,

respectively), undergoing WES or WGS testing displayed PMD mimics, representing pathologies that clinically resemble mitochondrial diseases. **Interpretation**: We reported the largest French cohort of patients suspected of PMD with pathogenic variants in nuclear genes. We have emphasized the clinical complexity of PMD and the challenges associated with recognizing and distinguishing them from other pathologies, particularly neuromuscular disorders. We confirmed that WES/WGS, instead of panel approach, was more valuable to identify the genetic basis in patients with "possible" PMD and we provided a genetic testing flowchart to guide physicians in their diagnostic strategy.

Introduction

Primary mitochondrial diseases (PMD) are among the most common metabolic diseases. The prevalence of childhood-onset and adult-onset PMD combined was initially estimated to be at least 20 in 100,000 but could be far above.[1] The combined lifetime risk of 249 autosomal recessive mitochondrial disorders was estimated at 48.4 (40.3–58.5)/100,000 in the European gnomAD dataset.[2] These disorders are due to pathogenic variants in the mitochondrial or nuclear genomes that primarily lead to alterations of Oxidative Phosphorylation (OXPHOS) or other mitochondrial dysfunctions. They display heterogeneous clinical and biochemical presentations with a combination of different modes of mendelian and maternal inheritance.

Clinical, biochemical, and genetic heterogeneities among individuals involve significant diagnostic challenges for clinicians. To assist clinicians in recognizing these pathologies, various criteria have been proposed. The most widely employed scoring system, the Nijmegen Mitochondrial Diagnostic Criteria (MDC) score, classifies pathologies into four groups: Score 1 corresponds to "unlikely PMD," scores 2 to 4 correspond to a "possible PMD," scores 5 to 7 correspond to a "probable PMD," and scores 8 to 12 correspond to a "definite PMD."[3] To complicate the issue further, many diseases, in particular multisystem disorders, mimic mitochondrial pathologies and OXPHOS deficiencies may be secondary events in neuromuscular or multisystem disorders with a non-mitochondrial genetic cause.[1,4] In this article, we referred to such disorders as "PMD-mimics." Therefore, the definitive diagnosis of PMD relies on identifying the causative pathogenic variant. Pathogenic variants in the mitochondrial DNA (mtDNA) are responsible for 5 to 20% of PMD depending on the patient population and age,[5-7] while variants in mitochondrially nuclear-encoded genes account for up to 50% in some cohorts.[8] In the last years, faced with the great heterogeneity of these pathologies, the diagnostic strategy has undergone substantial evolution.[9] Concerning nuclear genes, the Whole Exome Sequencing (WES) approach has demonstrated remarkable efficacy in exponentially identifying new mitochondrial genes and uncovering mimics.[6,10-12] More recently, a first-line approach using Whole Genome Sequencing (WGS) has been proposed, enabling the concurrent testing of mtDNA and nuclear genes. However, it's worth noting that this method is not yet widely available in all laboratory settings.[9]

Since 2000, the French Network of Diagnostic Laboratories for Mitochondrial Diseases, called MitoDiag, has been working in close collaboration with two mitochondrial disease national reference centers, CALISSON (https://www.mito-calisson.fr/) and CARAMMEL (https://carammel.org/), and the clinical Neuromuscular Network FILNEMUS (https://www.filnemus.fr//), to improve the diagnosis and patient care by sharing practices and knowledge on PMD. MitoDiag includes 11 diagnostic laboratories ensuring a complete national network (Angers, Bordeaux, Caen, Grenoble, Lille, Lyon, Nice, Paris (Necker, Bicêtre, Pitié-Salpêtrière) and Reims). Patients suspected of PMD were sequenced by either targeted gene panels, WES or WGS. We reported the first clinical and genetic description of a French cohort of 397 patients in whom a diagnosis could be confirmed by the identification of pathogenic variants in nuclearly-encoded genes. We showed the evolution of diagnoses with the development of NGS techniques from targeted gene panels to WGS and we investigated the clinical and biological characteristics of patients diagnosed with PMD or PMD mimics. From our experience, we have developed a genetic testing flowchart to guide physicians in their diagnostic strategy.

Methods

Standard protocol approvals, registrations, and patient consents

Patients with a positive molecular diagnostic testing were consented for inclusion in this cohort (Institutional Review Board Committee Authorization 2023 R004-004). Patients were referred to the different laboratories from MitoDiag, the French Network of Diagnostic Laboratories for Mitochondrial Diseases, (https://www.mitodiag.fr/home), both National Reference Centers, CALISSON and CARAMMEL, and the clinical Neuromuscular Network FILNEMUS (https://www.filnemus.fr/).

Cohort

A total of 397 patients, 294 children (age at first analysis ≤18) and 103 adults were reported, harboring variants either classified as likely pathogenic (class 4) or pathogenic (class 5), according to ACMG guidelines,[13] in nuclear-encoded genes. Clinical and biological data were collected through the patients' records. As 74% of our patients were below 18 years at the age of diagnosis, we used the mitochondrial disease criteria (MDC) initially designed for children to phenotype the patients.[3] Score 1 corresponds to "unlikely PMD," score 2 to 4, corresponds to a "possible PMD," score 5 to 7 corresponds to a "probable PMD" and score 8 to 12 corresponds to a "definite PMD." All included patients had either a PMDC score of at least 2, or a respiratory chain deficiency, or multiple mtDNA deletions. The clinical features have been adapted according to the HPO (Human Phenotype Ontology) classification (https://hpo.jax.org/app/). For all patients, mtDNA pathogenic variants were ruled out by whole mtDNA sequencing. Patients with isolated optic atrophies or carrying mtDNA pathogenic variants were excluded from the study, and are reported elsewhere.[5,14,15]

NGS technologies

NGS technology was sequentially and successively implemented, first by targeted gene panels with a limited number of genes then by increasing the panel size up to more than 400 genes. The targeted largest panel used in the MitoDiag network included the 482 nuclear genes of the PMD panel (Version 2.117) of the Genomics England PanelApp (https://panelapp.genomicsengland.co.uk/), and three genes of the Severe Paediatric Disorders panel (Version 1.127) (*NBAS, WDR45* and *GK*). We checked the mitochondrial localization of proteins using the MitoCarta 3.0 database (https://www.broadinstitute.org/files/shared/metabolism/mitocarta/human.mitocarta3.0.html). Note that the PMD panel (Version 2.117) contains a set of 11 genes that are not included in the MitoCarta database (*RRM2B, FA2H, PLA2G6, SLC3A1, SLC13A5, SLC19A2, SLC19A3, TANGO2, SACS, STXBP1, and WFS1*). In this study, we defined as "mitochondrial genes" the MitoCarta genes and *RRM2B* gene for which it has been clearly demonstrated to impact the mtDNA maintenance. All other genes, not included in MitoCarta, except for *RRM2B*, were categorized as "non-mitochondrial genes."

More recently, most laboratories have implemented WES in their diagnosis setting and since the implementation of the national sequencing core platforms SeqOIA and Auragen, a limited set of patients have had WGS.

Genomic DNA was extracted either from blood or fibroblast cells using standard laboratory protocols. Libraries were generated using an enzymatic DNA fragmentation approach following the manufacturer protocols (Thermo Fisher® and Illumina®). Samples were processed in mitochondrial centers from the French MitoDiag network with different library preparations and sequencing platforms (Thermo Fisher® and Illumina®). Variant annotation was based on the Ensembl human database (GRCh37 release). Copy number variants were called using different in-house pipelines based on a double normalization of depth coverage. Most patients were singleton and parental segregation of identified pathogenic variants were further confirmed by Sanger sequencing. Variants linked to the clinical phenotype were classified as pathogenic (class 5) or likely pathogenic (class 4) according to the ACMG classification, after a thorough analysis with the different prediction-based algorithms (Varsome: https://varsome.com/ and Franklin: https://franklin.genoox.com).

For mtDNA sequencing, the entire mtDNA was amplified in overlapping fragments using a combination of primers designed to avoid nuclear pseudogene amplification as previously described.[16]

OXPHOS spectrophotometric measurements

Enzymatic spectrophotometric measurements of OXPHOS respiratory chain complexes and citrate synthase were performed at 37°C on patient's muscle according to national standard procedures.[17]

Muscle histology analyses

Muscle histology was assessed as previously reported.[18] Reported abnormal results included the presence of ragged red fibers, negatively stained fibers for cytochrome c oxidase, mitochondrial proliferation with succinate dehydrogenase staining and excessive lipid accumulation.

Results

Demographics

We report a total of 397 unrelated patients, including 294 children (74%) and 103 adults (26%), in whom we have identified, by NGS, pathogenic or likely pathogenic nuclear variants according to the ACMG classification (SupTable S1 and SupTable S2, Fig. 1A). 37% and 48% of children and adults respectively were females (109/294 children and 50/103 adults). The percentage of consanguinity reached 38% (112/294) in children and 8% (8/103) in adults. Participants were scored using the MDC

(A)

	Children (N=294)	Adults (N=103)	Total (N=397)
MDC 1	1 (<1%)	7 (7%)	8 (2%)
MDC 2-4	210 (71%)	65 (63%)	275 (69%)
MDC ≥5	83 (28%)	31 (30%)	114 (29%)
Consanguinity	112 (38%)	8 (8%)	120 (30%)
Targeted panel	216 (73%)	76 (74%)	292 (73%)
WES	72 (25%)	27 (26%)	99 (25%)
WGS	6 (2%)	0	6 (2%)

(B)

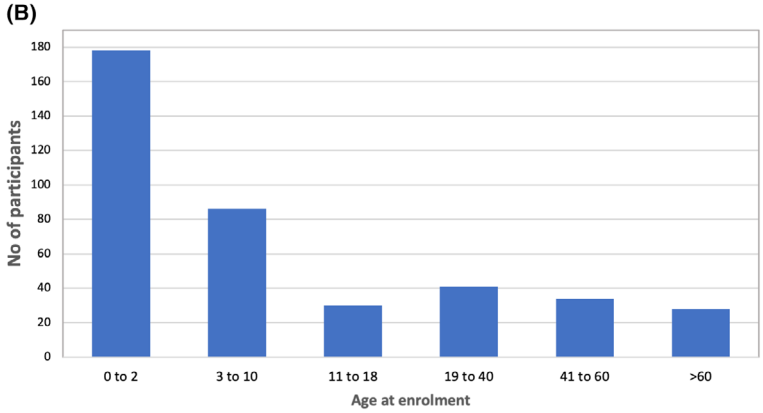


Figure 1. (A) Overview of patients according to MDC score, consanguinity and molecular testing (targeted panels, WES, WGS). Patients were classified according to the MDC score. Patients with a PMDC score of 1 had either an OXPHOS deficiency or multiple mtDNA deletions. *N*, number. (B) Age distribution of individuals at the time of enrolment.

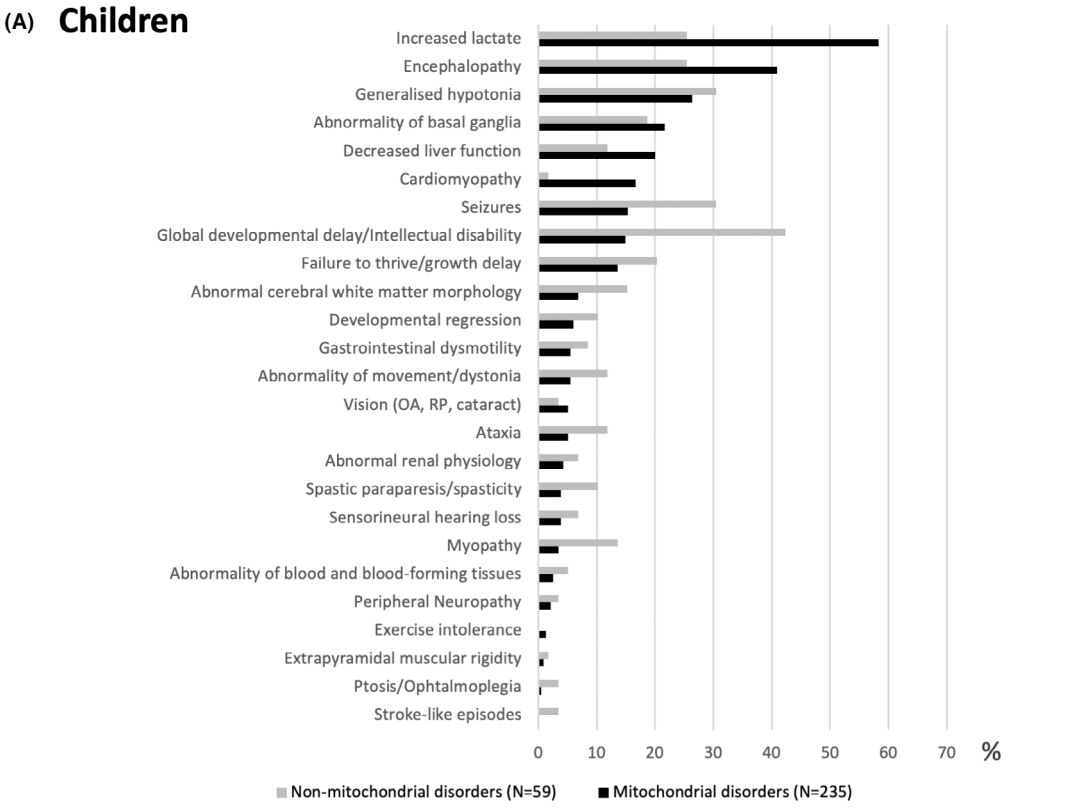
scale[3] (Fig. 1A). Most patients had a "possible PMD" (Score 2 to 4), 71% and 63% of children and adults respectively (210/294 children, 65/103 adults). The age of the patients at first analysis varied from birth to 81 years old, the most represented age category being children under 2 years old (45%, 178/397) (Fig. 1B).

Among the 397 patients, 292 patients (73%), subdivided into 216 children and 76 adults, were screened by mitochondrial targeted gene panels while 99 patients (25%) with 27 adults and 72 children, were tested by WES, and 6 children (2%) by WGS. We detected a total of 501 variants, of which 50% (253/501) were novel, within 172 different genes.

Unexpected number of patients with mitochondrial disorders mimics

The cohort was characterized by a broad spectrum of phenotypic presentations and variable severity and outcome ranging from lethal infantile to mild adult-onset disease (Fig. 2). Interestingly, among the 397 individuals, 322 (81%) had PMD and 75 (19%) had PMD mimics. 80% and 84% of children and adults, respectively (235/294 children, 87/103 adults) had PMD while 20% and 16% of children and adults, respectively (59/294 children, 16/103 adults) had PMD mimics. Among the 105 patients (78 children and 27 adults), who underwent WES or

Figure 2. Percentage of patients for clinical symptoms following the Human Phenotype Ontology (HPO) classification. Children clinical features are represented in the left panel (A), in black for children with primary PMD (*N* = 235) and in gray for children with PMD mimics (*N* = 59). Adults' clinical features are represented in the right panel (B), in black for adults with primary PMD (*N* = 87) and in gray for adults with PMD MIMICS (*N* = 16). OA, optic atrophy; RP, retinitis pigmentosa.



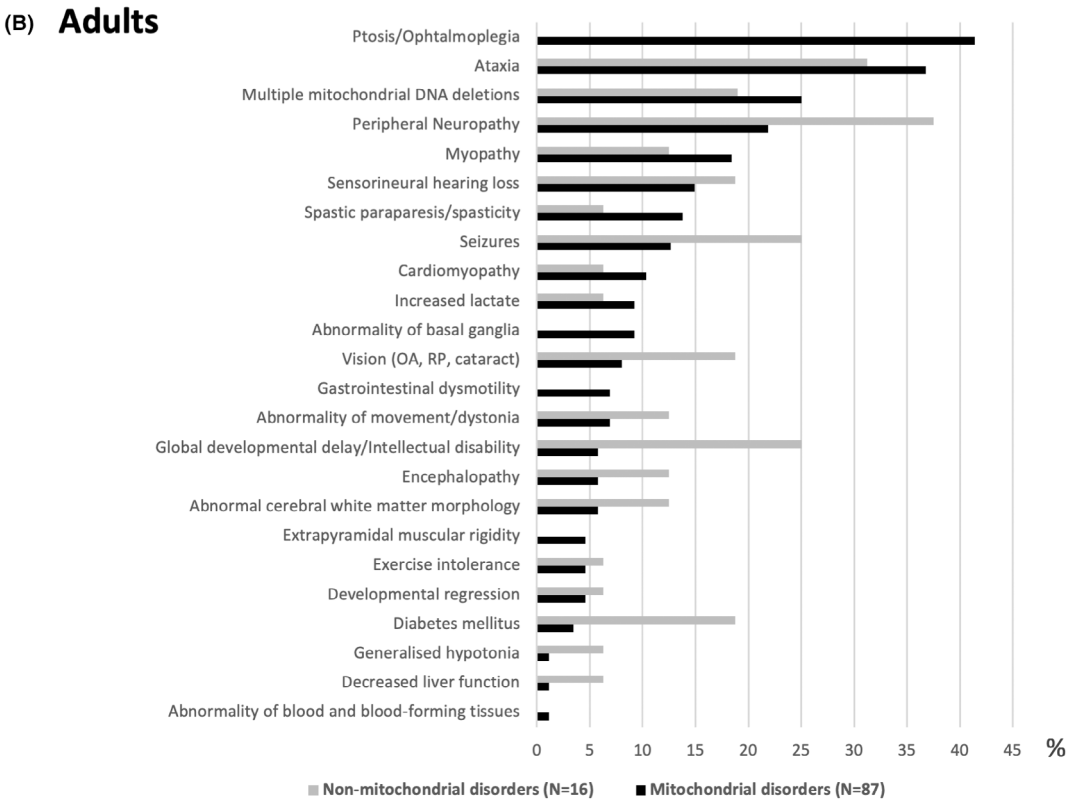


Table 1. Percentage of patients with PMD and PMD mimics among the 105 patients tested by WES or WGS according to the PMDC score.

MDC Score	Total (105)	MD	PMD MIMICS
Children	78	36 (46%)	42 (54%)
1–4	50	18 (36%)	32 (64%)
≥5	28	18 (64%)	10 (36%)
Adults	27	14 (52%)	13 (48%)
1–4	18	6 (33%)	12 (67%)
≥5	9	8 (89%)	1 (11%)

WGS analysis, 54% (42/78) and 48% (13/27) of children and adults respectively, had PMD mimics (Table 1). This percentage was higher if the analysis was restricted to patients with possible PMD (MD score 2–4), with 64% (32/50) and 67% (12/18) of children and adults respectively. The clinical and molecular data of these patients with PMD mimics are described in Sup Table S3.

Phenotypes in children

In children (Sup Table 1, Fig. 2A), the most common phenotype was Leigh syndrome characterized by the association of encephalopathy, psychomotor retardation or regression, lactic acidosis and abnormality of basal ganglia (20%, 60/294). Cardiomyopathies were over-represented in PMD compared to PMD mimics (17% versus 2%, $p < 0,01$). They were largely represented by hypertrophic cardiomyopathies and few dilated cardiomyopathies. Global developmental delay/intellectual disability and seizures were more frequent in PMD mimics (42% versus 15% $p < 0,01$, and 31% versus 15% $p < 0,01$, respectively). Other neurologic features such as abnormal movements or spastic paraplegia appeared more frequent in children with PMD mimics, but dystonia was probably underestimated as this term was often not mentioned in patients with Leigh syndrome.

Phenotypes in adults

In adults (Sup Table 2, Fig. 2B), the most common phenotype was Chronic Progressive External Ophthalmoplegia (CPEO) (35%, 36/103), isolated or associated with neuropathy and/or cerebellar ataxia, which was strongly suggestive of PMD, as all the 36 patients carried variants in mitochondrial genes. Although there seemed to be a higher prevalence of peripheral neuropathy, seizures, intellectual disability or diabetes in patients with PMD mimics, these differences were not significant.

Genotypes

In both children and adults, most patients had autosomal recessive disorders (87% and 65%, respectively) (Fig. 3A), but autosomal dominant disorders were more common in adults (33% versus 5% in children). About two-third were missense variants while loss-of-function variants (nonsense, splice site, and insertion/deletion (indel)) reached 30% and 2–3% were copy number variants (CNV) (Fig. 3B). We also identified one patient with uniparental disomy (UPD) in *GAN* gene. We identified 25 *de novo* variants (21/294) in children (7%) and 4/103 in adults (4%). Most of them (68%, 17/25) were involved in autosomal dominant disorders, 7/25 (28%) in X-linked disorders, mainly responsible for pyruvate dehydrogenase deficiency, and 1/25 (4%) was identified in *trans* of an inherited variant in the *PLA2G6* recessive gene. These 25 variants occurred in 18 different genes, the majority of which (67%, 12/18) were non-mitochondrial genes. Finally, we identified a complex multigenic disease, with more than a single gene defect involved, in 1% (4/397) of patients (3/294 children, 1/103 adults).

In children, we observed a very large genetic heterogeneity. Even among the most frequently mutated genes, *ECHS1, PDHA1, POLG, SLC19A3*, and *SURF1*, only accounted for 19% (56/294) of children. *ECHS1*, the most mutated gene represented 5% (14/294) (Fig. 3C). Among the 63 patients (60 children, 3 adults) diagnosed as Leigh syndrome, 22 (35%) carried mutations in genes encoding for structural or assembly factors for complex I subunits. The other genes most frequently mutated in Leigh syndrome encoded exclusively for mitochondrial proteins (*SURF1* (9%, 6/63), *ECHS1* (6%, 4/63) and *SERAC1* (5%, 3/63)) except *SLC19A3* (9%, 6/63) which encoded a thiamine transporter. Concerning children with PMD, genes were clustered according to mitochondrial functions as described in Mitocarta (Table 2). The genes were distributed in the different subgroups with the highest percentages in genes related to OXPHOS functions (respiratory chain subunits, assembly factors, and cofactors) (29%, 69/235), especially complex I subunits (15%, 35/235), and genes involved in mitochondrial protein synthesis translation and degradation (24%, 57/235) with a significant percentage of variants in genes encoding for tRNA synthetases and transferases (9%, 21/235).

In adults, the distribution was more homogeneous with eight common genes *POLG, TWNK, SPG7, RRM2B, AFG3L2, OPA1, TK2*, and *TYMP* responsible for 52% of cases (54/103). *POLG* was the most frequently mutated gene (16.5%, 17/103), mainly with a recessive mode of inheritance (82%, 14/17). In patients with PMD, almost half of patients (48%, 42/87) had variants in genes involved in mtDNA maintenance, replication and nucleotide metabolism.

In patients with PMD mimics, we also observed a large genetic heterogeneity. Apart from the *SLC19A3* gene, which is the primary differential diagnosis for

Table 2. Mutated mitochondrial genes are classified according to mitochondrial functions as described in Mitocarta and their repartition among children and adult patients.

Gene functions (children/adults)	Genes	Children (*N* = 235)	Adults (*N* = 87)
Mitochondrial translation (18%/9%)			
ARS genes	*AARS2, CARS2, DARS2, EARS2, FARS2, IARS2, KARS, NARS2, PARS2, RARS2, SARS2, TARS2, VARS2, YARS2*	21 (9%)	7 (8%)
Others	*GATB, GFM1, MTFMT, MTO1, RMND1, TSFM*	21 (9%)	1 (1%)
Mitochondrial protein metabolism (6%/18%)			
Mitochondrial metabolism	*DLD, GCDH, HIBCH, MCCC2, FASTKD2, PDSS1, PYCR2*	9 (4%)	2 (2%)
Chaperone	*FXN, HSPD1*	1 (<1%)	1 (1%)
Others	*AFG3L2, DNAJC19, LRPPRC, NFU1, PARK7, SPG7, XPNPEP3*	5 (2%)	13 (15%)
RC subunits, assembly factors, cofactors (29%/12%)			
Complex I	*NDUFA10, NDUFA12, NDUFA2, NDUFA4, NDUFA2, NDUFA4, NDUFAF2, NDUFAF4, NDUFAF6, NDUFB11, NDUFB8, NDUFS1, NDUFS3, NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFV1*	**35 (15%)**	1 (1%)
Others subunits	*ATP5G3, COX15, UQCRC2*	6 (2%)	1 (1%)
Assembly factors	*ACAD9, BCS1L, LYRM7, SCO2, SDHAF1, SURF1, TMEM126B, TMEM70*	23 (10%)	7 (8%)
Others	*ADCK3, COQ2, COQ6*	5 (2%)	2 (2%)
mtDNA maintenance replication and nucleotide metabolism (11%/48%)			
mtDNA maintenance	*ATAD3A, FBXL4, RRM2B*	6 (2%)	6 (7%)
mtDNA replication	*POLG, POLG2, TWNK*	12 (5%)	**30 (34%)**
Nucleotide metabolism	*DGUOK, TK2, TYMP*	10 (4%)	6 (7%)
Mitochondrial dynamics (2%/7%)	*CHCHD10, MFN2, OPA1, QIL1 (C19ORF70), VPS13D*	6 (2%)	6 (7%)
Lipids metabolism (13%/1%)	*AGK, ECHS1, ETFDH, SERAC1, TAZ*	30 (13%)	2 (2%)
Carbohydrate metabolism (8%/0)	*PC, PDHA1, PDHB, PDHX*	18 (8%)	0
Metals and cofactors (2%/0)	*ISCA1, ISCA2, NAXE, PANK2*	4 (2%)	0
MtRNA metabolism (2%/0)	*ELAC2, PNPT1, TRMU*	5 (2%)	0
Small molecule transport (2%/1%)	*MPV17, SFXN4, SLC25A3, SLC25A20, SLC25A4 (ANT1)*	6 (2%)	2 (2%)
TCA cycle (4%/0)	*ACO2, FH, PMDH2, SUCLA2, SUCLG1*	9 (4%)	0
Others (1%/0)	*ETHE1, HPDL, CPS1*	3 (1%)	0

The highest percentage are shown in bold.
N, number.

"mitochondrial" Leigh syndrome, there was no recurrent gene. The main affected functions included transcription/translation factors, ionic channels, transporters, or proteins responsible for myopathies such as laminin, titin, or myosin.

Histologic findings

Histology investigations were available in 37 children and 50 adults (Fig. 4A). Abnormalities suggestive of mitochondrial disease such as COX-negative fibers, ragged red fibers (RRF), mitochondrial proliferation, or lipid accumulation were more common in adults (84%, 41/50) than in children (57%, 21/37) and were clearly over-represented in patients with PMD compared with PMD mimics. Most children had COX negative fibers (57%, 12/21) while RRF were rare (14%, 3/21), observed in 2 patients with *SURF1* variants and one carrying *ANT1* variant. Lipid accumulation was more common in children (57%, 12/21 vs 12%, 5/41) while not specific of PMD and was also observed in three children with variants in the non-mitochondrial genes including *ADCY5, CACNA1A, and MEGF10*. Among the 41 adults, 4 patients harbored pathogenic variants in non-mitochondrial genes (*KIF1A, LAMA2, SYNE1, and WFS1*), the presence of COX negative fibers could be secondary to patient age.[19,20]

Biochemical findings

A total of 166 children and 42 adults had tissue biopsies allowing the measurements of respiratory chain (RC) enzyme activities (Fig. 4B) (131 muscle, 12 liver and 73 skin biopsies). OXPHOS deficiencies in muscle or liver tissues and fibroblasts were more common in children (79%, 131/166) than in adults (48%, 20/42). They were clearly over-represented in children with PMD (88%, 115/

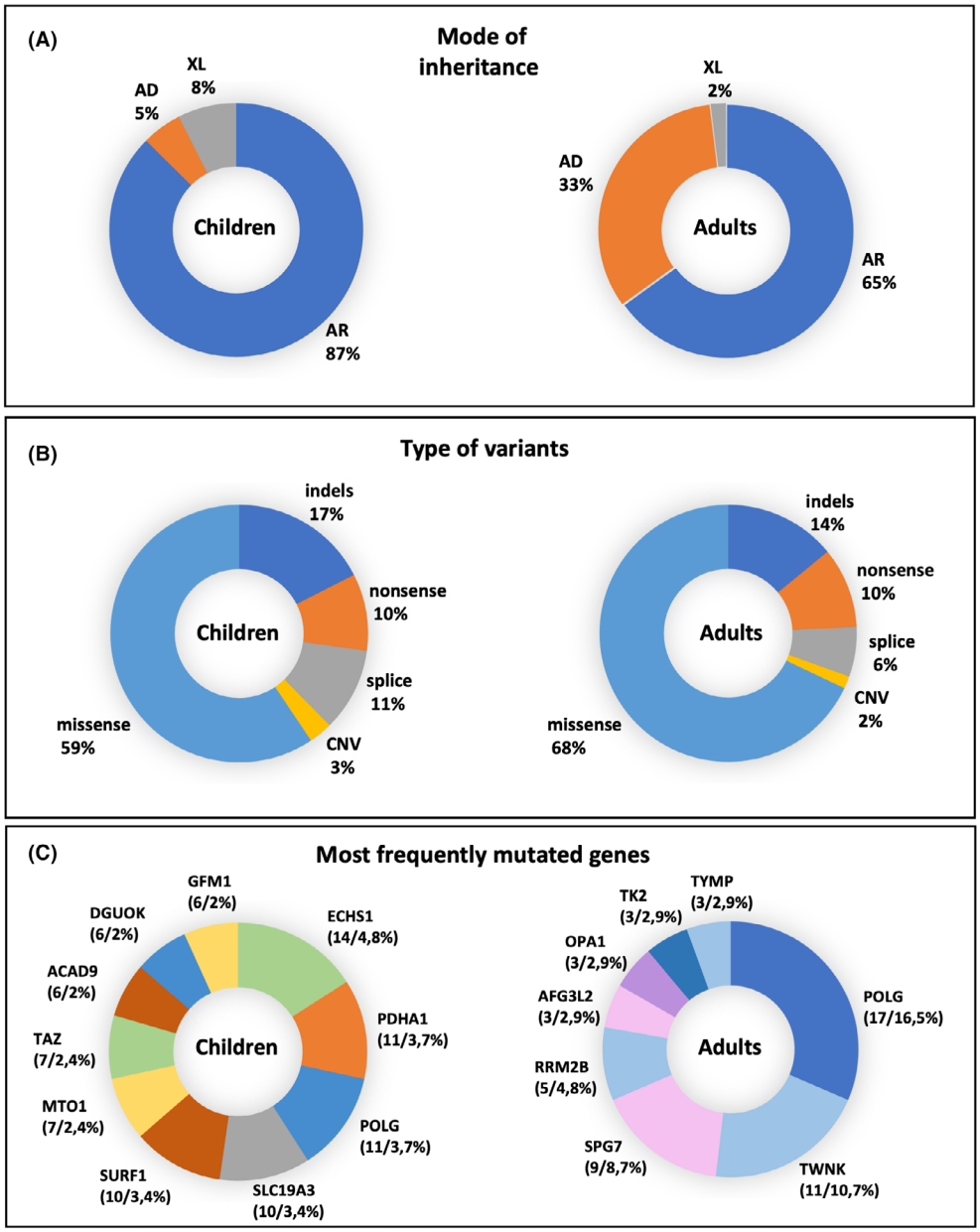


Figure 3. Analysis of pathogenic variants identified in the 294 children (left panels) and the 103 adults (right panels). (A) Mode of inheritance, autosomal dominant (AD), autosomal recessive (AR), X-linked (XL) are indicated with the percentage. (B) Molecular classification according to the type of variants (Missense; indels:Insertion/deletion; nonsense; splice variant; CNV: Copy number variation. (C) The most frequently mutated genes are listed if pathogenic variants were identified at least 3 times in adults, and 6 times in children. In brackets: absolute numbers/frequencies. The mitochondrial functions are represented by colors: blue: mtDNA maintenance, green: lipids metabolism, orange: carbohydrate metabolism, brown: assembly factors, yellow: mitochondrial translation, pink: mitochondrial protein metabolism, purple: mitochondrial dynamics, gray: non-mitochondrial gene.

131) compared to PMD mimics and none has been showed in adults with PMD mimics. Most patients with PMD had combined RC deficiencies (Fig. 4C). Complex I deficiency was over-represented in children (23%, 26/115) whereas complex IV was over-represented in adults (28%, 6/21). Other mitochondrial enzyme deficits were much rarer. Complex I deficient patients harbored mostly pathogenic variants in genes encoding structural or assembly factors for complex I subunits, but also in genes such as *MTO1* and *ECHS1*, involved respectively in protein

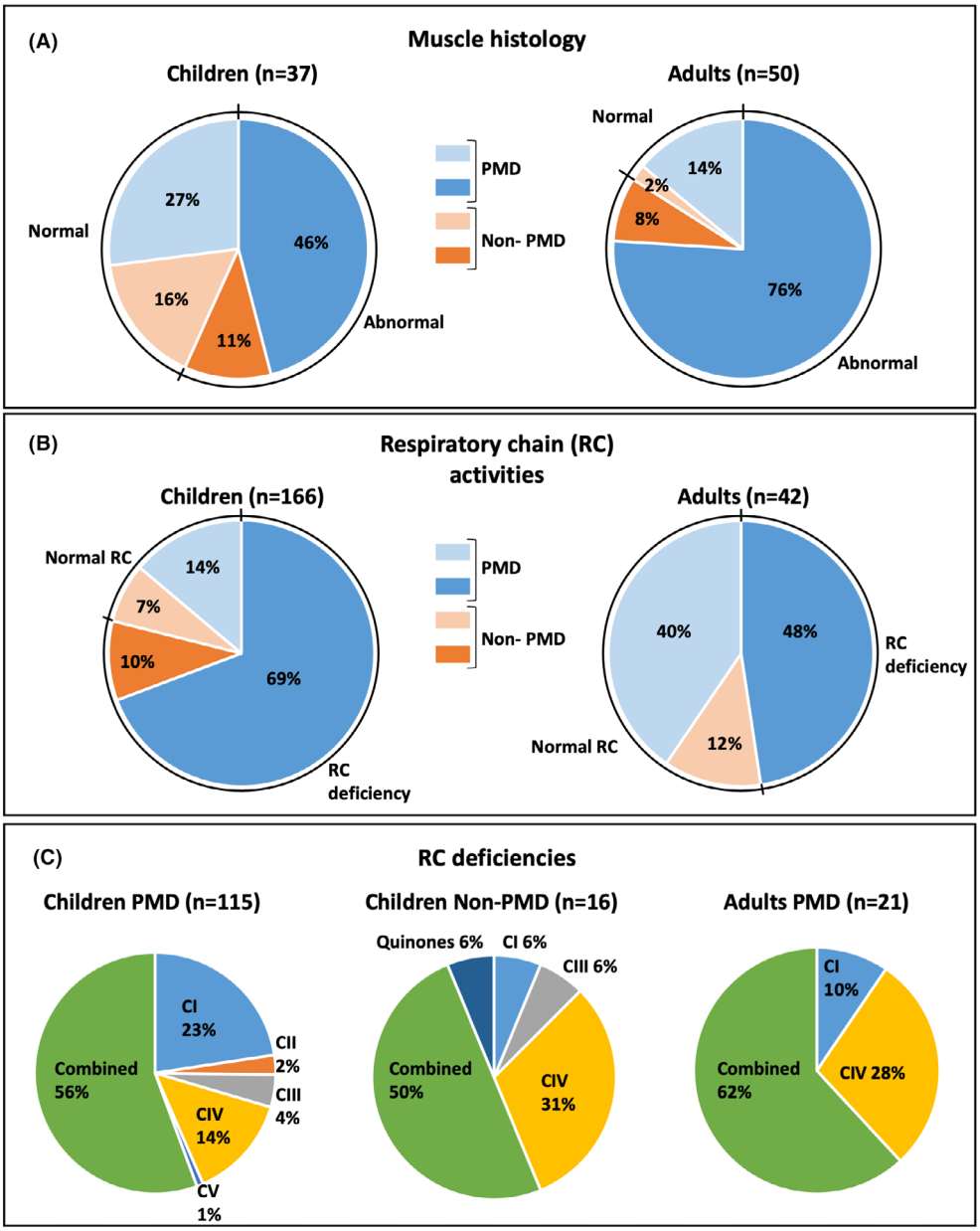


Figure 4. Histology and biochemical characterization of the patient cohort according to age, children and adults with mitochondrial (MD) and PMD mimics (PMD mimics). (A) Histology analysis. Pie charts showing the patient number (n) and percentages with normal (light blue and light orange) or abnormal, including ragged-red fibers, COX negative fibers, mitochondrial proliferation or excessive lipids accumulation, (dark blue and dark orange) muscle histology in children and adults (B) Pie charts depicting the number (*n*) of patients with normal (light blue and light orange) or abnormal (dark blue and dark orange) respiratory chain (RC) activities. (C) Summary of the outcome of respiratory chain activity with the percentages of complexes of the respiratory chain; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V; or combined or quinone deficiencies.

translation and lipid metabolism, which usually are responsible for combined mitochondrial deficiencies, or *ATAD3A* for which a role in mitochondrial dynamics has been suggested.[21] Among the 46 patients with complex IV deficiency, the most frequently mutated genes were *SURF1* or *SCO2* encoding for complex IV assembly factors (30%, 14/46).

In children with PMD mimics ($N = 28$), over half exhibited a RC deficiency (57%, 16/28). Most of them had combined enzyme deficiencies (50%, 8/16), followed

by complex IV deficiency (31%, 5/16). Genes responsible for these deficits were connected to a wide variety of functions including ion channels, receptors or various enzymes. Two patients showed isolated deficiencies in complex I and III, secondary to pathogenic variants in the *UFM1* and *CHRND* genes respectively. One patient had quinones deficiency secondary to a pathogenic variant in the *SIAT9* gene.

Discussion

We described the largest French cohort of patients suspected of PMD, in whom we identified a nuclear disease-causing genetic defect by either targeted gene panels, WES or WGS. The patient cohort encompassed 397 individuals (294 children and 103 adults) of whom 322 (81%) had PMD and 75 (19%) had PMD mimics. Our data confirmed the large clinical heterogeneity of PMD and the high number of phenocopies that make them difficult to recognize.[22,23]

Genetic heterogeneity was more marked in children with a large variety of genes. Even *ECHS1*, the most mutated gene accounted only for 5% of cases (14/294). However, looking at the child cohort with PMD, a high percentage of pathogenic variants occurred in genes related to OXPHOS functions (RC subunits, assembly factors, and cofactors) (29%, 69/235), especially targeting complex I subunits (15%, 35/235), and genes involved in mitochondrial protein synthesis, translation and degradation (18%, 42/235) with a high prevalence of variants in genes encoding for tRNA synthetases and transferases (9%, 21/235). Our data confirmed that patients carrying pathogenic variants in mitochondrial aminoacyl-tRNA synthetases (ARSs) exhibited a broad disease spectrum with a high intrafamilial variability in children as well in adults, like patient 29 who harbored pathogenic variants in the *NARS2* gene (SupTable S2).[24] According to previous reports, Leigh syndrome was the most common phenotype[25] highly suggestive of primary mitochondrial dysfunction. Only the non-mitochondrial gene, *SLC19A3*, which encodes a thiamine transporter, was responsible for a very comparable phenotype. Patients carrying mutations in *SLC19A3* could benefit from a specific therapy with thiamine and this gene was included early in the mitochondrial gene panels.[26]

In adults, pathogenic variants were mainly identified in genes involved in mtDNA maintenance, replication, and nucleotide metabolism, among patients with PMD (48%, 42/87). CPEO, the most common phenotype, isolated or associated with peripheral neuropathy and/or cerebellar ataxia, was mainly due to primary mitochondrial dysfunction. Compared to the large cohort of Northeast of England described by Gorman et al,[27] French adults harbored mainly pathogenic variants in the four most common genes *POLG, TWNK*, *SPG7*, and *RRM2B*. However, unlike the UK cohort, recessive and dominant variants of *POLG* (16.5%, 17/103) were more common than recessive variants in *SPG7* (8.7%, 9/103). Patients with *RRM2B* variants had recessive or dominant CPEO. Ataxia and peripheral neuropathy were frequently identified in patients with mitochondrial or PMD mimics, often associated with multiple mtDNA deletions. However, mtDNA deletions were frequently observed in the elderly due to aging and may erroneously point to PMD. Conversely, the absence of mtDNA deletions did not rule out a mutation in genes involved in mtDNA stability. For example, a 70-year-old patient (SupTable S2 patient 90) had a Parkinson syndrome with ptosis without apparent mtDNA multiple deletions while harboring a pathogenic variant in the *TWINKLE* gene (c.1120C>T, p.Arg374Trp).

POLG was the most mutated gene (28/397), and the clinical features formed a *continuum* rather than distinct phenotypes as previously suggested.[28] Children showed a combination of different symptoms, mainly hepatopathy and seizures while adults showed mainly CPEO, ataxia and neuropathy. Rarely described,[29,30] two patients also exhibited parkinsonism.

Diabetes was not a common presentation in PMD linked to nuclear genes and could be more suggestive of mtDNA variants. Two adult patients suffered from Wolfram syndrome which mimicked a PMD due to the association of late onset diabetes, deafness and optic atrophy to neuropathy and ataxia. Stroke-like episodes were also rarely reported in our cohort and even if it was probably underestimated, especially in patients with *POLG* variants, they are also more suggestive of mtDNA variants.

Among the patients with PMD mimics, there was also a large clinical heterogeneity. These patients often presented with a multisystemic disease, sometimes including lactic acidosis or suggestive MRI abnormalities mimicking PMD. The most common disorders were metabolic disorders, epileptic encephalopathies, abnormal movements disorders, myopathies, and spastic paraplegia. Note that *de novo* variants were rare in PMD and that most of these variants occurred in PMD mimics (67%, 12/18) with almost half of these patients (44%, 11/25) who had seizure or epileptic encephalopathy which shows the interest of a trio approach for these phenotypes.[31,32]

Moving toward a pangenomic strategy in suspected mitochondrial disorders

From 2015 to 2022, within the MitoDiag network, NGS technology was sequentially and successively implemented, first with limited gene panels then by increasing the

panel size up to more than 400 genes. During the last 3 years, following the various publications showing a better performance of WES compared with large gene panels,[6,10,33] WES gradually replaced targeted gene panels. More recently, the implementation of WGS within both national genomic sequencing platforms SEQOIA and AURAGEN has facilitated the access of WGS for patients when trio analysis was possible. The diagnostic yield, within the MitoDiag network, varied between 15 to 35% of patients depending on the gene panel size used, age and phenotype of the patients tested (unpublished data). Higher yields were observed for pediatric populations. Although we cannot have a precise estimate, these yields were close to that described in other large cohort studies using WES (31–39%).[7,34,35] Several studies using WES showed higher yield reaching 54% of positive diagnoses but were conducted on more selected patients or preferentially pediatric population.[6,10,33] Recently studies using WGS identified definitive genetic diagnosis for 31% to 55% depending on recruitment criteria used.[7,36,37] Surprisingly, WGS did not increase the diagnostic yield as much as expected. This could be attributed to the challenges in interpreting intronic variants without RNA sequencing and the current limitations of in silico bioinformatics tools for detecting complex Structural Variations (SV). In our cohort, the number of WGS cases was still limited to draw any conclusions, and the contribution of WGS will need to be reassessed.

We confirmed in our cohort that WES/WGS, instead of targeted gene panel approach, was more valuable to identify the genetic basis in clinically heterogeneous patients with suspected PMD (MDC score 2–4). Indeed, looking at the 105 patients tested by WES/WGS, among the 68 patients with PMDC score ≤4, more than half suffered from a PMD mimics, 64% of children (32/50) and 67% of adults (12/18). In patients with probable or definite PMD (MDC score ≥5), this percentage was lower as expected. Mutated genes, in patients with PMD, were all included in our large panel of 485 genes. Also, for patients who have a strong suspicion of PMD (MDC score ≥5), this panel can be used as first *in silico* filter on WES/WGS data to facilitate variant interpretation or can be discussed in laboratories, which do not have the possibility of carrying out WES. Moreover, a limited gene panel mitochondrial DNA maintenance disorder including 28 genes (Version 3.0 https://panelapp.genomicsengland.co.uk/), can also be efficiently used to filter NGS data in adult patients with CPEO as for the 36 patients suffering from CPEO, all mutated genes were included in this panel.

Concerning respiratory chain (RC) deficiencies, the definition itself of PMD is unclear and these data confirmed that RC defect was not specific to PMD and are frequently associated with pathogenic variants in non-mitochondrial genes. Indeed, a RC deficiency was identified in more than half of children with PMD mimics (57%, 16/28) and genes responsible for these deficits were connected to a wide variety of functions including ion channels, receptors, or various enzymes. Most defects were combined or involved complex IV deficiency. Interestingly, one child (Patient 282) suffering from encephalopathy and highly suspected of primary PMD, because of an isolated complex I deficiency, was finally diagnosed, after a long diagnostic odyssey, with a pathogenic variant in the *UFM1* gene encoding a ubiquitin-like modifier.

Another advantage of the WES/WGS approach was the possibility of identifying multiple genetic diseases in one patient. This is particularly true for consanguineous patients, with an increased risk of having several autosomal recessive disorders, and for PMD, which are characterized by great clinical heterogeneity and frequent multisystem involvement. For this reason, WES/WGS data should always be completely analyzed when the patient's phenotype is not fully explained. In our cohort, four patients (1%, 4/397) had multiple genetic diseases including two children for whom the association of two pathogenic variants in two non-mitochondrial genes mimicked a PMD (patients 211 and 241, table S1). It is also important to note that complex PMD can be linked to the combination of nuclear and mtDNA variants as for patient 73 (SupTable S2) who presented with a spastic paraplegia due to a variant in the *SPG7* gene and diabetes, deafness and was associated with the m.3243A>G pathogenic variant with 44% mutant load in muscle. Also, mtDNA sequencing should always be done when the patient's phenotype is not completely explained.

Finally, a pangenomic strategy allowed a fast identification for potentially treatable pathologies. For example, disorders linked to aminoacyl tRNA synthetase (ARS) deficiencies have shown a great phenotypic heterogeneity and overlaps between the different mitochondrial and cytoplasmic ARS genes. As targeted ARS therapeutic trials are taking place and have shown a beneficial effect of supplementation of the ARS-specific amino acid in patients,[38,39] it is important not to waste time to confirm the molecular diagnosis for these patients.

From our experience, we have developed a genetic testing flowchart to guide physicians in their diagnostic strategy (Fig. 5). Since it is premature for the routine diagnostic application of WGS, we have chosen not to detail it in this flowchart. To avoid diagnostic delay, mtDNA sequencing and WES should be performed simultaneously when possible, especially in critical illness. However, to reduce the costs and to adapt to the

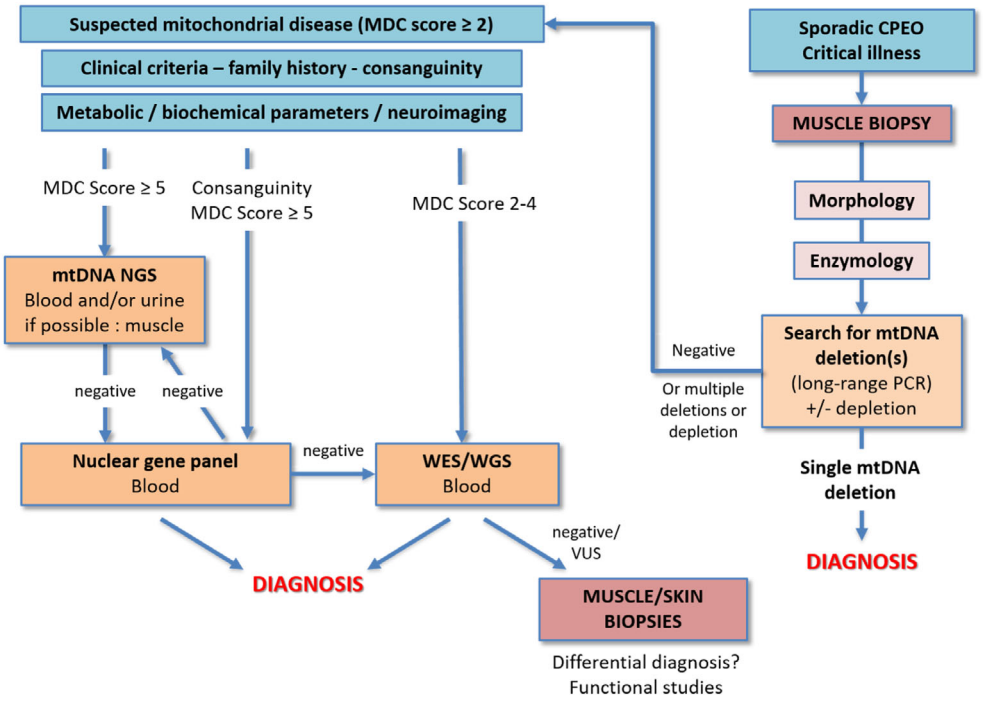


Figure 5. Diagnostic approach for mitochondrial disorders. Score 2–4: possible PMD, score ≥5 probable PMD.

current organization of the MitoDiag laboratories, we suggested performing mtDNA testing first, followed by targeted mitochondrial encoded gene panel when PMDC score is ≥5 (probable PMD). In the absence of available muscle, mtDNA testing should be carried out preferentially from uroepithelial cells, which is more sensitive for mtDNA variant detection than blood. In a context of consanguinity, nuclear genes testing should be performed first. Muscle biopsy is no longer used in routine first-line diagnostic but remains essential for accurate genetic diagnosis of sporadic large scale mtDNA deletions, for instance due to CPEO, and mtDNA mutations restricted to skeletal muscle. Muscle biopsy is also recommended for critical illness requiring a prompt diagnosis. Moreover, given the significant increase in the number of identified variants of unknown signification (VUS), muscle biopsy and/or skin biopsies are essential to perform functional studies to support the variant pathogenicity. Finally, it also can be useful to find additional arguments for the diagnosis of PMD when genetics is inconclusive.

Finally, given the complexity of PMD, the growing number of VUS and potential mtDNA /nuclear genes interactions, the MitoDiag network has developed innovative solutions in term of improving the molecular diagnosis. It has developed Mitomatcher, a French national database collecting genetic and clinical data for PMD patients.

Strengths and weaknesses of the study

Our study had several strengths. It is the largest French cohort of patients suspected of PMD carrying pathogenic nuclear genes-causing described so far. Individuals were recruited nationwide highlighting the usefulness of the MitoDiag network and national reference centers, CALISSON and CARAMMEL. The description of clinical features and natural history of PMD patients is fundamental to better understand the genotype/phenotype correlations, to improve diagnostic strategies and interpretations of VUS, essential to reduce the burden of patient's diagnostic odysseys.

One of the weaknesses of the paper was first that this retrospective study was not designed to investigate the positive yield in PMD since we did not include patients with mtDNA pathogenic variants and patients with optic neuropathies already reported in previous studies.[5,14,15] We could not define the exact number of tested patients which was estimated around 2000. Secondly, we took advantages of the different databases of national centers, however without a unique database with systematic and homogeneous implementation of HPO terms, it was possible that clinical information was not exhaustive and the patient PMDC scoring was potentially underestimated. However, clinical data obtained were consistent with the literature[7,34,35] and a national database will henceforth be systematically implemented with HPO features, to improve the clinical data quality.

Conclusion

The MitoDiag network and reference centers for PMD worked together to facilitate a consistent standard of care. The implementation of clinical and genetic databases allows improving diagnostic strategies, establishing genotype–phenotype correlations and may help the development of clinical trials. The study showed, a larger genetic heterogeneity in children, with a high prevalence of pathogenic variants in genes related to OXPHOS functions and mitochondrial translation, while in adults, pathogenic variants occurred with a high prevalence in genes related to mtDNA maintenance. More than half of patients initially suspected of possible PMD (MDC score $\leq$4), tested by WES/WGS, were classified as PMD mimics. MitoDiag has integrated these technologies into the diagnostic strategy to avoid multiplication of gene testing and reduce the diagnostic wandering of PMD patients.

Author Contributions

C.R., V.Pr. and E.P. contributed to the design and conception of the study. Members of the MITODIAG network and CALISSON and CARAMMEL reference centers for mitochondrial diosrders contributed to the acquisition and data analysis. C.R., V.Pr., E.P., A.C. and V.Pa. contributed to drafting the text or preparing the figures.

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Conflict of Interest

The authors declare no competing financial interest.

Data Availability Statement

Anonymized data not published within this article will be made available by request from any qualified investigator.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1.
Table S2.
Table S3.
Table S4.