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Research Article

Clinical and genetic spectrum in a cohort of patients with suspected mitochondrial disease

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Abstract

Introduction: Mitochondrial diseases (MDs) are a heterogeneous group of genetic disorders affecting oxidative phosphorylation, characterized by diverse neuromuscular and multisystemic manifestations, whose clinical variability and genetic complexity necessitate a comprehensive, multidisciplinary diagnostic approach.

Aim: This study aims to explore the phenotypic and genotypic spectrum of patients with suspected MD, focusing on mitochondrial genome variations to improve diagnostic accuracy and support genetic counseling.

Materials and methods: We analyzed 240 patients with a Nijmegen mitochondrial disease score (NMDS) ≥3, with 90 individuals undergoing comprehensive clinical, biochemical, neuroimaging, and genetic assessments. Descriptive statistics were applied to summarize clinical and biochemical data, while inferential analyses, including chi-square tests and regression models, were employed to explore genotype-phenotype associations.

Results: The study delineated a multifaceted clinical spectrum, with neuromuscular dysfunction as the predominant hallmark, evidenced by a high prevalence of developmental delay (88.9%), hypotonia (77.8%), and exercise intolerance (63.3%). Patients with mitochondrial involvement displayed a significantly greater burden of neurological (seizures: 71.9% vs. 43.1%, p=0.009) and cardiac abnormalities (37.5% vs. 15.5%, p=0.018), underscoring their diagnostic relevance. Genetic analysis identified gene variants associated with the patients' phenotypes in 35.6% of cases, with Complex I being the most frequently affected, emphasizing its pivotal role in mitochondrial dysfunction and disease severity.

Conclusion: Our findings highlight the genetic and clinical heterogeneity of MDs, emphasizing the importance of integrating molecular, biochemical, and neuroimaging data to enhance early diagnosis and facilitate personalized treatment strategies.

Keywords

mitochondrial disease, Nijmegen mitochondrial disease score, qPCR-HRM, Sanger sequencing

Introduction

Mitochondrial diseases (MDs) are a heterogeneous group of genetic disorders primarily impairing oxidative phosphorylation, constituting the most common inherited metabolic conditions and a major subset of genetic neurological disorders. Epidemiological data suggest a prevalence of 5-15 per 100,000 in children[1] and around 12.5 per 100,000 in adults[2]. However, their marked clinical heterogeneity often hinders prompt and accurate diagnosis, leading to diagnostic delays and underestimation of disease burden.

The pathophysiology of MDs reflects the mitochondria's

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central role in cellular energy homeostasis, resulting in multisystem involvement that may emerge at any age and affect virtually any organ. High-energy-demand tissues, such as the brain, muscles, heart, and liver, are particularly vulnerable.[3] The progressive course and high morbidity and mortality of MDs emphasize the critical need for improved diagnostic strategies and disease-specific therapies.[4] While both nuclear and mitochondrial genomes contribute to MD pathology, this study specifically focuses on cases related to mitochondrial DNA (mtDNA) to better elucidate their clinical and biochemical profiles.

mtDNA mutations give rise to a wide phenotypic spectrum, from mild, late-onset presentations to severe, early-onset disorders. Although later-onset forms are more typical, certain mtDNA variants are associated with severe, pediatric-onset phenotypes.[5] Pathogenic variants typically include point mutations or large-scale rearrangements, frequently affecting mitochondrial protein synthesis, particularly through defects in transfer RNA (tRNA) genes[6], or impairing subunits of the electron transport chain (ETC)[7], leading to early-onset, rapidly progressive disease with marked morbidity.

Aim

This study aims to investigate the phenotypic and genotypic spectrum of patients with suspected mitochondrial disease, with a focus on mitochondrial genome variants. By characterizing the genetic landscape, we seek to refine diagnostic accuracy and facilitate informed genetic counseling. This represents the largest cohort analyzed to date in the Moldovan population, providing valuable insights into the genetic basis and clinical heterogeneity of mitochondrial diseases.

Materials and methods

The research was conducted between March 2021 and October 2024. A total of 90 unrelated patients were selected from a large pool of individuals referred for assessment of underlying metabolic disorders to the Institute of Mother and Child, a national referral center in the Republic of Moldova. Inclusion was based on the modified Nijmegen mitochondrial disease scoring system (NMDS), with all patients scoring ≥3. The NMDS is a validated diagnostic tool incorporating clinical features (muscular, central nervous system, and multisystem involvement), biochemical and imaging findings, and muscle biopsy morphology to estimate the likelihood of mitochondrial disease. As muscle biopsy was unavailable, analysis focused on the remaining NMDS components. According to the NMDS, a score of 1 indicates mitochondrial disease is unlikely, 2–4 suggests a possible disorder, 5–7 signifies a probable disorder, and ≥8 confirms a definitive mitochondrial disease diagnosis.[8]

All patients underwent a comprehensive set of assessments and investigations, including the following:

Clinical workup

Each patient underwent a detailed clinical history review and neurological examination. Initial laboratory tests included complete blood count, liver and kidney function tests, electrolytes, blood gases, lactate, ammonia, creatine kinase, and uric acid. To exclude other inborn metabolic disorders, secondary screening included plasma amino acids, acylcarnitine profiles, and urinary organic acids. Neurophysiological assessments (audiometry, electromyography, and electroencephalography), brain imaging (MRI or CT), and electrocardiography were performed as clinically indicated.

Molecular genetic testing

The molecular-genetic analysis followed a two-step protocol. Initially, a total of 240 patients with NMDS ≥3 were screened using quantitative PCR high-resolution melting (qPCR-HRM) (Applied Biosystems 7500 Real-Time PCR System, USA), with customized primers targeting seven predefined pathogenic point mutations commonly associated with mitochondrial disorders (m.3243A>G, m.8344A>G, m.8993T>G/C, m.13513G>A, m.3460G>A, m.11778G>A, m.14484T>C). For mutations identified via qPCR-HRM, heteroplasmy levels were assessed by PCR-restriction fragment length polymorphism (PCR-RFLP) and densitometric analysis of electrophoresis gels using ImageJ software for semi-quantitative evaluation. Following the qPCR-HRM screening, patients with NMDS ≥6 or positive qPCR-HRM results were selected for further evaluation. In total, 90 patients underwent comprehensive mtDNA sequencing. In this second phase, Sanger sequencing by capillary electrophoresis (Applied Biosystems 3500 Dx Genetic Analyzer, USA) was performed using customized primers targeting mitochondrial genes encoding ETC components, including both protein-coding and RNA genes. Only genetic variants cataloged in the ClinVar database were selected for detailed analysis. Pathogenicity classification of all identified variants was done according to American College of Medical Genetics and Genomics (ACMG) guidelines.[9]

DNA was extracted from peripheral blood using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). Informed consent was obtained from adult patients and parents of affected children, and authorizations for individual data protection were secured.

Statistical analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics, v. 27.0). Descriptive statistics summarized demographic, clinical, and genetic data; continuous variables are presented as mean ± standard deviation (SD) and categorical as frequencies. Chi-square tests compared categorical variables. ANOVA assessed differences in continuous variables across groups, with post hoc tests as needed. Binary logistic regression identified significant predictors of

diagnostic outcomes, reported as odds ratios (OR) with 95% confidence intervals (CI). Significance was defined at $p<0.05$.

Results

Patient selection and demographics

A total of 240 patients with NMDS ≥3 were initially enrolled to capture individuals meeting the minimum criteria for suspected MDs. From this cohort, 90 were selected for detailed analysis based on the availability of comprehensive clinical, biochemical, instrumental, and genetic data. Of these, 32 patients demonstrated confirmed mitochondrial involvement based on molecular and/or functional criteria, whereas 58 remained undiagnosed at the molecular level despite comprehensive evaluation.

Among the 90 selected patients (54 males, 36 females), the mean age at symptom onset was 14.34±2.00 months, with onset ranging from early infancy to late childhood or adolescence. The mean age at enrollment was 44.80±5.73 months, indicating a delay between initial symptoms and definitive evaluation. During the study period, 8 patients (8.9%) died at a mean age of 31.00±8.50 months, most presenting with severe neuromuscular and multisystem involvement.

Clinical phenotype and multisystemic involvement

Clinical presentation was dominated by muscular (96.7%) and neurological (95.6%) impairments, with developmental delay most frequent (88.9%), followed by hypotonia (77.8%) and exercise intolerance (63.3%). Seizures affected over half of the patients (53.3%), with varied semiologies: generalized tonic-clonic seizures (38.9%), myoclonic episodes (10.0%), polymorphic seizures (7.8%), absence seizures (2.2%), and treatment-resistant forms (31.1%). Additional neurological findings included ataxia (22.2%), brainstem involvement (34.4%), peripheral neuropathy (18.9%), and extrapyramidal signs (16.7%) observed in a subset of patients, characterized by dyskinesia, dystonia, and rigidity.

Regarding neuromuscular manifestations, the mitochondrial-afflicted group showed significantly higher rates of marked neuromuscular involvement (37.5% vs. 13.8%, χ^2=6.70, p=0.010), seizures (71.9% vs. 43.1%, χ^2=6.86, p=0.009), and loss of acquired skills (28.1% vs. 5.17%, χ^2=9.40, p=0.002). Beyond neuromuscular features, a wide range of multisystem abnormalities was documented, with 83 patients (92.2%) showing multisystem involvement. Endocrine and growth abnormalities were the most prevalent (73.3%), with growth impairment (70.0%) and short stature (47.7%) being the most frequent findings, alongside subclinical hypothyroidism (11.1%) and pubertal disorders (3.3%).

Gastrointestinal dysfunction was present in nearly one-third of patients (33.3%), including poor appetite (60.0%), feeding difficulties (37.8%), dysphagia (15.6%), vomiting (24.4%), constipation (11.1%), diarrhea and gastroesophageal reflux (each 4.4%), and, rarely, intestinal obstruction (2.2%).

Hepatic involvement was noted in 26.7% of patients, mainly as unexplained hepatic dysfunction (18.9%), moderate-to-severe transaminase elevations (22.2%), and severe hepatic failure (4.4%).

Ophthalmic involvement was observed in 41.1% of patients with optic nerve atrophy (25.6%), strabismus (17.8%), nystagmus (13.3%), ophthalmoplegia (6.7%), and cortical blindness (5.6%) being the predominant findings. Auditory impairment was identified in 4.4% of cases.

Hematological alterations were observed in 28.9% of patients, with coagulation abnormalities (22.2%) being most frequent, followed by macrocytic anemia (10.0%), thrombocytopenia (8.9%), neutropenia (6.7%), and cerebrovascular hemorrhages (3.3%).

Cardiac abnormalities were observed in 21 patients (23.3%), including conduction disturbances (16.7%) and hypertrophic and dilated cardiomyopathies (each 2.2%). ECG anomalies (18.8%) included premature ventricular contractions, ventricular hypertrophy, and atrial dilation.

Renal involvement (10%) comprised acute kidney failure (3.3%), glomerulopathy (2.2%), tubulointerstitial nephritis (2.2%), nephrotic syndrome (2.2%), and tubular dysfunction (1.1%).

Dysmorphic features were observed in 44.4% of patients, with the most common traits being prominent ears and earlobes (12.2%), transverse palmar creases (7.8%), almond-shaped eyes (8.9%), hypotelorism (7.8%), hypertelorism (5.6%), thin upper lip (7.8%), and frontal bossing (5.6%).

Significant differences in multisystemic manifestations were observed between groups. Ocular involvement (optic atrophy, nystagmus) was more frequent in the mitochondrial-afflicted group (65.6% vs. 27.6%; χ^2 = 12.32, p<0.001), as was cardiovascular involvement (heart failure, cardiomyopathies) at 37.5% vs. 15.5% (χ^2 = 5.57, p=0.018). Conversely, endocrine and growth disturbances were more common in the undiagnosed group (81.0% vs. 59.4%; χ^2 = 4.94, p=0.026).

Notable differences in NMDS scores were also observed between the mitochondrial-afflicted and undiagnosed groups (χ^2 = 9.69, p=0.008). In the mitochondrial involvement group, 68.8% had probable MD scores (6-7), and 28.1% had definite MD (8). Conversely, in the undiagnosed group, 93.1% had probable MD, with only 6.9% reaching definite MD scores.

Metabolic and instrumental findings

Metabolic analyses showed elevated blood lactate levels (mean 3.16±1.6 mmol/L) in 96.6% (87 patients), a finding

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likely attributable to the inclusion of patients with high NMDS scores. ANOVA indicated significant lactate differences across NMDS groups ($F(2,87) = 3.91$, $p=0.024$), with the highest mean lactate levels observed in the high NMDS score group (4.23±2.59 mmol/L). Plasma alanine was elevated in 15.6% of cases, abnormal acylcarnitine profiles in 13.3%, and increased urinary tricarboxylic acid cycle intermediates in 7.8%.

Neuroimaging data were available for 66 patients (73.3%), with abnormalities detected in 66.6%. These findings were significantly more prevalent in the mitochondrial involvement group (81.3% vs. 31.0%; $\chi^2 = 21.30$, $p<0.01$). Cerebral and cerebellar atrophy (46.8% vs. 13.8%; $\chi^2 = 11.86$, $p<0.01$), basal ganglia pathology (18.8% vs. 3.4%; $\chi^2 = 5.96$, $p=0.015$), and white matter abnormalities (21.9% vs. 6.9%; $\chi^2 = 4.31$, $p=0.038$) were notably more frequent in the mitochondrial-afflicted cohort. Furthermore, bilateral basal ganglia lesions characteristic of Leigh syndrome were exclusively observed in the mitochondrial involvement group (3/32; $\chi^2 = 5.62$, $p=0.018$).

Electromyography abnormalities were detected in 15.6% (14/90), with the most common finding being reduced motor nerve conduction in the lower limbs. Electroencephalography abnormalities were significantly more frequent in the mitochondrial involvement group (59.4% vs. 32.8%; $\chi^2 = 8.53$, $p=0.014$), with epileptiform discharges in 25% versus 10.3% of the undiagnosed cohort.

To integrate these multi-systemic and instrumental observations into a predictive framework, a binary logistic regression was performed. The model identified elevated plasma alanine (OR: 9.83, 95% CI: 1.89–51.14, $p=0.007$), loss of acquired skills (OR: 7.45, 95% CI: 1.45–38.21, $p=0.016$), neuroimaging abnormalities (OR: 6.35, 95% CI: 1.98–20.37, $p=0.003$), and cardiovascular manifestations (OR: 3.92, 95% CI: 1.12–13.63, $p=0.032$) as significant predictors of mitochondrial diagnosis. The model explained 49.2% of the variance in diagnosis and correctly classified 77.25% of cases.

Molecular results

Molecular investigations followed a stepwise approach. All 90 patients underwent qPCR-HRM screening for 8 pathogenic mutations, identifying 8 positive cases (8.9%). Subsequent targeted mtDNA sequencing in 82 patients revealed 24 additional variants (29.3%), yielding a total of 32 cases with confirmed mitochondrial involvement (35.6%). The genetic variants identified, in conjunction with the corresponding phenotypic manifestations, are comprehensively presented in **Table 1**.

Among the identified variants, the most frequently affected genes encoded Complex I subunits (MT-ND; 25.0%), followed by mitochondrial RNA genes (21.9%), Complex V subunits (MT-ATP; 15.6%), and Complex III (MT-CYB) and Complex IV (MT-CO2), each at 3.1%. Notably, 31.3% exhibited alterations across multiple ETC complexes, although no significant association with clinical severity was detected ($\chi^2 = 3.44$, $p=0.064$). The distribution of these variants, stratified by gene involvement and detection methodology, is comprehensively illustrated in **Fig. 1**.

Pathogenic or likely pathogenic mutations were confirmed in 19 patients (59.4%), while phenotype-associated variants of uncertain significance (VUS) were detected in 13 (40.6%). Most VUS localized to Complex I (34.1%), followed by Complex V (29.2%) and Complex IV (17.0%). Notably, Complex V variants significantly correlated with higher NMDS scores ($\chi^2 = 4.54$, $p=0.033$), highlighting their potential role in modulating disease severity.

Discussion

This study offers a detailed characterization of the clinical and genetic landscape of MDs in a Moldovan cohort. The clinical manifestations observed in our cohort, particularly the high incidence of neurological and muscular impairments, are consistent with the known pathophysiology of MDs.[10,11] The high frequency of developmental delay, hypotonia, and seizures reflects mitochondrial dependence of high-energy tissues. Importantly, 31.1% of patients exhibited treatment-resistant epilepsy, emphasizing the severe, often refractory neurological impact and the essential role of mitochondrial integrity in neurological function.[12] These observations concur with prior reports documenting similar neurological involvement patterns in MD.[13,14]

Multisystemic involvement extended beyond the central nervous system, manifesting as endocrine/growth abnormalities, ophthalmic impairments, gastrointestinal dysfunction, and cardiac pathology, underscoring the broad systemic impact of mitochondrial dysfunction.

Significant differences in NMDS scores between the mitochondrial involvement and undiagnosed groups underscore its utility in stratifying candidates for genetic testing, with higher scores correlating strongly with confirmed MD diagnoses, thereby reinforcing the value of standardized diagnostic scoring systems. Molecular analysis validated qPCR-HRM as an efficient, rapid screening method for common pathogenic mtDNA mutations, predominantly affecting complex I and tRNA genes, consistent with previous reports.[15,16]

A key finding relates to variants affecting mitochondrial complex V subunits, which showed a significant association with higher NMDS, indicating a strong link between impaired ATP synthesis and increased clinical severity. This underscores the central role of energy metabolism in disease burden and reinforces complex V dysfunction as a key diagnostic and prognostic marker in mitochondrial pathology.

Pathogenic mtDNA mutations identified, such as m.3243A>G (MT-TL1), m.8993T>G (MT-ATP6), m.3460G>A (MT-ND1), and m.11778G>A (MT-ND4), are well-established variants linked to classic mitochondrial disorders, including MELAS, NARP, Leigh syndrome, and LHON. In contrast, the numerous VUS identified across

Table 1. Integrated clinical and genetic profiling of patients with mitochondrial DNA variants

Case ID	Sex / Onset Age	Clinical phenotype	Identified variants, Heteroplasmy and Genetic classification (ACMG, ClinVar)
P1	M / 7 months	EI, hypotonia, brainstem involvement, tonic-clonic seizures, AFH	*MT-TL1* – m.3243A>G, Hetero (blood 56.1%); P (MELAS, LS, MIDD)
P2	F / 3 months	Hypotonia, DD, optic nerve atrophy, loss of skills, feeding issues, tonic-clonic seizures	*MT-ATP6* – m.8993T>G, Hetero (blood 83.2%); P (NARP, LS)
P3	M / 15 years	Painless central vision loss, color vision deficit, EI, optic nerve atrophy, migraine, AFH	*MT-ND1* – m.3460G>A, Hetero (blood 96%); P (LHON)
P4	M / 14 years	Visual impairment, central scotoma, optic nerve atrophy, EI, migraine, nystagmus, AFH	*MT-ND4* – m.11778G>A, Homo; P (LHON)
P5	M / 6 months	DD, hypotonia, EI, PN, dysarthria, tonic-clonic seizures, nystagmus, cerebellar ataxia	*MT-ATP6* – m.8993T>G, Hetero (blood 91.8%); P (NARP, LS)
P6	M / At birth	Facies myopathica, tonic-clonic seizures, DD, EI, spasticity, optic atrophy, RVH, PN, strabismus, renal issues, intellectual disability	*MT-TL1* – m.3243A>G, Hetero (blood 62.6%); P (MELAS, LS, MIDD)
P7	F / 20 years	Bilateral sequential vision loss, migraine, optic nerve atrophy, nystagmus, AFH	*MT-ND1* – m.3460G>A, Hetero (blood 92.0%); P (LHON)
P8	F / 2 years	Ophthalmoplegia, stroke-like episodes, EI, DD, rhabdomyolysis, loss of skills, dysarthria, ataxia, tonic-clonic seizures, strabismus, RVH	*MT-TL1* – m.3243A>G, Hetero (blood 70.3%); P (MELAS, LS, MIDD)
P9	F / 1 month	Hypotonia, EI, neurodevelopmental delay, spastic cerebral palsy, startle-type seizures, myoclonus, growth impairment, SNHL	*MT-ND4* – m.12018C>G, Homo; VUS (Hypotonia, LS, Epilepsy, SNHL, MC) *MT-TH* – m.12201T>C, Hetero; LP (SNHL)
P10	M / At birth	Impaired psychomotor development, hypertonia, PN, spasticity, tonic-clonic seizures, optic nerve atrophy, nystagmus, growth impairment, LVH, PN	*MT-ATP6* – m.8612T>C, Homo; VUS (LS) *MT-ND3* – m.10237T>C, Homo; VUS (LHON) *MT-ND6* – m.14466T>C, Homo; VUS (LS)
P11	F / At birth	EI, hypotonia, DD, tonic-clonic seizures, repetitive episodes of vomiting, feeding difficulties, growth impairment, conduction defects, dysmorphic features	*MT-CO2* – m.8241T>G, Homo; VUS (MIDD) *MT-ATP8* – m.8514C>T, Homo; VUS (LS) *MT-ND4* – m.10932C>T, Homo; VUS (LS)
P12	F / 3 years	Ophthalmoplegia, DD, nystagmus, tonic-clonic seizures, spasticity, optic atrophy, strabismus	*MT-TN* – m.5667G>A, Homo; P (External ophthalmoplegia)
P13	M / 1 month	Hypotonia, EI, DD, DCM, RVH, hematological alterations, feeding difficulties	*MT-CYB* – m.14849T>C, Homo; VUS (EI, MC, LS, Septo-optic dysplasia)
P14	F / 2 years	Ophthalmoplegia, hypotonia, DD, PN, brainstem involvement, nystagmus, AFH	*MT-ND1* – m.4180A>G, Homo; VUS (LS) *MT-ND4L* – m.10663T>C, Hetero; LP (LHON)
P15	M / At birth	Severe hypotonia, DD, polymorphic seizures, optic nerve atrophy, hyperkinesis	*MT-ND3* – m.10254G>A, Homo; LP (LS)
P16	M / At birth	EI, DD, pyramidal signs, myoclonus, recurrent vomiting, optic nerve atrophy, strabismus, nystagmus, intellectual disability	*MT-CO2* – m.8202T>C, Homo; VUS (LS) *MT-CO2* – m.8252C>T, Hetero; VUS (LS) *MT-CO3* – m.9247G>A, Homo; VUS (LS)
P17	M / 1 month	Ophthalmoplegia, hypotonia, brainstem involvement, cerebellar ataxia, DD, flaccid cerebral palsy, febrile seizures, nystagmus, PN.	*MT-ATP6* – m.8873G>C, Homo; VUS (LS) *MT-CYB* – m.14792C>T, Homo; VUS (LS) *MT-CYB* – m.15117T>C, Hetero; VUS (LS)
P18	F / 6 months	Dystonia, PN, EI, dysarthria, tonic-clonic seizures, spasticity, optic nerve atrophy, cortical blindness, DD, nystagmus, AFH	*MT-ATP8* – m.8558C>T, Homo; VUS (MC) *MT-ATP6* – m.8785C>T, Homo; VUS (LS) *MT-ATP6* – m.8873G>C, Homo; VUS (LS)
P19	F / 4 months	DD, facies myopathica, severe hypotonia, dystonia, dysarthria, reactive pancreatitis, tonic-clonic seizures, PN, cerebellar ataxia	*MT-CO2* – m.7698T>C, Homo; VUS (LS) *MT-ATP6* – m.8615T>C, Homo; VUS (LS) *MT-ND5* – m.12545C>T, Homo; VUS (LS)

Case ID	Sex / Onset Age	Clinical phenotype	Identified variants, Heteroplasmy and Genetic classification (ACMG, ClinVar)
P20	F / 6 months	EI, hypotonia, DD, stroke-like episodes, polymorphic seizures, strabismus, dyskinetic cerebral palsy, dystonia, optic nerve atrophy	*MT-ND1* – m.4171C>A, Homo; VUS (LHON, LS); m.4193 T>C, Homo; VUS (LS) *MT-CO2* – m.7698T>C, Homo; VUS (LS)
P21	F / 1 month	Rhabdomyolysis, hypotonia, EI, tonic-clonic seizures, DD, dysphagia, recurrent vomiting.	*MT-ND3* – m.10197G>A, Homo; LP (LS)
P22	F / 3 months	Hypotonia, global developmental delay, tonic-clonic seizures, spastic cerebral palsy, DCM, strabismus, feeding difficulties.	*MT-ATP6* – m.9125C>T, Homo; VUS (LS) *MT-ND6* – m.14423G>A, Hetero; VUS (LS) *MT-CYB* – m.15132T>C, Homo; VUS (MC)
P23	F / 4 months	Hypotonia, DD, HCM, polymorphic seizures, recurrent vomiting, optic atrophy, strabismus	*MT-ATP8* – m.8528T>C, Hetero; LP (HCM) *MT-ATP6* – m.9194A>G, Homo; VUS (LS)
P24	M / 1.5 years	EI, hypotonia, loss of skills, SNHL, dysarthria, recurrent vomiting, diarrhea, RVH, ataxia	*MT-TL1* – m.3264T>C, Homo; VUS (MIDD)
P25	M / 1 month	Hypertonia, neurodevelopmental delay, spastic cerebral palsy, RVH, tonic-clonic seizures, cortical blindness, optic nerve atrophy	*MT-ND1* – m.4184T>C, Homo; LP (LHON) *MT-ND4* – m.11360A>G, Homo; VUS (LS, LHON, seizures)
P26	F / At birth	Facies myopathica, DD, RVH, myoclonus, tonic-clonic seizures, optic atrophy	*MT-ATP8* – m.8536A>T, Homo; LP (Sporadic myoclonus epilepsy)
P27	M / 10 months	EI, severe hypotonia, global developmental delay, loss of skills, dystonia, subclinical hypothyroidism, stereotypies, AFH	*MT-TK* – m.8328G>A, Homo; VUS (MM, EI) *MT-ATP8* – m.8373A>T, Hetero; VUS (LS) *MT-ATP8* – m.8418T>C, Homo; VUS (ON)
P28	F / 11 months	EI, hypotonia, ataxia, dysarthria, papilloretinal coloboma, SNHL, DD, ataxia	*MT-RNR1* – m.1494C>T, Homo; LP (SNHL)
P29	M / 7 years	Loss of skills, DD, ataxia, PN, EI, hypotonia, rhabdomyolysis, dysphagia, dysarthria	*MT-ND1* – m.4142G>A, Homo; LP (LS)
P30	M / 9 months	Severe hypotonia, EI, tonic-clonic seizures, dysarthria, RVH, DD, dysmorphic features, intellectual disability, cerebellar ataxia, AFH	*MT-ND2* – m.4953A>G, Homo; VUS (LS) *MT-ND4* – m.10931T>C, Homo; VUS (LS) *MT-TE* – m.14687A>G, Homo; VUS (MM)
P31	M / 3 months	Hypotonia, DD, polymorphic seizures, cortical blindness, optic nerve atrophy, nystagmus	*MT-ND2* – m.5038T>C, Homo; VUS (LS) *MT-ND6* – m.14482C>G, Homo; LP (LHON)
P32	F / 3 months	Tonic-clonic seizures, DD, EI, hypotonia, dysphagia, feeding difficulties, growth impairment, intellectual disability, PN	*MT-CO2* – m.7746A>G, Homo; VUS (LS) *MT-ATP6* – m.9125C>T, Homo; VUS (LS) *MT-ND5* – m.12479T>C, Homo; VUS (LS)

ACMG: American College of Medical Genetics and Genomics; AFH: aggravated family history; DCM: dilated cardiomyopathy; DD: developmental delay; EI: exercise intolerance; F: female; HCM: hypertrophic cardiomyopathy; Hetero: heteroplasmic; Homo: homoplasmic; LHON: Leber's hereditary optic neuropathy; LP: likely pathogenic variant; LS: Leigh syndrome; LVH: left ventricular hypertrophy; M: male; MC: mitochondrial cardiomyopathy; MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MIDD: maternally inherited diabetes and deafness; MM: mitochondrial myopathy; NARP: neuropathy, ataxia, and retinitis pigmentosa; ON: optic neuropathy; P: pathogenic variant; PN: peripheral neuropathy; RVH: right ventricular hypertrophy; SNHL: sensorineural hearing loss; VUS: variant of uncertain significance

genes such as MT-ND5, MT-CO2, and MT-ATP6 underscore the inherent complexity of mitochondrial genomics and highlight the existing gaps in genotype-phenotype correlations. While these variants do not yet meet the criteria for definitive pathogenicity, the recurrent presence of these VUS in patients with overlapping clinical phenotypes suggests they may act as early markers of disease susceptibility or modifiers that influence severity and progression – for instance, m.12479 T>C (MT-ND5) and m.14466 T>C (MT-ND6).

The clinical significance of these VUS is complicated by variable phenotypic expression influenced by tissue-specific heteroplasmy, environmental factors, and nuclear gene interactions. Accurately clarifying the pathogenicity of these recurrent VUS remains a key priority, requiring future integration of transcriptomics and functional validation studies to bridge the gap between genomic discovery and practical clinical application in diagnostic routines.

Despite our study's strengths, certain limitations must be acknowledged. First, muscle biopsy and enzymatic assays were not performed, which we acknowledge as a significant limitation for the functional validation of the

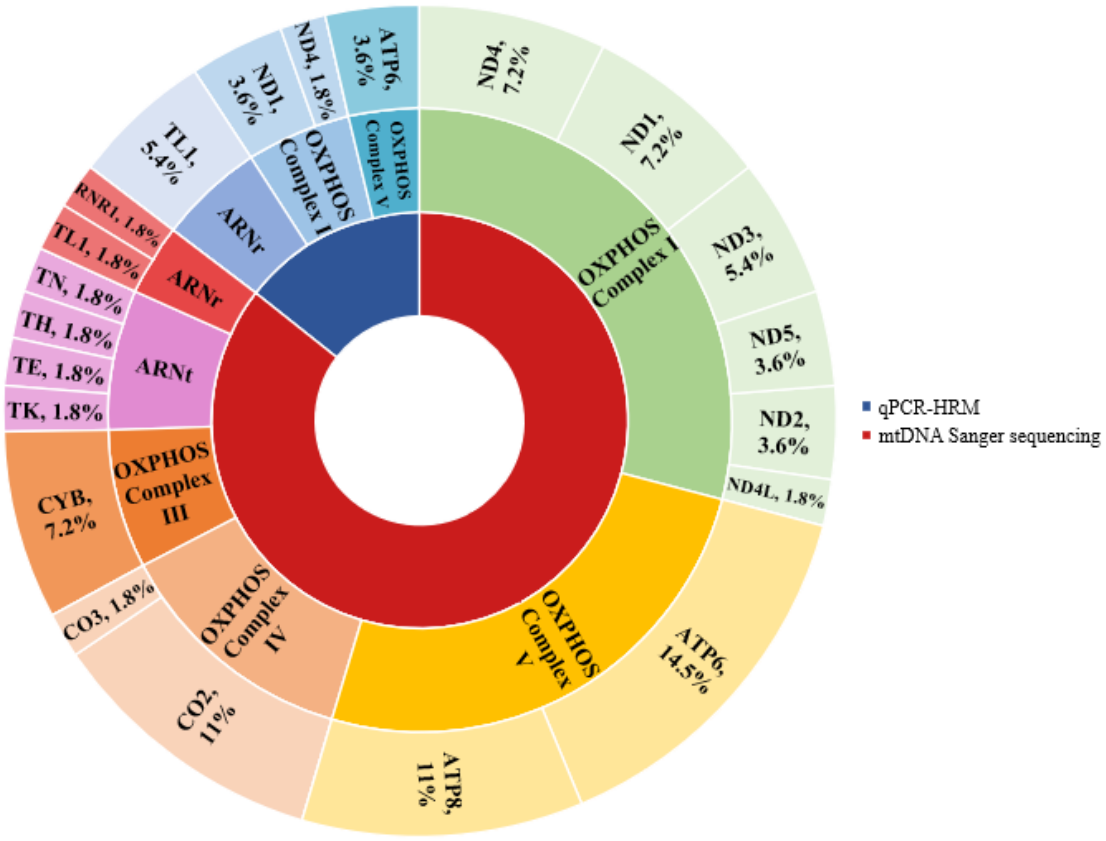

Figure 1. Distribution of identified mitochondrial variants by gene locus and detection method (qPCR-HRM vs. mtDNA Sanger sequencing).

identified genetic variants. While these remain important for confirming mitochondrial dysfunction, in the current era of advanced molecular diagnostics, a "genomics-first" approach is increasingly adopted to avoid invasive procedures, particularly in pediatric cohorts.[17,18] Second, other limitations include the inability to determine heteroplasmy levels in sequenced patients and the reliance on blood samples, which may fail to capture the significant tissue-specific variability inherent to mitochondrial disorders. Moving beyond these technical constraints, the study was also characterized by a limited sample size, which may affect generalizability, and a focus solely on mtDNA mutations. Although preliminary nuclear genomic screenings are ongoing for select cases, this study was intentionally designed to focus on mtDNA variants, leaving nuclear-mitochondrial interactions for a subsequent analysis.

Furthermore, the high proportion of undiagnosed cases (64.5%) reflects diagnostic challenges due to genetic complexity and undetected nuclear mutations. This is further compounded by the fact that in the Republic of Moldova, next-generation sequencing investigations are not yet covered by the public health system, representing a significant financial barrier for families. However, advanced methods, such as whole-genome or nuclear exome sequencing, coupled with functional studies, could further enhance diagnostic accuracy.[19,20]

Conclusion

This study is the first comprehensive analysis of mitochondrial diseases in the Moldovan population, revealing notable clinical and genetic heterogeneity. Integrating detailed phenotyping, standardized diagnostics, and advanced genetic testing, we identified variants correlating with phenotypes in 35.6% of cases. Pathogenic mutations were mainly linked to complex I deficiencies, consistent with their known role in mitochondrial pathology, while complex V variants correlated with more severe phenotypes and higher Nijmegen Mitochondrial Disease Score, emphasizing ATP synthase dysfunction's impact. The high prevalence of variants of uncertain significance highlights challenges in interpreting mitochondrial genetics and the need for further functional studies. These results underscore the value of a multidisciplinary approach combining thorough clinical and genetic assessment to improve diagnostic accuracy, genetic counseling, and enhanced patient outcomes.

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References

1. Gorman GS, Chinnery PF, DiMauro S, et al. Mitochondrial diseases. Nat Rev Dis Primers 2016;2:16080.
2. Gorman GS, Schaefer AM, Ng Y, et al. Prevalence of nuclear and mitochondrial DNA mutations related to adult mitochondrial disease. Ann Neurol 2015;77(5):753–9.
3. Klopstock T, Priglinger C, Yilmaz A, et al. Mitochondrial disorders. Dtsch Arztebl Int 2021;118(44):741–8.
4. Zong Y, Li H, Liao P, et al. Mitochondrial dysfunction: mechanisms and advances in therapy. Signal Transduct Target Ther 2024;9(1):124.
5. Saneto RP, Sedensky MM. Mitochondrial disease in childhood: mtDNA encoded. Neurotherapeutics 2013;10(2):199–211.
6. Chujo T, Tomizawa K. Mitochondrial tRNA modifications: functions, diseases caused by their loss, and treatment strategies. RNA 2025;31(3):382–94.
7. Fernandez-Vizarra E, Zeviani M. Mitochondrial disorders of the OXPHOS system. FEBS Lett 2021;595(8):1062–106.
8. Morava E, Van Den Heuvel L, Hol F, et al. Mitochondrial disease criteria: Diagnostic applications in children. Neurology 2006;67(10): 1823–6.
9. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17(5):405–24.
10. Nascimento A, Ortez C, Jou C, et al. Neuromuscular manifestations in mitochondrial diseases in children. Semin Pediatr Neurol 2016;23(4):290–305.
11. Kuusik B, Mithal DS. Updates on neurologic manifestations of mitochondrial disease. Curr Opin Pediatr 2025;37(1):107–11.
12. Finsterer J, De Carvalho EHT . Cerebral manifestations of mitochondrial disorders. Can J Neurol Sci 2017;44(6):654–63.
13. Falk MJ. Neurodevelopmental manifestations of mitochondrial disease. J Dev Behav Pediatr 2010;31(7):610–21.
14. Lax NZ, Gorman GS, Turnbull DM. Review: Central nervous system involvement in mitochondrial disease. Neuropathol Appl Neurobiol 2017;43(2):102–18.
15. Richter U, McFarland R, Taylor RW, et al. The molecular pathology of pathogenic mitochondrial tRNA variants. FEBS Lett 2021;595(8):1003–24.
16. Ryzhkova A, Sazonova M, Sinyov V, et al. Mitochondrial diseases caused by mtDNA mutations: a mini-review. Ther Clin Risk Manag 2018;14:1933–42.
17. Witters P, Saada A, Honzik T, et al. Revisiting mitochondrial diagnostic criteria in the new era of genomics. Genet Med 2018;20(11): 1478–81.
18. Hinojosa JC, Bhai S. Diagnostic testing in suspected primary mitochondrial myopathy. Muscles 2023;2(1):75–85.
19. Theunissen TEJ, Nguyen M, Kamps R, et al. Whole exome sequencing is the preferred strategy to identify the genetic defect in patients with a probable or possible mitochondrial cause. Front Genet 2018;9:400.
20. Davis RL, Kumar KR, Puttick C, et al. Use of whole-genome sequencing for mitochondrial disease diagnosis. Neurology 2022;99(7): 730–42.

Additional information

Ethical statements

The study was approved by the Research Ethics Committee of the "Nicolae Testemițanu" State University of Medicine and Pharmacy (Decision No. 03 of September 9, 2020).

- The authors declared that no clinical trials were used in the present study.
- The authors declared that no experiments on humans or human tissues were performed for the present study.
- The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.
- The authors declared that no experiments on animals were performed for the present study.
- The authors declared that no commercially available immortalized human and animal cell lines were used in the present study.

Conflict of interest

The authors have declared that no competing interests exist.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI. No AI tools were used in the preparation of this manuscript.

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Author contributions

DS, UN, and SV contributed to the conception and design of the study. DS, BD, UN, and SV were involved in data collection. DS and BD performed the data analysis and processing. DS, AN, and CD conducted the analyses described in the manuscript and played a key role in interpreting the

results and drafting the article. All authors critically revised the manuscript for important intellectual content and approved the final version.

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Data availability

All of the data that support the findings of this study are available in the main text.