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CIÊNCIAS MÉDICAS

# A Personalized Medicine Approach for Mitochondrial Respiratory Chain Disorders in Childhood in The New Era of Genome Sequence

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D

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# A PERSONALIZED MEDICINE APPROACH FOR MITOCHONDRIAL RESPIRATORY CHAIN DISORDERS IN CHILDHOOD IN THE NEW ERA OF GENOME SEQUENCE

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# Author statement

I, Maria Margarida Paiva Coelho, hereby declare that this thesis, titled "A Personalized Medicine Approach for Mitochondrial Respiratory Chain Disorders in Childhood in the New Era of Genome Sequencing," is my original work and has been conducted independently, except where otherwise acknowledged.

This dissertation is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) at Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto (ICBAS-UP). It comprises a cumulative body of research, incorporating the following peer-reviewed manuscripts:

1. Paiva Coelho, M., Martins, E., Vilarinho, L. "**Diagnosis, Management, and Follow-up of Mitochondrial Disorders in Childhood: A Personalized Medicine in the New Era of Genome Sequence.**" European Journal of Pediatrics 178, no. 1 (January 2019): 21–32. https://doi.org/10.1007/s00431-018-3292-x.
2. Paiva Coelho, M., Correia, J., Dias, A., Nogueira, C., Bandeira, A., Martins, E., Vilarinho, L. "**Iron-sulfur Cluster ISD11 Deficiency (LYRM4 Gene) Presenting as Cardiorespiratory Arrest and 3-Methylglutaconic Aciduria**." JIMD Reports 49, no. 1 (September 2019): 11–16. https://doi.org/10.1002/jmd2.12058.
3. Pelayo, G., Paiva Coelho, M., Correia, J., Bandeira, A., Nogueira, C., Vilarinho, L., Martins, E. "**Phenotyping Mitochondrial Glutamyl-tRNA Synthetase Deficiency (*EARS2*): A Case Series and Systematic Literature Review.**" Neurobiology of Disease 200 (October 2024): 106644. https://doi.org/10.1016/j.nbd.2024.106644.
4. Paiva Coelho, M., Costa, T., Dias, A., Graça, I.C.R., Rocha, H., Vilarinho, L., Martins, E., Rodrigues, J.E., Gil, A.M. "**NMR-based Urinary Biomarkers in Pediatric Primary Mitochondrial Disorders and Chronic Kidney Disease: Shared Mitochondrial Dysfunction, Diverging Biosignatures.**" Metabolomics (*in press*) PREPRINT available at Research Square. https://doi.org/10.21203/rs.3.rs-6758787/v3

I am the first author of all manuscripts included in this thesis. In the third manuscript, I share first authorship with Dr. Gonçalo Pelayo, reflecting equal contributions to the study. This work originated from Dr. Pelayo's Master's Thesis in Medicine (*Mestrado Integrado em Medicina*, Instituto de Ciências Biomédicas Abel Salazar – University of Porto), entitled *"Fenotipagem da deficiência de glutamil-tRNA sintetase mitocondrial (EARS2): uma revisão sistemática e dois novos casos"*, which I supervised and was publicly defended on July 4, 2024. Specific contributions of all

co-authors and collaborators are explicitly acknowledged in the respective publications through CredIT (Contributor Roles Taxonomy) authorship statements. The full-text reproduction of each manuscript is available in Appendix, along with the corresponding authorizations and copyright permissions.

This thesis adheres to the ethical and academic integrity standards set forth by U. Porto, and all necessary ethical approvals were obtained for the research conducted herein. All references to other authors' ideas, statements, or data are duly acknowledged in the text and in the reference lists, in accordance with accepted academic citation standards.

I confirm that this work has not been previously submitted, in whole or in part, for the award of any other degree or qualification at this or any other institution.

I take full responsibility for the content of this thesis and its findings.

Porto, 15 October 2025

Maria Margarida Paiva Coelho

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# Declaração de Honra

Declaro que a presente tese é da minha autoria e que não foi utilizada previamente, no todo ou em parte, em qualquer outra unidade curricular ou curso desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, encontrando-se devidamente indicadas no texto e nas respetivas listas bibliográficas, de acordo com as normas de referenciação em vigor. Estou ciente de que a prática de plágio ou auto-plágio constitui um ilícito académico.

Porto, 15 de outubro de 2025

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# List of Abbreviations

**3-MGA-uria:** 3-methylglutaconic aciduria

**3-MGA:** 3-methylglutaconic acid

**AA:** Amino Acids

**aaRS:** Aminoacyl-tRNA synthetases

**ACMG:** American College of Medical Genetics and Genomics

**AD:** Autosomal Dominant

**ADHD:** Attention Deficit Hyperactivity Disorder

**ADP:** Adenosine Diphosphate

**AI:** Artificial intelligence

**AR:** Autosomal Recessive

**ATP:** Adenosine Triphosphate

**ATPase:** Adenosine Triphosphatase

**BBGD:** Biotin-Responsive Basal Ganglia Disease

**BCKDH:** Branched-Chain α-Ketoacid Dehydrogenase Complex

**BN-PAGE:** Blue Native Polyacrylamide Gel Electrophoresis

**CK:** Creatine Kinase

**CKD:** Chronic Kidney Disease

**CNS:** Central Nervous System

**CNV-seq:** Copy Number Variant Sequencing (se usado no método genético)

**CNV:** Copy Number Variant

**CoQ10:** Coenzyme Q10

**COX:** Cytochrome c Oxidase

**CS:** Citrate Synthase

**CSF:** Cerebrospinal Fluid

**DMA:** Dimethylamine

**DMD:** Duchenne Muscular Dystrophy

**DNA:** Deoxyribonucleic **Acid**

**dNTPs:** Deoxyribonucleotide Triphosphates

***EARS2*:** Glutamyl-tRNA Synthetase Gene

**EEG:** Electroencephalography

**ETC:** Electron Transport Chain

**F:** Female

**FAD:** Flavin Adenine Dinucleotide

**FADH2:** Flavin Adenine Dinucleotide (reduced form)

**Fe–S cluster:** Iron-Sulfur cluster

**FGF-21:** Fibroblast Growth Factor 21

**FLAIR:** Fluid Attenuated Inversion Recovery

**FTT:** Failure to Thrive

**GDF-15:** Growth and Differentiation Factor 15

**HSCT:** hematopoietic stem cell transplantation

**ICBAS:** Instituto de Ciências Biomédicas Abel Salazar

**ICU:** Intensive Care Unit

**iPSCs:** Induced Pluripotent Stem Cells

**KD:** ketogenic diet

**KSS:** Kearns-Sayre Syndrome

**LBSL:** Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Lactic Acidosis

**LC-MS:** Liquid Chromatography–Mass Spectrometry

**LDH:** Lactate Dehydrogenase

**LHON:** Leber Hereditary Optic Neuropathy

**LP:** Likely Pathogenic

**LS:** Leigh Syndrome

**LSS:** Leigh Syndrome Spectrum

**LTBL:** Leukoencephalopathy with Thalamus and Brainstem Involvement with High Lactate

**M:** Male

**m:** Months

**MAM:** mitochondrial-associated membrane

**MAT:** Mitochondrial Augmentation Therapy

**MAVS:** Mitochondrial Antiviral Signalling Protein

**MD:** Mitochondrial Disorder

**MDC:** Mitochondrial Disease Criteria

**MDS:** mtDNA Depletion Syndromes

**MELAS:** Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like Episodes

**MERRF:** Myoclonic Epilepsy with Ragged Red Fibers

**MILS:** Maternally Inherited Leigh Syndrome

**ML:** Machine Learning

**MMR:** Mismatch Repair

**MRI:** Magnetic Resonance Imaging

**MRS:** Magnetic Resonance Spectroscopy

**mtARS:** Mitochondrial Aminoacyl-tRNA Synthetases

**mtDNA:** Mitochondrial DNA

**mtRNA:** Mitochondrial RNA

**$NAD^+$/NADH:** Nicotinamide Adenine Dinucleotide (oxidized/reduced form)

**NARP:** Neurogenic Muscle Weakness, Ataxia, and Retinitis Pigmentosa

**nDNA:** Nuclear DNA

**NDUFV2:** NADH-Ubiquinone Oxidoreductase Flavoprotein 2

**NER:** Nucleotide Excision Repair

**NF-L:** Neurofilament Light Chain

**NGS:** Next-Generation Sequencing

**NMR:** Nuclear Magnetic Resonance (spectroscopy)

**NR:** Normal Range

**NSHL:** Neurosensory Hearing Loss

**OCR:** Oxygen Consumption Rate

**OR:** odds ratio

**OXPHOS:** Oxidative Phosphorylation

**P:** Pathogenic

**PCR:** Polymerase Chain Reaction

**PDHc:** Pyruvate Dehydrogenase Complex

**PEO:** Progressive External Ophthalmoplegia

**PMD:** Primary Mitochondrial Disorder

**POLG:** DNA Polymerase Gamma

**PQN:** Probabilistic Quotient Normalization

**QC:** Quality Control

**RC:** Respiratory Chain

**RCT:** randomized clinical trial

**RNA:** Ribonucleic Acid

**ROS:** Reactive Oxygen Species

**RRF:** Ragged-Red Fibers

**SANDO:** Sensory Ataxic Neuropathy, Dysarthria, and Ophthalmoparesis

**SD:** Standard Deviation

**SDH:** Succinate Dehydrogenase

**SMA:** Spinal Muscular Atrophy

**SMD:** Secondary Mitochondrial Dysfunction

**SusMD:** Suspected Mitochondrial Disorder

**tRNA:** Transfer RNA

**TSA:** Total Spectral Area (usado na normalização NMR)

**uOA:** Urinary Organic Acids

**VUS:** Variant of Uncertain Significance

**WES:** Whole Exome Sequencing

**WGS:** Whole Genome Sequencing

**Y:** Years

# Abstract

**Background:** Paediatric mitochondrial disorders are characterized by marked clinical and genetic heterogeneity with frequent phenotypic overlap with other genetic syndromes. Despite advances in genomic sequencing, many children remain without a molecular diagnosis. Validated biomarkers for diagnosis, prognosis, and follow-up are still lacking.

**Objectives:** This thesis aimed to (*i*) characterize the diagnostic trajectory and clinical spectrum of children with suspected mitochondrial disease in a single centre longitudinal cohort, (*ii*) contribute detailed genotype–phenotype correlations in ultra-rare mitochondrial disorders, and (*iii*) assess untargeted urinary $^{1}$H NMR metabolomics for exploratory biomarker discovery**.**

**Patients and Methods:** A single-centre cohort study included 178 children with suspected PMD who underwent targeted NGS panels, whole-exome sequencing (WES), and/or mtDNA analysis. A subset of patients was included in an untargeted urinary $^{1}$H NMR metabolomics study.

**Results:** A genetic diagnosis was established in 37% of the cohort (38 PMD; 28 non-PMD), while two were diagnosed with acquired causes and 110 remained undiagnosed. A novel phenotype of ISD11 (*LYRM4*) deficiency was delineated, confirming pathogenicity and expanding its clinical spectrum. The *EARS2*-related disease spectrum was refined through combined cohort analysis and literature synthesis, highlighting atypical presentations beyond classical disease. Untargeted urinary $^{1}$H-NMR metabolomics identified biosignatures distinguishing PMD from disease- and healthy-controls, independent from renal impairment.

**Discussion:** This work contributes to refine the diagnostic approach to paediatric mitochondrial disease by demonstrating the value of longitudinal phenotyping, systematic genetic reanalysis, and multidisciplinary evaluation in improving diagnostic yield and clarifying genetic mimics. It expands the clinical and molecular spectrum of ultra-rare disorders such as *LYRM4* and *EARS2* deficiencies. Importantly, applying untargeted urinary $^{1}$H NMR metabolomics in a paediatric cohort, identifies an exploratory multimetabolite biosignature model with potential diagnostic utility. These findings support the integration of metabolomics into the diagnostic and research framework of mitochondrial medicine and highlight its promise as a tool for biomarker discovery with translational potential.

**Conclusion:** This thesis underscores the importance of integrating genomics, metabolomics, and longitudinal phenotyping into clinical practice, paving the way toward a personalized medicine approach for mitochondrial respiratory chain disorders in childhood in the new era of genome sequencing.

**Keywords**: Primary Mitochondrial Disorder; Mitochondrial Mimics; Next-Generation Sequencing (NGS); Whole-Exome Sequencing (WES); Urinary Metabolomics; $^{1}$H NMR Metabolomics; Biomarkers; Precision Medicine

# Resumo

**Introdução:** As doenças mitocondriais caracterizam-se pela sua heterogeneidade clínica e genética e frequente sobreposição fenotípica com outras patologias genéticas. Apesar dos avanços na sequenciação genética, muitos casos mantêm-se sem diagnóstico definitivo. A ausência de biomarcadores específicos e validados para diagnóstico, prognóstico e seguimento contribuiu para os desafios nesta área da medicina.

**Objetivos:** Esta tese teve como objetivos: (i) caracterizar a trajetória diagnóstica e o espectro clínico de doentes pediátricos com suspeita de doença mitocondrial numa coorte unicêntrica; (ii) contribuir com descrições de correlações genótipo–fenótipo em doenças mitocondriais ultra-raras; e (iii) avaliar a metabolómica urinária por $^{1}$H NMR não direcionada como ferramenta exploratória para identificação de biomarcadores.

**Material e Métodos:** Foi realizado um estudo de coorte unicêntrico, que incluiu 178 crianças com suspeita de doença mitocondrial primária. Os doentes foram submetidos a painéis NGS, sequenciação do exoma completo (WES) e/ou análise de mtDNA. Um subgrupo de doentes foi incluído num estudo exploratório de metabolómica urinária por $^{1}$H NMR não direcionada.

**Resultados:** Foi estabelecido um diagnóstico genético em 37% da coorte (38 doença mitocondrial primária; 28 não-mitocondriais), enquanto dois foram diagnosticados com causas adquiridas e 110 permaneceram sem diagnóstico. Contribui para a descrição da deficiência de LYRM4. Através da análise dos casos desta coorte e da revisão da literatura (re)delineou-se o espectro fenotípico da deficiência de EARS2. O de estudo de metabolómica urinária por $^{1}$H NMR identificou uma bio-assinatura capaz de distinguir doença mitocondrial de grupos controlo patológicos ou saudáveis, independentemente do atingimento renal.

**Discussão:** Esta tese contribui para aperfeiçoar a abordagem diagnóstica das doenças mitocondriais, demonstrando melhoria da acuidade diagnóstica através da fenotipagem longitudinal, da reanálise genética sistemática e da avaliação multidisciplinar. Para além disso traduz o impacto da elevada frequência de mimetismos mitocondriais. Salienta-se a aplicação da metabolómica urinária por $^{1}$H NMR numa coorte pediátrica, que permitiu apresentar um painel de metabolitos discriminativos. Estes dados apoiam a integração da metabolómica na abordagem diagnóstica e de investigação em medicina mitocondrial, nomeadamente na descoberta de biomarcadores, com potencial de aplicação translacional em medicina de precisão.

**Conclusão:** Este estudo reforça a importância de integrar a genómica, a metabolómica e caraterização fenotípica longitudinal na prática clínica, permitindo uma abordagem de medicina personalizada nas doenças mitocondriais com início em idade pediátrica, nesta nova era da sequenciação genómica.

**Palavras-chave:** Doença Mitocondrial Primária; Mimetizadores Mitocondriais; Next-Generation Sequencing (NGS); Whole-Exome Sequencing (WES); Metabolómica Urinária; Espectroscopia por $^{1}$H NMR; Biomarcadores; Medicina de Precisão.

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# Publications

The following publications are included this thesis:

1. Paiva Coelho, M., Martins, E., & Vilarinho, L. (2019). **Diagnosis, management, and follow-up of mitochondrial disorders in childhood: A personalized medicine in the new era of genome sequence.** *European Journal of Pediatrics*, *178*(1), 21–32. https://doi.org/10.1007/s00431-018-3292-x

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2. Paiva Coelho, M., Correia, J., Dias, A., Nogueira, C., Bandeira, A., Martins, E., & Vilarinho, L. (2019). **Iron-sulfur cluster ISD11 deficiency (*LYRM4* gene) presenting as cardiorespiratory arrest and 3-methylglutaconic aciduria**. *JIMD Reports*, *49*(1), 11–16. https://doi.org/10.1002/jmd2.12058

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1. Lopes, T., Coelho, M., Bordalo, D., Bandeira, A., Bandeira, A., Vilarinho, L., Fonseca, P., Carvalho, S., Martins, C., & Oliveira, J. G. (2018). **Síndrome de Leigh: A propósito de um caso clínico com mutação no DNA mitocondrial.** *Revista Paulista de Pediatria*, *36*(4), 519–523. https://doi.org/10.1590/1984-0462/;2018;36;4;00003

   Impact factor (JIF): 2.0, PEDIATRICS (Q2)

2. Paiva Coelho, M., Soares, A. R., Magalhães, C., & Martins, E. (2020). **Genes, Children and Pediatricians.** *NASCER E CRESCER - BIRTH AND GROWTH MEDICAL JOURNAL*, 131-133 Pages. https://doi.org/10.25753/BIRTHGROWTHMJ.V29.I2.17550

   Not indexed in ISI Web of Knowledge. Indexed in SciELO.

3. Nogueira, C., Pereira, C., Silva, L., Laranjeira, M., Lopes, A., Neiva, R., Rodrigues, E., Campos, T., Martins, E., Bandeira, A., Coelho, M., Magalhães, M., Damásio, J., Gaspar, A., Janeiro, P., Gomes, A. L., Ferreira, A. C., Jacinto, S., Vieira, J. P., … Vilarinho, L. (2024). **The genetic landscape of mitochondrial diseases in the next-generation sequencing era: A Portuguese cohort study.** *Frontiers in Cell and Developmental Biology*, *12*, 1331351. https://doi.org/10.3389/fcell.2024.1331351

   Impact factor (JIF): 4.3, DEVELOPMENTAL BIOLOGY (Q1)

4. Baldo, M. S., Azevedo, L., Coelho, M. P., Martins, E., & Vilarinho, L. (2024). **A Comprehensive Approach to the Diagnosis of Leigh Syndrome Spectrum.** *Diagnostics*, *14*(19), 2133. https://doi.org/10.3390/diagnostics14192133

   Impact factor (JIF): 3.3, MEDICINE, GENERAL & INTERNAL (Q1)

5. Neugebauer, J., Reinson, K., Bellusci, M., Park, J. H., Hikmat, O., Bertini, E., Schiff, M., MetabERN PM-MD Consortium authors[2], & Rahman, S. (2025). **Current global vitamin and cofactor prescribing practices for primary mitochondrial diseases: Results of a European reference network survey.** *Journal of Inherited Metabolic Disease*, *48*(1), e12805. https://doi.org/10.1002/jimd.12805

   Impact factor (JIF): 3.8, MEDICINE, RESEARCH & EXPERIMENTAL (Q1)

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[1]Joint first authorship.

[2]As a Consortium author

*“Science and everyday life cannot and should not be separated.”*

*- Rosalind Franklin*

# *Chapter I.* ***Introduction***

# 1. Mitochondria function and their role in health and disease

Mitochondria are dynamic, multifunctional organelles essential to the maintenance of cellular and organism homeostasis. Historically described as the "powerhouses of the cell" for their role in adenosine triphosphate (ATP) synthesis[1], mitochondria are now increasingly recognized as central signalling platforms that orchestrate a wide array of cellular processes including metabolism, apoptosis, immunity, calcium regulation, and redox balance[2,3]. They have thus been likened not only to power stations, but also to the cell's "CEO" or even "motherboard," integrating internal and external cosigns to drive adaptive responses[4,5].

These pleiotropic roles are mediated by an intricate structural organization, dual genetic control (nuclear and mitochondrial DNA), and capacity for dynamic remodelling. Disruption in mitochondrial structure or function can impair energy production, deregulate signalling cascades, and drive cellular dysfunction. Such dysfunction underlies a broad spectrum of diseases, from primary mitochondrial disorders (PMD) to cancer, neurodegeneration, and metabolic syndromes[6].

## 1.1. Mitochondrial functions

Mitochondria are a central hub in cellular metabolism, with energy production as primary function through ATP generation via oxidative phosphorylation (OXPHOS) or β-oxidation of fatty acids. Mitochondria are multifunctional organelles that contribute to numerous cellular processes essential for homeostasis, schematically illustrated in Figure 1. These include intermediate metabolism - amino acid, organic acids and nucleotide metabolism, and biosynthesis, such as iron–sulfur (Fe–S) cluster assembly. Proper organelle function is maintained through coordinated mitochondrial biogenesis - and dynamic remodelling processes such as fusion, fission, and mitophagy. In addition, mitochondria regulate redox homeostasis, calcium buffering, and apoptotic signalling, and form contact sites with the endoplasmic reticulum and nucleus to mediate metabolic integration and stress responses.

Comprehensive knowledge of mitochondrial functions is crucial for contextualising the clinical presentations and metabolic profiles observed in PMD. Disruption in any of these pathways, whether due to nuclear (nDNA) or mitochondrial DNA (mtDNA) defects, can result in multisystem disease with variable severity[7].

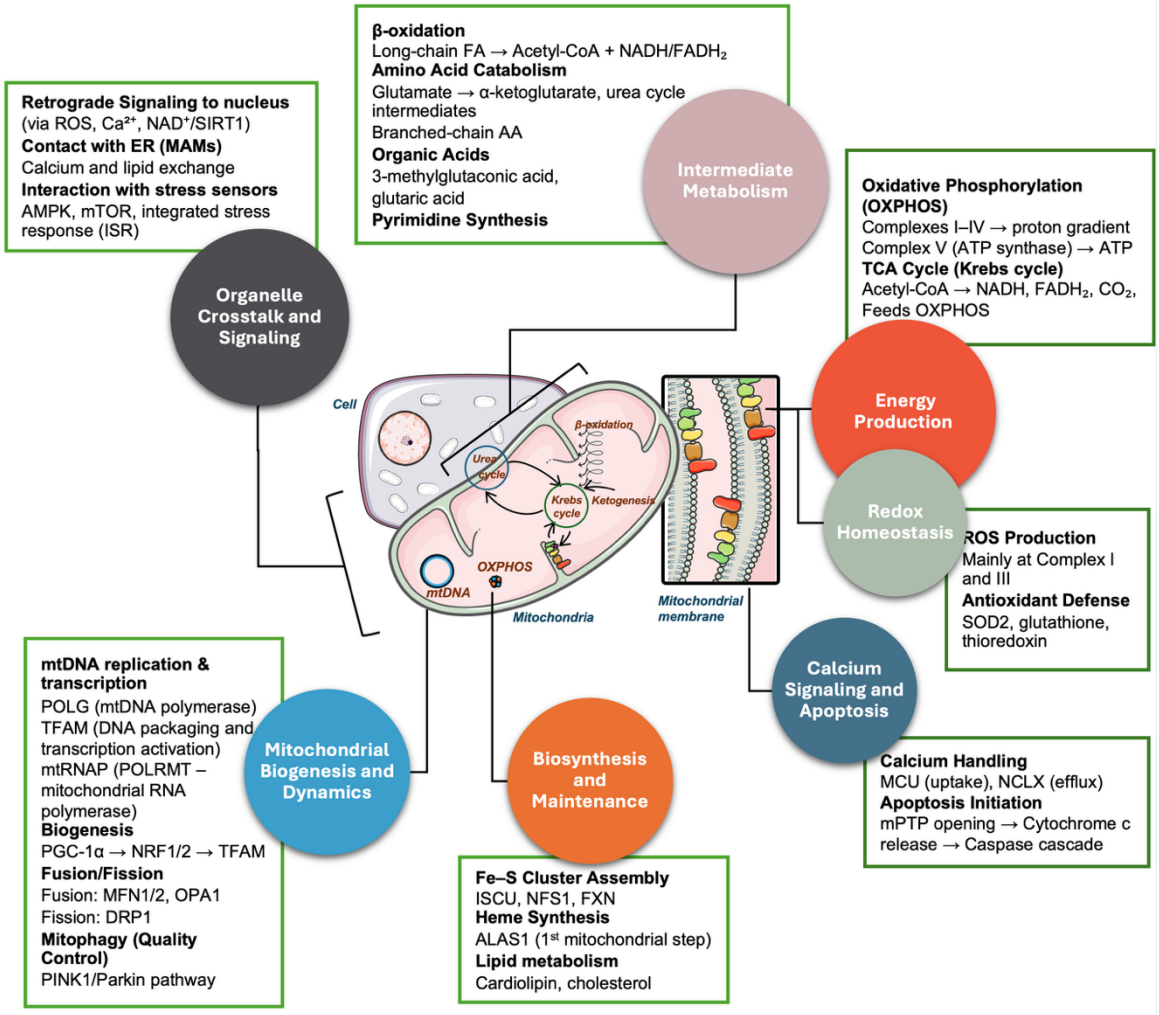


***Figure 1. Overview of mitochondrial functions and their integration into cellular homeostasis.***

### 1.1.1. Energy Production

The core bioenergetic function of mitochondria is OXPHOS, the process responsible for generating ATP, the universal cellular energy currency. In OXPHOS, electrons derived from NADH and $FADH_2$ are transferred through the electron transport chain (ETC) to drive proton translocation across the inner mitochondrial membrane, creating an electrochemical gradient that enable ATP synthesis from the rotation of ATP synthase (complex V). Coenzyme Q10 (CoQ10) and cytochrome c (Cyt c) act as mobile electron carriers, shuttling electrons between the major respiratory complexes[8].

OXPHOS is primarily fuelled by the Krebs cycle, located in the mitochondrial matrix. The cycle oxidizes acetyl-CoA, derived from glycolysis, into $CO_2$, while generating reducing equivalents (NADH and $FADH_2$) that feed the ETC. In addition to glucose-derived acetyl-CoA, fatty acid β-oxidation provides an important alternative energy substrate, particularly under fasting conditions. Long-chain fatty acids are catabolized to yield acetyl-CoA, NADH, and $FADH_2$, which converge on the Krebs cycle and OXPHOS. Similarly, the catabolism of amino acids contributes to mitochondrial acetyl-CoA and reducing equivalents, ensuring alternative sources in energy

production[9]. These interconnected processes are illustrated in Figure 2, which depicts how glycolysis, β-oxidation, and the TCA cycle converge to supply substrates for OXPHOS.

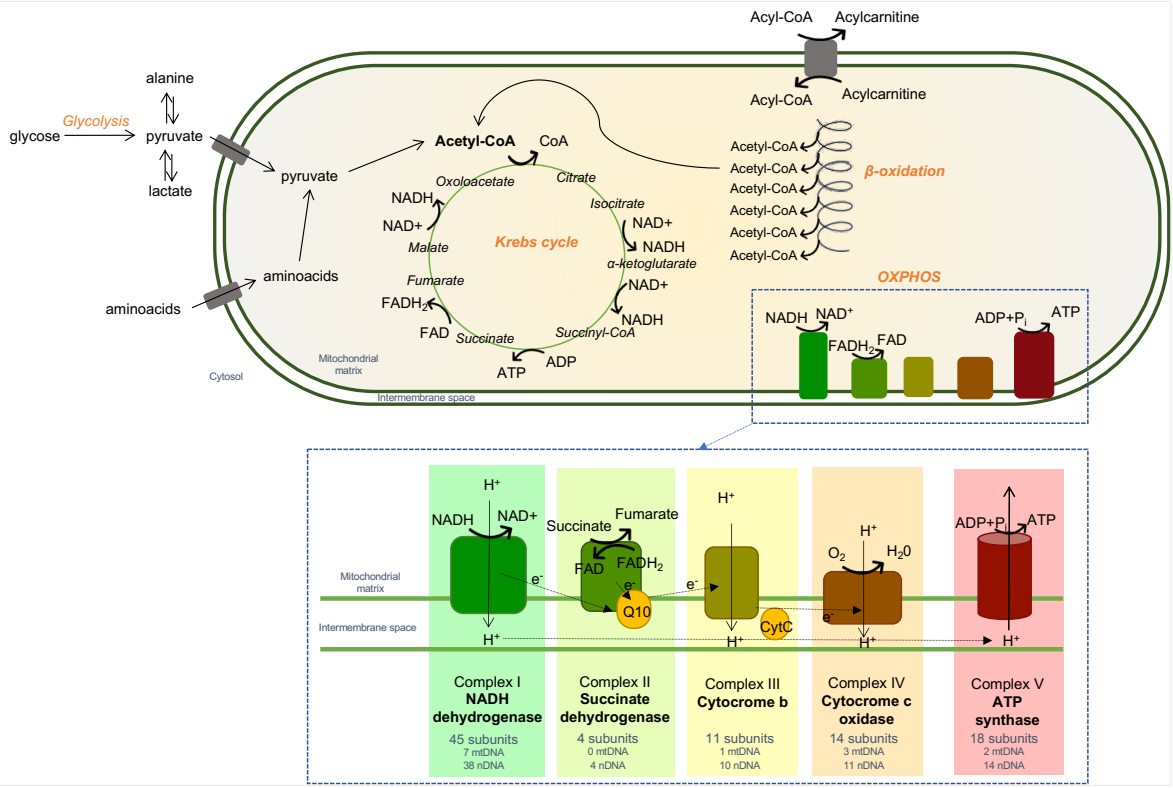


***Figure 2. Integration of glycolysis, Krebs cycle, β-oxidation, and oxidative phosphorylation.*** *This schematic illustrates the convergence of major metabolic pathways that fuel mitochondrial ATP production. Pyruvate, derived from glycolysis, and acetyl-CoA, generated through β-oxidation of fatty acids and amino acid catabolism, enter the Krebs cycle within the mitochondrial matrix. Reducing equivalents (NADH and $FADH_2$) donate electrons to the electron transfer chain (ETC), which drives proton translocation and enables ATP synthase (complex V) to complete oxidative phosphorylation (OXPHOS). Each complex (except complex II) comprises both nuclear-encoded and mitochondrial-encoded subunits, underscoring the dual genomic control of respiratory chain function.*

Oxidation of one NADH generates approximately 2.5 molecules of ATP, whereas one $FADH_2$ yields about 1.5 molecules of ATP, resulting in a net production of ~10 ATP equivalents per acetyl-CoA oxidized through the tricarboxylic acid cycle. Accordingly, the complete oxidation of one glucose molecule through glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation produces approximately 30–32 molecules of ATP[8].

Defects in OXPHOS cause decreased ATP production, lactate and Krebs cycle intermediates accumulation, and excess reactive oxygen species (ROS) generation.

### 1.1.2. Redox Regulation and Reactive Oxygen Species (ROS) Detoxification

Mitochondria are a major source of ROS, primarily generated at complexes I and III of the ETC. Physiological ROS levels play roles in signalling and adaptation (mitohormesis), but their excess induces oxidative damage[10,11]. Antioxidant systems including manganese superoxide dismutase, glutathione, and peroxiredoxins mitigate ROS toxicity[12].

### 1.1.3. Biosynthesis

Beyond its canonical role in generating NADH and $FADH_2$ the Krebs cycle acts as a metabolic hub for diverse biosynthetic pathways, amino acid and organic acid metabolism, including branched-chain amino acid degradation, urea cycle intermediates, and anaplerotic reactions critical for metabolic homeostasis[2,3,13]. Citrate exported to the cytosol provides acetyl-CoA for lipid and cholesterol synthesis, α-ketoglutarate gives rise to glutamate and glutamine, and oxaloacetate is transaminated to aspartate, a precursor for nucleotide biosynthesis. These cataplerotic outflows are counterbalanced by anaplerotic reactions, which replenish cycle intermediates to sustain both energy production and biosynthesis[9,13].

Mitochondria also serve as biosynthetic hubs for heme, steroids, and pyrimidines, and are essential for the formation of Fe–S clusters and biosynthesis and remodelling of phospholipids such as cardiolipin, crucial for mitochondrial membrane structure and function[14–20].

### 1.1.4. Calcium Homeostasis and Apoptosis

Mitochondria modulate cytosolic calcium levels via the mitochondrial calcium uniporter (MCU) and $Na^+/Ca^{2+}$ exchangers[21]. Calcium uptake stimulates dehydrogenases and enhances metabolism, but calcium overload induces permeability transition pore (mPTP) opening, cytochrome c release, and apoptosis[21,22].

### 1.1.5. Organelle Dynamics and Quality Control

Mitochondria continuously undergo fusion and fission to maintain structural integrity, distribute mtDNA, and segregate damaged regions[23]. Mitophagy, regulated by PINK1/Parkin, eliminates dysfunctional mitochondria[24]. Biogenesis is controlled by PGC-1α, NRF1/2, TFAM, and POLG, ensuring adaptation to energy demands and maintenance of mitochondrial mass[25–27].

### 1.1.6. Mitochondrial Signalling and Inter-Organelle Crosstalk

Beyond their metabolic and energetic roles, mitochondria act as signalling platforms that integrate physiological and stress-related signals, often mediated by ROS levels[28]. They participate in processes such as inflammation and innate immunity, exemplified by the mitochondrial antiviral signalling protein (MAVS), which triggers interferon responses upon recognition of viral RNA[26,29].

Retrograde signalling from mitochondria to the nucleus influences gene expression profiles, while mitochondria-to-endoplasmic reticulum contact sites (mitochondria-associated membranes, or MAMs) facilitate calcium and lipid exchange[30,31]. Mitochondrial status modulates cytosolic pathways including AMPK, mTOR, and sirtuins, affecting autophagy, growth, and metabolic adaptation[24].

Recent insights also highlight the role of mitochondria in innate immune responses (e.g., via MAVS), epigenetic regulation (through metabolite-driven chromatin remodelling), and cellular differentiation. Their ability to sense redox changes and transmit metabolic signals positions them as central hubs in cell fate determination[11,26].

## 1.2. Mitochondrial Heterogeneity and Functional Specialization

Emerging evidence highlights that mitochondria are not homogeneous entities: within tissues or even single cells, distinct mitochondrial populations exhibit differential proteomes, metabolic outputs, and stress responses. Recent studies demonstrate that regionally specialized mitochondria may exist within a single cell type, with functional implications for energy production, calcium buffering, ROS handling, and response to signalling inputs[32].

This spatial and contextual heterogeneity underpins tissue-specific susceptibility to mitochondrial dysfunction and contributes to the variable phenotypic expression seen in mitochondrial disease and other complex disorders[3,32].

Collectively, mitochondria represent multifunctional organelles whose disruption leads to wide-ranging systemic consequences. Their dual role as metabolic engines and regulatory hubs makes them central to understanding both rare and common disease mechanisms.

## 1.3. Mitochondrial Dysfunction

Primary mitochondrial dysfunction refers to impaired oxidative phosphorylation that arises directly from defects in the mitochondrial genome or nuclear genes encoding components of the respiratory chain, mitochondrial translation machinery, or organelle maintenance systems. In contrast, secondary mitochondrial dysfunction (SMD) arises via indirect mechanisms that converge on mitochondrial dysfunction as a secondary effect that occurs as a consequence of other genetic, epigenetic, or acquired conditions, in the absence of pathogenic variants in genes directly involved in the mitochondrial respiratory chain or mtDNA maintenance[33–35].

SMD encompasses a wide spectrum of aetiologies, including monogenic diseases unrelated to canonical mitochondrial genes, chromosomal and epigenetic abnormalities, and acquired conditions such as chronic inflammation, metabolic disorders, malignancy, infection, and exposure to mitochondrial toxins. SMD is also a well-established key factor in ageing[36]. A structured overview of etiological categories, representative examples, and proposed pathogenic mechanisms is presented in Table 1.

***Table 1. Causes of secondary mitochondrial dysfunction.***

| Etiologic group | Representative Examples | Proposed Mechanisms | Ref. |
|---|---|---|---|
| **Primary (Genetic) causes** | | | |
| **Other Inherited Errors of Metabolism** | Maple syrup urine disease, propionic acidemia, methylmalonic acidemia, galactosemia, Carnitine palmitoyl transferase 1 deficiency | Organic acid toxicity, secondary inhibition of respiratory chain, mtDNA damage | 12,37–39 |
| | Wilson disease, Niemann–Pick type C, Glycosylation disorders, ceroid lipofuscinosis type 2 and 3 | Accumulation of toxic metabolites interfering with mitochondrial function | 12,40–43 |
| **Neurogenetic and neuromuscular genetic diseases** | Spinal muscular atrophy, Charcot-Marie-Tooth, muscular dystrophies, congenital myasthenic syndromes, channelopathies, inclusion body myositis | Altered mitochondrial transport, axonal degeneration, energy depletion | 34,39–42,44–48 |
| **Other monogenic disorders** | *HPDL, MECP2, PNPLA4, WFS1* | ER stress, membrane instability, glycosylation defects, energy failure | 35,42,49 |
| **Chromosomal** | Trisomy 21, 1p36 deletion, dup5q, 22q11.2 deletion | Transcriptional dysregulation, oxidative stress, abnormal mitochondrial dynamics | 50–54 |
| **Acquired (non-genetic) or multifactorial causes** | | | |
| **Endocrine & Metabolic** | Hypothyroidism, diabetes mellitus, PCOS, obesity | Hyperglycaemia, insulin resistance, mitochondrial uncoupling, increased ROS, reduced biogenesis | 55–57 |
| **Neurologic** | Autism spectrum disorder, Parkinson and Alzheimer's disease | | 58–60 |
| **Inflammatory & Immune-mediated** | Lupus erythematosus systemic, rheumatoid arthritis, Inflammatory bowel disease | Cytokine-mediated mitochondrial stress, impaired bioenergetics, antigen presentation via mitochondria | 61–63 |
| **Neoplastic & Hematologic** | Leukaemia, lymphoma, myelodysplastic syndromes, glioblastoma, breast cancer | Warburg effect, oncometabolites, mtDNA instability, resistance to apoptosis | 64,65 |
| **Infectious** | CMV, EBV, HIV, congenital rubella, tuberculosis, malaria, sepsis | Viral proteins targeting mitochondria, mtDNA depletion, nitric oxide-mediated inhibition of respiration | 12,26,29 |
| **Toxic & Pharmacologic** | Valproate, isoniazid, azathioprine, statins, aminoglycosides, linezolid, doxorubicin, propofol, arsenic | Direct inhibition of OXPHOS enzymes, depletion of cofactors, mtDNA damage, increased lactate | 66,67 |
| **Nutritional** | Malnutrition, thiamine, riboflavin, selenium, copper, carnitine, vitamin B12 deficiency; refeeding syndrome | Cofactor-dependent enzyme inactivation (e.g., PDH, α-KGDH), impaired FAO, oxidative damage | 12,42 |

*Causes are classified in genetic (inborn) versus acquired, with representative examples and proposed mechanisms. Adapted from Videira (2021)[68], with expanded data from recent literature on mitochondrial phenocopies and secondary mitochondrial dysfunction. Ref: reference.*

These conditions may impair mitochondrial function through diverse mechanisms such as oxidative stress, cofactor depletion, impaired mitophagy, disrupted mitochondrial dynamics, or direct inhibition of respiratory chain complexes[12,40]. Importantly, mitochondrial dysfunction may contribute significantly to the clinical phenotype in such disorders, particularly in tissues with high energy demand[37,69].

## 2. Mitochondrial genetic regulation

The regulation of mitochondrial function relies on the coordinated interaction between two distinct genomes: the mtDNA, inherited maternally and residing within the organelle, and the nDNA, which encodes the majority of mitochondrial proteins. This dual genetic control is essential for maintaining oxidative phosphorylation (OXPHOS) and other mitochondrial processes, including dynamics, biogenesis, mitophagy, and metabolism of amino acids, lipids, and nucleotides.

### 2.1. Human mitochondrial DNA

The human mitochondrial DNA is a circular double-stranded molecule of 16,569 base pairs encoding 37 genes: 13 for essential subunits of the respiratory chain complexes (I, III, IV, and V), 22 for mitochondrial tRNAs, and 2 for mitochondrial rRNAs (Figure 3)[28,70]. Unlike nuclear DNA, mtDNA lacks introns and protective histones, and is particularly susceptible to damage from ROS generated within mitochondria. Moreover, mtDNA replication and transcription are independent from the cell cycle and are regulated by nuclear-encoded proteins such as POLG, TFAM, and TFB2M [28].

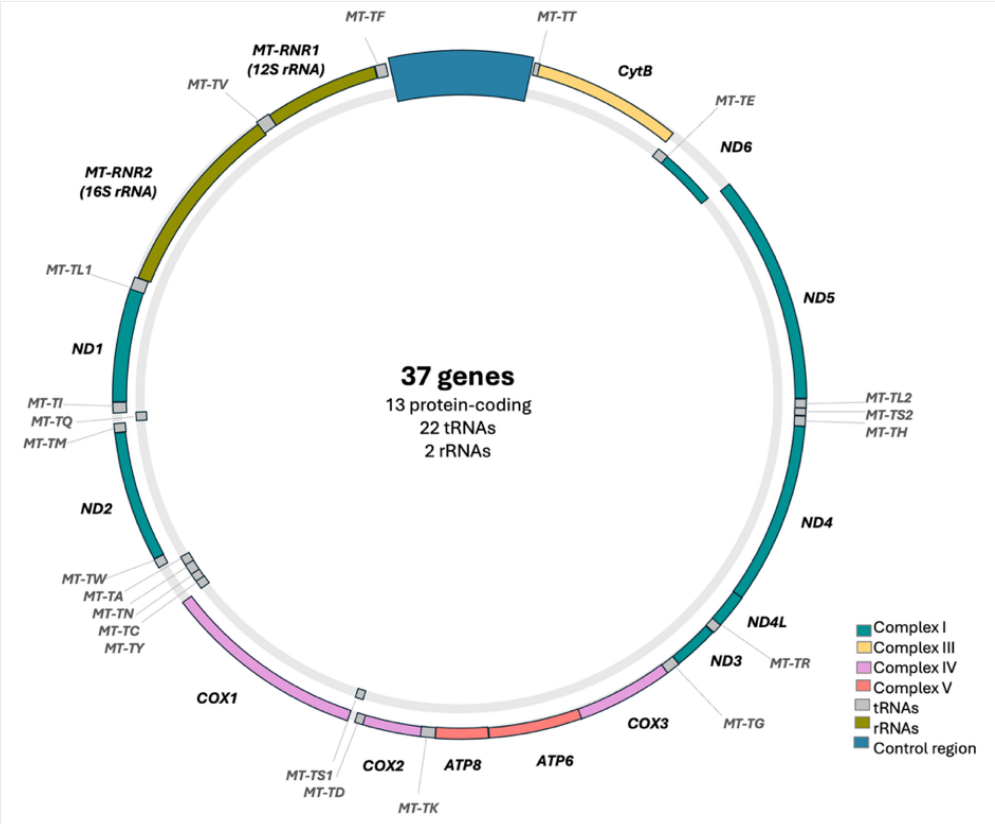


***Figure 3. Human mitochondrial DNA (mtDNA) genome map.*** *The circular genome encodes 13 protein-coding genes involved in oxidative phosphorylation (OXPHOS), including subunits of Complex I (ND1–ND6, ND4L), Complex III (CytB), Complex IV (COX1–3), and Complex V (ATP6, ATP8); 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (12S and 16S rRNA). Most genes are encoded on the heavy (H) strand, whereas ND6 and a subset of tRNA genes are encoded on the light (L) strand.*

Human mtDNA is exclusively maternally inherited, as mitochondria are transmitted to the offspring through the oocyte, with no paternal contribution under physiological conditions.

Each cell contains hundreds to thousands of mtDNA copies, and pathogenic mutations may be present in a proportion of these copies - a condition known as heteroplasmy or even in all copies (homoplasmy). Heteroplasmy levels tend to correlate with disease severity[71,72].

The clinical threshold for phenotypic expression depends on the mutation type, tissue-specific energy demand, and heteroplasmy level[6,70,71,73]. Both point mutations (e.g., *m.3243A>G*, *m.8344A>G*, *m.8993T>G*) and large-scale rearrangements (specially single large deletions) have been associated with a wide spectrum of mitochondrial disorders partially dependent to heteroplasmy levels[71,74–78].

The proportion of mutant mtDNA in oocytes may vary substantially due to the mitochondrial genetic bottleneck occurring during oogenesis[79,80]. This phenomenon contributes to the wide intrafamilial phenotypic variability observed in maternally inherited mitochondrial disorders, as illustrated in Figure 4.

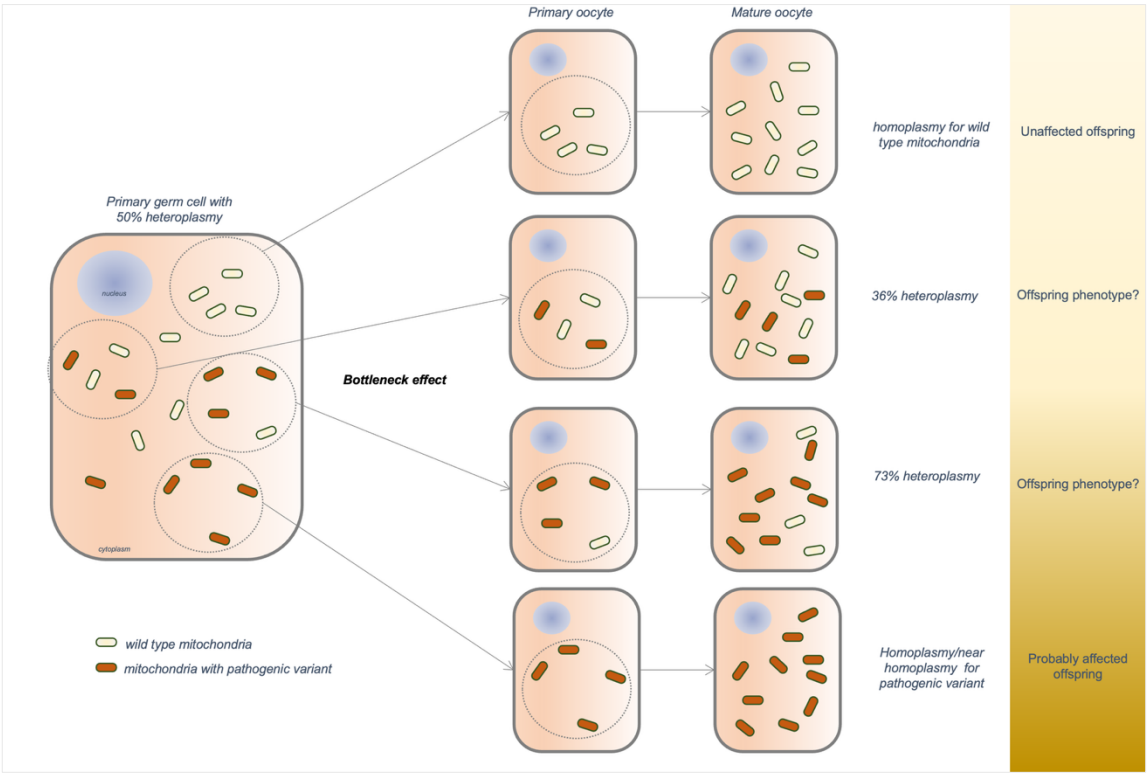


***Figure 4. Mitochondrial genetic bottleneck during oogenesis and impact on heteroplasmy.*** *A primary germ cell containing both wild-type and mutant mitochondria (50% heteroplasmy) gives rise to oocytes with variable levels of heteroplasmy due to stochastic segregation of mtDNA. This phenomenon contributes to the phenotypic variability observed among offspring from the same maternal lineage. Reproduced with permission from Springer Nature[81].*

Another distinct feature of mtDNA is the effect of random *post*-mitotic segregation. During cell division, mtDNA molecules are distributed to daughter cells through a mitotic segregation, which occurs in a stochastic and non-Mendelian manner, potentially leading to shifts in heteroplasmy levels across tissues and over time[82,73,83]. A positive selection may favour the propagation of mitochondria harbouring wild-type mtDNA during oogenesis, and eventually during neurogenesis,, leading to a progressive reduction of pathogenic heteroplasmy in specific cell types or during development, as described in m.3243A>G patients[80,82]. Moreover, heteroplasmy levels correlate with nuclear methylation signatures linked to cardiometabolic traits and mortality, underscoring a cross-talk between mitochondrial genome dosage, heteroplasmy, and epigenetic regulation[83].

Mitochondrial DNA copy number varies widely across tissues and developmental stages and is tightly regulated to ensure adequate mitochondrial function. Copy number of mtDNA varies with age and also contributes to disease heterogeneity[84]. Lower mtDNA content has been associated with an increased burden of somatic heteroplasmy and altered gene expression, particularly in tissues with high metabolic demand[73]. Disease burden and rate of progression have also been negatively correlated with mtDNA copy number[82].

## 2.2. Dual genetic regulation

Although the mitochondrial genome encodes 13 essential proteins for OXPHOS, over 1,100 mitochondrial proteins are encoded by nDNA and imported into the organelle to sustain its structure, dynamics, and function. These include components of the OXPHOS system, proteins required for mtDNA replication and maintenance, mitochondrial translation machinery, and factors regulating dynamics, mitophagy, metabolite transport, and intracellular signalling[85,86].

Mitochondrial translation is a specialized process that synthesizes the 13 essential polypeptides encoded by the mitochondrial genome, all of which are core subunits of the OXPHOS system. This machinery relies on unique mitoribosomes, mitochondrial tRNAs and rRNAs, and a set of nuclear-encoded factors that regulate initiation, elongation, and termination. Accurate mitochondrial translation requires coordinated expression of both the mitochondrial and nuclear genomes, as the majority of OXPHOS subunits are nuclear-encoded and must assemble with their mitochondrially translated counterparts. Defects in mitochondrial translation can arise from mutations in mtDNA-encoded tRNAs or rRNAs, as well as in nuclear genes encoding ribosomal proteins, translation factors, or RNA-modifying enzymes. These impairments result in combined OXPHOS deficiencies, frequently associated with severe clinical phenotypes including encephalopathy, cardiomyopathy, and multisystem mitochondrial disease[87–89].

Mitochondrial membranes are highly dynamic structures whose synthesis, remodelling, and inter-organelle trafficking are essential for organelle function and cellular homeostasis. As

mitochondria lack complete lipid biosynthetic pathways, they rely on import from the endoplasmic reticulum and other organelles, with critical contributions from contact sites such as mitochondria–ER membranes (MAMs). Among mitochondrial lipids, cardiolipin plays a pivotal role in maintaining cristae structure and stabilizing respiratory chain super complexes, while its remodelling by tafazzin exemplifies the pathogenic consequences of lipid dysregulation, as seen in Barth syndrome. Membrane remodelling is tightly coupled to mitochondrial dynamics, including fusion, fission, and mitophagy, which collectively influence lipid distribution and organelle quality control. Defects in lipid synthesis, remodelling, or trafficking have been increasingly recognized as contributors to metabolic and mitochondrial diseases, suggesting that perturbations of mitochondrial lipid biology may represent an underdiagnosed mechanism of mitochondrial dysfunction[90].

Mitochondrial dynamics encompass the continuous and tightly regulated processes of fusion, fission, biogenesis, and mitophagy that collectively maintain mitochondrial morphology, distribution, and quality control. Fusion, mediated by MFN1, MFN2, and OPA1, enables content exchange and functional complementation, whereas fission, primarily driven by DRP1 and its adaptors, facilitates segregation of damaged organelles and mitochondrial proliferation. These processes are closely integrated with mitophagy, through PINK1/Parkin-dependent and independent pathways, to ensure selective clearance of dysfunctional mitochondria, while biogenesis, coordinated by PGC-1α and TFAM, replenishes the mitochondrial pool. By orchestrating turnover and remodelling, mitochondrial dynamics serve as a central mechanism for adapting to metabolic demands and oxidative stress. Dysregulation of these pathways contributes to mtDNA instability, impaired bioenergetics, and heightened susceptibility to neurodegeneration, metabolic syndromes, cardiovascular disease, and primary mitochondrial disorders[11,23].

Despite being independent of the nuclear cell cycle, replication of mtDNA relies on replication machinery encoded in the nuclear genes, as illustrated in Figure 5. Dysfunctions in this machinery can impair mtDNA synthesis and lead to secondary mtDNA depletion or accumulation of multiple deletions[91].

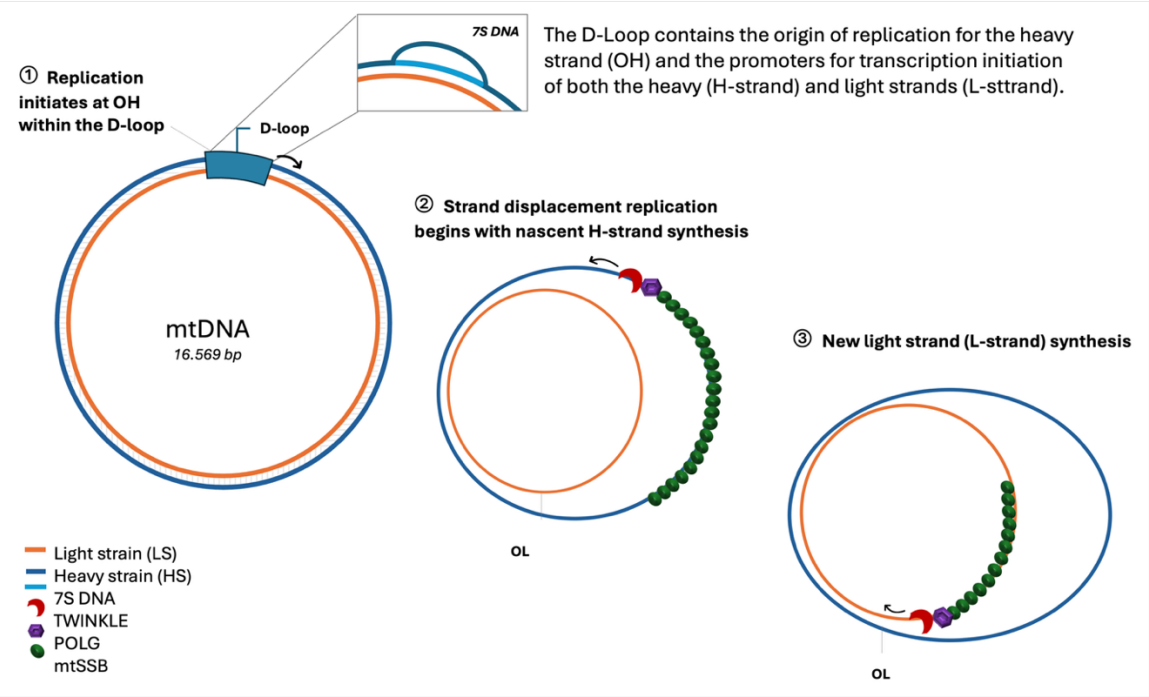


***Figure 5. Schematic representation of human mitochondrial DNA (mtDNA) replication.*** *Replication initiates at the origin of the heavy strand (OH), located within the D-loop region, which also contains the promoters for transcription of both the heavy (HSP) and light (LSP) strands. Replication of the heavy strand (H-strand) begins at OH with displacement of the parental strand. (1) The TWINKLE helicase unwinds the duplex, while DNA polymerase-gamma (POLG) synthesizes the nascent H-strand with support from mitochondrial single-stranded binding proteins (mtSSBs). (2) Upon exposure of the light strand origin (OL), replication of the light strand (L-strand) initiates in the opposite direction. (3). The diagram also shows 7S DNA (a short triple-stranded DNA segment within the D-loop), TWINKLE (purple), POLG (green), and mtSSB (dark green spheres).*

Mitochondrial DNA is particularly vulnerable to damage due to its proximity to the respiratory chain, the absence of protective histones, and limited DNA repair capacity. Unlike the nucleus, mitochondria lack nucleotide excision repair, and nuclear-encoded enzymes are responsible for base excision repair (BER), the predominant pathway safeguarding mtDNA integrity. In addition, mitochondria appear to possess specialized mismatch repair (MMR) activity as well as recombination-based mechanisms such as homologous recombination. When DNA damage exceeds the repair capacity, mitochondria rely on genome degradation and quality control processes, including fusion–fission dynamics and mitophagy, to maintain cellular homeostasis. Defective mtDNA repair contributes to the accumulation of mutations and deletions, with implications for aging, neurodegeneration, and primary mitochondrial disease[92].

Furthermore, mtDNA is organized into nucleoids, and its distribution and segregation during mitochondrial division are tightly regulated. Defects in genes regulating mitochondrial dynamics (e.g., *OPA1*, *MFN2*) or genome maintenance and repair (e.g., *MGME1*, *DNA2*) have been associated with distinct clinical syndromes.

## 3. Mitochondrial disorders

Considering dual genomic regulation of mitochondrial function, defects in either genome may result in impaired mitochondrial function and are associated with disease, often with multisystemic clinical features and variable onset[70,93].

### 3.1. Definitions

PMD constitute a diverse group of genetic diseases that arise from pathogenic variants affecting mitochondrial or nuclear genes, that directly compromise mitochondrial function or structure. Pathogenic mechanisms include disrupted mitochondrial ultrastructure; defects in respiratory chain activity; impaired biosynthesis of cofactor or vitamins, and abnormalities in key mitochondrial metabolic pathways, such as the tricarboxylic acid (TCA) cycle and pyruvate metabolism[93].

Therefore PMD may result from mutations in genes not directly encoding OXPHOS subunits but involved in related processes such as mitochondrial translation, cofactor biosynthesis, protein import, and quality control[3,25,91,27].

Mitochondrial DNA maintenance and replication ensure the stability and integrity of the mitochondrial genome, preserving adequate deoxynucleotide pools and the integrity of the replication machinery. Mutations in these genes are associated with mtDNA instability, resulting in qualitative or quantitative changes in mtDNA.

A definitive diagnosis of mitochondrial disease requires genetic confirmation, via identification of causative variants in nDNA or mtDNA, and in the latter can be due to point mutations or large-scale rearrangements.

### 3.2. Prevalence and incidence

The estimated prevalence of PMD in the general population ranges from 1.3 to 20 cases per 100,000 individuals, based on genetically confirmed cohorts[94,95]. Reported incidence rates are consistently higher in childhood: national data from Spain indicate a paediatric prevalence of 0.63 per 100,000 per year, and an adult incidence of 0.14 per 100,000 per year, both based on molecular diagnoses[96]. The underlying genetic aetiology also differs by age group: nuclear DNA variants account for 80–90% of paediatric cases, while mitochondrial DNA mutations predominate in adults, representing approximately 60–80% of molecularly confirmed diagnoses[97,95,94].

## 3.3. Genetic basis of mitochondrial disorders

### 3.3.1. mtDNA point mutations

Pathogenic point mutations in mtDNA represent a major cause of PMD, following maternal inheritance and displaying a variable phenotype. Phenotypic expression is influenced by the above mentioned factors such as heteroplasmy, or energy demand of affected tissues, and additional modifying factors, such as haplogroups[73,82,98,99]. Even within the same maternal lineage, clinical heterogeneity is frequently observed, ranging from asymptomatic individuals to severe disease, complicating both diagnosis and genetic counselling[75].

Point mutations in mitochondrial genes are a frequent cause of mitochondrial disease and are often associated with multisystemic phenotypes[93]. Among the most well-characterized mtDNA point mutations are m.3243A>G in the *MT-TL1* gene, commonly recognized as associated with the phenotype of Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS) and maternally-inherited diabetes and deafness (MIDD); m.8344A>G in *MT-TK*, most frequently presenting as Myoclonic Epilepsy with Ragged Red Fibers (MERRF); and m.8993T>G in *MT-ATP6*, typically associated with (Neuropathy, Ataxia, and Retinitis Pigmentosa NARP) and Leigh syndrome (LS)[93].

Molecular diagnosis of mtDNA point mutations is commonly performed using targeted sequencing or whole mtDNA analysis via next-generation sequencing (NGS). Low-level heteroplasmic mutations may be undetectable in blood, particularly in adults, reinforcing the need to test other tissues such as urine or muscle when clinical suspicion remains high[100].

When clinical suspicion is high and standard screening is performed as first tier and is inconclusive, whole mtDNA sequencing should be considered even in the presence of low heteroplasmy levels[39,100–102].

### 3.3.2. mtDNA rearrangements

Large-scale mtDNA rearrangements, particularly single large deletions, represent a well-established cause of primary mitochondrial disease and are typically sporadic. Large-scale deletions typically arise through recombination events at short direct repeats, which serve as hotspots for breakpoints. Large-scale deletions typically arise at specific breakpoints, often flanked by short direct repeats that act as hotspots for rearrangements. Such structural instability predisposes the mitochondrial genome to recombination events, providing a molecular basis for the observed deletions and duplications[103,104].

These deletions can span several kilobases and result in loss of genomic regions spanning protein-coding genes, tRNAs, or rRNAs, thereby impairing mitochondrial translation and OXPHOS

function[103,104].The clinical presentation is highly variable and includes distinct phenotypes such as progressive external ophthalmoplegia (PEO), Pearson syndrome in infancy or Kearns–Sayre syndrome (KSS), often forming a *continuum* over time[76,77]. Severity of these phenotypes may reflect different degrees of heteroplasmy and tissue distribution but do not correlate with the length of the deletion[104,103].

Although less frequent, mtDNA duplications may co-occur with deletions, contributing to the phenotypic variability observed in some patients[105,106]. Duplications tend to be maternally inherited opposed to sporadic large deletions[103].

Of note, multiple mtDNA deletions, variable in length, are usually secondary to nuclear gene defects affecting mtDNA replication and maintenance, most notably in *POLG* and *TWNK* (as previously explained). Unlike single deletions, these are typically inherited in an autosomal dominant or recessive manner[91,107]. Rearrangements are also observed in Parkinson, cancer, inclusion body myositis and normal ageing, usually somatic and in much lower heteroplasmy levels[103,107].

### 3.3.3. Nuclear genome

Unlike maternally inherited mtDNA mutations, nDNA-related mitochondrial disorders follow Mendelian inheritance, most often autosomal recessive (AR), but also autosomal dominant (AD) or X-linked (dominant or recessive)[108].

The recognition of nuclear gene involvement has expanded dramatically in recent years, driven by next-generation sequencing (NGS) technologies[109,110]. Nuclear mutations now account for a large proportion of paediatric mitochondrial diagnoses, particularly in severe early-onset phenotypes[93]. Nuclear-encoded mitochondrial genes frequently exert multifunctional roles that extend beyond their canonical pathways. For example, proteins initially classified as factors of mtDNA maintenance or nucleotide metabolism (e.g., *ATAD3A*, *MFN2* or *DNM1L*) have also been implicated in processes such as inner membrane stability, nucleoid organization, reactive oxygen species homeostasis, and respiratory chain complex assembly. This pleiotropy contributes to the phenotypic diversity observed in mitochondrial disorders, as disruption of a single nuclear gene may simultaneously compromise mtDNA replication, OXPHOS complex stability, and metabolic signalling. The recognition of these non-canonical functions highlights the complexity of nuclear–mitochondrial crosstalk and underscores the challenges of establishing straightforward genotype–phenotype correlations in mitochondrial disease.[90]

#### OXPHOS structural subunits and assembly factors

Mutations in nuclear-encoded structural subunits or assembly factors of the respiratory chain can lead to isolated or combined complex deficiencies. These typically manifest as early-onset

encephalomyopathies, cardiomyopathy, or LS, but the clinical spectrum is wide, ranging from fatal neonatal disease to milder myopathies. Examples include *NDUFS1*, *NDUFAF2* (complex I), *SDHA*, *SDHB* (complex II), *BCS1L* (complex III), *SURF1*, *SCO2* (complex IV), and *ATP12* (ATP synthase). Defects in assembly factors are particularly important, as they compromise the correct formation and stability of OXPHOS complexes[111].

### *Cofactor biosynthesis pathways*

Mitochondrial enzymes require specific cofactors for proper function, and defects in their biosynthesis or recycling cause distinct clinical syndromes. Coenzyme Q10 (ubiquinone) deficiency due to mutations in *COQ2*, *COQ6*, *PDSS2*, or other COQ genes is an illustrative example, with presentations ranging from nephrotic syndrome to encephalomyopathy, ataxia, or isolated myopathy. Defects in lipoic acid metabolism (e.g., *LIAS*, *LIPT1*, *LIPT2*) result in combined respiratory chain deficiency and metabolic crises, while iron–sulfur cluster assembly defects (e.g., *ISCU*, *NFU1*, *BOLA3*) are more commonly associated with myopathy, lactic acidosis, and severe encephalopathy.

### *Mitochondrial maintenance and replication*

These disorders result from autosomal recessive mutations in nuclear genes involved in mtDNA replication (e.g., *POLG*, *TWNK*) or nucleotide metabolism and salvage pathways (e.g., *TK2*, *DGUOK*, *RRM2B*) and can lead to qualitative and/or quantitative defects in mtDNA. Qualitative defects in mtDNA refer to multiple deletions in mtDNA that can be variable in length and heteroplasmy. The quantitative reduction in mtDNA content (i.e. copy number), termed mtDNA depletion, leads to a group of clinically heterogeneous and often severe disorders collectively known as mitochondrial DNA depletion syndromes (MDS) [91,112,113].

MDS are defined by a significant (<30%) reduction in mtDNA copy number (typically <10% of reference levels). Reduced mtDNA content leads to impaired synthesis of OXPHOS subunits and consequent energy failure. These conditions typically present in infancy or early childhood and can be systemic or tissue-specific (liver, skeletal muscle, brain). Clinical presentations range from isolated myopathy to multisystemic syndromes with hepatic failure, encephalopathy, or peripheral neuropathy. The resulting clinical phenotypes - myopathic, hepatocerebral, or encephalomyopathic - vary depending on the tissue and genetic background[112,93].

*POLG* mutations are one of the most frequent causes of nuclear-encoded mitochondrial disease leading to quantitative defects in childhood presentation and qualitative in older forms, with a phenotype spectrum ranging from Alpers–Huttenlocher syndrome, SANDO to PEO[114–116].

Interestingly, there are other pathophysiological pathways that can lead to MDS such as those involved in mitochondrial inner membrane stability (e.g., *MPV17*) or mitochondrial biogenesis (e.g. *OPA1*) [117].

#### *Mitochondrial gene expression*

A large group of nuclear disorders affect transcription, RNA processing, translation, or ribosome assembly within mitochondria. Pathogenic variants in nuclear-encoded mitochondrial aminoacyl-tRNA synthetases (e.g., *DARS2*, *RARS2*, *AARS2*) are responsible for a wide array of syndromes, often with leukoencephalopathy or ovarian failure[118]. Mutations in genes involved in mitochondrial transcript processing and modification (*PNPT1*, *ELAC2*, *MTO1*) can lead to encephalomyopathy or hypertrophic cardiomyopathy[25]. Defects in mitoribosomal subunits (*MRPS16*, *MRPL44*) or ribosome assembly factors result in impaired mitochondrial translation and severe multisystemic disease[89].

#### *Mitochondrial protein import and quality control*

Proper mitochondrial function depends on the import of nuclear-encoded proteins via specialized translocases. Defects in components of the TIM/TOM complexes (*TIMM50*, *DNAJC19*) can cause cardiomyopathy, encephalopathy, or metabolic crises. Similarly, disorders of mitochondrial chaperones and proteases (*LONP1*, *CLPP*, *AFG3L2*) lead to accumulation of misfolded proteins, resulting in syndromes with deafness, optic atrophy, and neurodegeneration[90].

#### *Mitochondrial carriers and metabolite transport*

Nuclear-encoded carrier proteins located in the inner mitochondrial membrane regulate the exchange of metabolites essential for energy metabolism. Mutations in SLC25 family members, such as *SLC25A21* (Mitochondrial inner membrane oxodicarboxylate carrier, causing spinal atrophy-like syndrome) or *SLC25A12* (aspartate-glutamate carrier deficiency), illustrate the centrality of transport processes[93,119].

### 3.4. Classification

Given the increasing number of nuclear and mitochondrial genes implicated in mitochondrial pathology, classification by biological pathway and molecular mechanism provides a rational framework for clinical and diagnostic decision-making[93,120,121]. Many nuclear-encoded defects result in downstream dysfunction of the OXPHOS system or mtDNA instability, despite not involving OXPHOS genes directly. It distinguishes between disorders of respiratory chain complexes, cofactor biosynthesis, mitochondrial gene expression, and mtDNA maintenance, among others[93,120]. The classification of mitochondrial disorders according to the current

International Classification of Inherited Metabolic Disorders (ICIMD) is summarized in Table 2, where these disorders are placed within the broader group of *Intermediary Metabolism: Energy*, the same level of disorders of Krebs cycle and pyruvate (as the subcategory of *Disorders of Energy Substrate Metabolism*). This expert-driven nosologic framework takes into consideration several factors such as the affected biochemical pathways and the underlying pathomechanism[121,122].

***Table 2. Mitochondrial disorders classification.***

| Classification of mitochondrial disorders | |
|---|---|
| **Mitochondrial DNA-related disorders** | Disorders of mtDNA-encoded oxidative phosphorylation proteins<br>▪ Complex I subunits<br>▪ Complex III subunits<br>▪ Complex IV subunits<br>▪ ATP synthase subunits |
| | Disorders of mtDNA-encoded tRNAs and rRNAs |
| **Nuclear-encoded disorders of oxidative phosphorylation** | Complex I subunits and assembly factors |
| | Complex II subunits and assembly factors |
| | Complex III subunits and assembly factors |
| | Complex IV subunits and assembly factors |
| | ATP synthase subunits and assembly factors |
| **Disorders of mitochondrial cofactor biosynthesis** | Coenzyme Q10 biosynthesis |
| | Lipoic acid and iron-sulfur cluster metabolism |
| | Cytochrome c biosynthesis |
| **Disorders of mtDNA maintenance and replication** | Nucleotide pool maintenance |
| | mtDNA replication and maintenance |
| **Disorders of mitochondrial gene expression** | Transcript processing and modification |
| | Aminoacyl-tRNA synthetases |
| | Mitoribosome biogenesis<br>▪ Mitoribosome subunit deficiencies<br>▪ Mitoribosome modification and assembly defects |
| **Other disorders of mitochondrial function** | Mitochondrial carriers and shuttles |
| | Mitochondrial protein import |
| | Protein quality control |
| | Miscellaneous mitochondrial dysfunction syndromes |

*Based on the current International Classification of Inherited Metabolic Disorders (ICIMD)[121]*

Within the ICIMD framework, disorders of energy substrate metabolism encompass defects in pathways that channel amino acid, carbohydrate, and lipid catabolites into the mitochondria to fuel oxidative phosphorylation. This group includes disorders of pyruvate metabolism, the Krebs cycle, and creatine metabolism, reflecting their central role in maintaining the supply of reducing equivalents for ATP synthesis[121].

## 3.5. Phenotype

Mitochondrial diseases are clinically heterogeneous disorders that can affect nearly every organ system, presenting with variable severity and onset across the lifespan. The phenotypic expression may be acute, episodic, chronic, or progressive, often involving multiple systems simultaneously or evolving over time[93].

This wide spectrum reflects the ubiquitous role of mitochondria in cellular energy metabolism, redox balance, calcium homeostasis, and apoptosis, the phenotype spectrum for any genetic disease plus the above explained characteristics of mtDNA, nuclear-mitochondrial interactions and other disease modifiers[122].

### 3.5.1. Clinical manifestations

PMD may present with static or progressive symptoms, and in some cases, with acute metabolic decompensation triggered by fever, fasting, or infection. Table 3 outlines possible manifestations by organ system, including both clinical signs and neuroimaging findings or metabolic findings.

***Table 3. Possible manifestations of mitochondrial disorders[35,93,123–133]***

| System | Clinical manifestation |
|---|---|
| **Central nervous system** | Encephalopathy and/or coma*; epilepsy*; myoclonus; ataxia*; dystonia; migraine*; developmental delay or regression; behavioural disorders; stroke or stroke-like episodes*; T2/FLAIR hyperintensities in basal ganglia, thalamus, cerebellum; white matter lesions; lactate peak on MR spectroscopy |
| **Neuromuscular** | Axonal or demyelinating peripheral neuropathy (may be subclinical); hypotonia; proximal and symmetrical muscle weakness*; myalgias*; exercise intolerance; rhabdomyolysis/myoglobinuria*; elevated creatine kinase (CK); altered Pi/PCr ratio on muscle MR spectroscopy |
| **Cardiac** | Hypertrophic or dilated cardiomyopathy; arrhythmias (especially AV block); heart failure* |
| **Hepatology** | Elevated transaminases; liver failure*; Reye-like syndrome*; cholestasis |
| **Gastrointestinal** | Failure to thrive; recurrent vomiting*; pancreatic dysfunction; gastric dysmotility; intestinal pseudo-obstruction* |
| **Endocrine** | Diabetes mellitus (type 1 or 2); glucose intolerance; hypothyroidism; hypoparathyroidism; adrenal insufficiency; short stature; growth hormone deficiency; primary or secondary hypogonadism; premature ovarian failure |
| **Kidney** | Proximal tubulopathy; generalized hyperaminoaciduria; steroid-resistant nephrotic syndrome; tubulointerstitial nephritis; chronic kidney disease (CKD) of unclear origin |
| **Haematological** | Sideroblastic anaemia; aplastic anaemia; neutropenia; thrombocytopenia |
| **Audiology** | Progressive or congenital sensorineural hearing loss (independent of aminoglycoside exposure) |
| **Ophthalmologic** | Ptosis; external ophthalmoplegia; retinitis pigmentosa; optic atrophy; cataract |
| **Cutaneous and subcutaneous** | Hypertrichosis; multiple lipomas; *livedo reticularis* |

| | |
|---|---|
| **Metabolic** | Hypoglycaemia*; metabolic acidosis*; persistent or recurrent hyperlactacidemia* (>/= 2 separate measurements with lactate >2.0 mmol/L; may worsen with fasting or glucose challenge) |

**Commonly observed in acute presentations or metabolic crises*

Disease onset may be acute, fulminant, chronic, or progressive, and can occur at any stage of life. Clinical presentation is highly heterogeneous, ranging from oligosymptomatic to multisystemic involvement, from mild to mild to life-threatening severity and from static to progressive courses.

In children, the phenotype often includes seizures, acute encephalopathy, ataxia, lactic acidosis, cardiomyopathy or liver failure, whereas in adults, progressive external ophthalmoplegia, myopathy, or neuropathy may predominate[35,134,135]. Phenotypic overlap with other neurometabolic or genetic diseases is frequent, reinforcing the need for integrative diagnostic strategies that combine clinical, biochemical, imaging, histological, and genetic data[35,136].

### *3.5.2. Classic syndromes*

A subset of mitochondrial disorders can be classified into well-recognized clinical syndromes, often referred to as "classic mitochondrial syndromes." These phenotypes are defined by characteristic constellations of signs and symptoms with preferential involvement of specific systems and show frequent associations with specific mitochondrial or nuclear gene defects[137–140].

Recent efforts of expert-base consensus have focused on harmonizing syndromic definitions and nomenclature. The North American Mitochondrial Disease Consortium (NAMDC) proposed refined diagnostic criteria for classic mitochondrial syndromes, establishing specific clinical and laboratory thresholds for inclusion (e.g., mandatory stroke-like episodes for MELAS)[138]. Leigh syndrome was redefined as the Leigh syndrome spectrum (LSS), with consensus-based clinical and neuroradiological criteria, encompassing a broader range of phenotypes beyond the classic presentation. This effort was supported by expert curation of 113 primary mitochondrial disease genes, thereby providing a more comprehensive and standardized framework for diagnosis[139]. The European Reference Network for rare neuromuscular diseases (EURO-NMD) also redefined the concept of primary mitochondrial myopathy (PMM), introducing subgroups based on the presence or absence of Progressive External Ophthalmoplegia (PEO) [140]. These updates aim to improve consistency in diagnosis across publications, clinical trials and registries.

The table below (Table 4) summarizes key classic mitochondrial syndromes, their main clinical features, associated genes, and references[137–140] .

***Table 4. Classic syndromes: characteristics and gene basis.***

| Syndrome | Main clinical features | Common gene(s) |
|---|---|---|
| **Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes (MELAS)** | Stroke-like episodes before age 40, seizures, encephalopathy, lactic acidosis, myopathy | m.3243A>G (*MT-TL1*), *POLG,* others |
| **Myoclonic Epilepsy with Ragged-Red Fibers (MERRF)** | Myoclonus, epilepsy, ataxia, myopathy, RRF on biopsy | m.8344A>G (*MT-TK*), others |
| **Leigh syndrome spectrum (LSS)** | Psychomotor regression, brainstem and basal ganglia lesions, lactic acidosis | *SURF1*, *MT-ATP6*, *PDHA1*, others |
| **Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP)** | Peripheral neuropathy, ataxia, retinitis pigmentosa | m.8993T>G (*MT-ATP6*), m.8993T>C |
| **Chronic Progressive External Ophthalmoplegia (CPEO)** | Bilateral ptosis and ophthalmoplegia, sometimes limb weakness or dysphagia | *POLG, TWNK*, Single large-scale mtDNA deletion |
| **Kearns–Sayre Syndrome (KSS)** | CPEO, pigmentary retinopathy, cardiac conduction defects | Single large-scale mtDNA deletion |
| **Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE)** | GI dysmotility, cachexia, ptosis, peripheral neuropathy, leukoencephalopathy | *TYMP* |
| **Alpers-Huttenlocher syndrome** | Early-onset epilepsy, psychomotor regression, liver failure | *POLG* |

While these labels can aid diagnostic orientation, they do not fully reflect the genetic and phenotypic heterogeneity of mitochondrial disease. Although many patients present with non-syndromic or overlapping features, several "classic" mitochondrial syndromes have been described, often associated with specific genetic variants or mutation types (e.g., mtDNA point mutations, single deletions, or nDNA variants affecting mitochondrial proteins). Recognition of these phenotypes may be important for diagnostic genetic investigation and variant prioritization[123,100,141–143].

### 3.5.3. Biochemical Markers in Mitochondrial Disease

Biochemical markers remain central in the diagnostic process of suspected MDs, especially in paediatric settings where clinical heterogeneity often complicates the initial approach. While many classical biomarkers such as lactate, pyruvate, and alanine continue to be used, their specificity is limited, and interpretation must consider age, sample quality, and concurrent illnesses.

Ketonemia may occur in mitochondrial disorders, usually during episodes of metabolic decompensation, with elevations of β-hydroxybutyrate (βOHB) and acetoacetate (AcAc). Beyond absolute levels, the βOHB/AcAc ratio provides additional information on the redox state of mitochondria: a high ratio indicates an increased $NADH/NAD^+$ balance, consistent with impaired oxidative phosphorylation and reduced respiratory chain activity. Thus, ketone abnormalities

reflect both compensatory energy metabolism and disturbed mitochondrial redox homeostasis, although they are generally transient and not specific for primary mitochondrial disease[132,144,145].

Lactate and pyruvate, when elevated in blood or cerebrospinal fluid (CSF), are traditional indicators of impaired oxidative metabolism. However, their diagnostic yield is modest, with elevated levels found in various metabolic and non-metabolic conditions[132]. Additionally, normal lactate levels do not exclude mitochondrial dysfunction, especially in chronic or non-neurological phenotypes. The lactate-to-pyruvate ratio (L/P) can aid in distinguishing respiratory chain defects (typically >20) from primary pyruvate dehydrogenase deficiency (<10), yet its sensitivity and specificity are limited, and interpretation must always be made in context and never in isolation[146,147,145].

Alanine is frequently elevated due to transamination of accumulating pyruvate and can provide a supportive clue to mitochondrial dysfunction, especially in chronic presentations. It can confirm true hyperlactacidemia and can be high even in the absence of hyperlactacidemia. However, normal alanine levels, particularly in neonates with transient lactic acidaemia, may argue against a mitochondrial aetiology[148–150].

Cerebrospinal fluid analyses offer increased specificity compared to blood, with elevated lactate, pyruvate, and alanine serving as useful indicators in the context of encephalopathy.

Citrulline has been proposed as a supportive biomarker in mitochondrial disorders, particularly in Leigh syndrome associated with ATP synthase defects particularly the m.8993T>G mutation[75,151,152]. Hypocitrullinemia in this context reflects the ATP-dependent impairment of carbamoyl phosphate synthetase I (CPS1) activity, compromising urea cycle function particularly in the intestine [151,153,154]. It is not a common feature of PMDs but appears to be a specific biomarker for *MT-ATP6* pathogenic variants that can present asymptomatically, even in newborn period[143].

Elevated plasma tyrosine has been repeatedly reported in infants with MDS, especially in hepatocerebral forms due to *DGUOK* or *MPV17* variants. In most cases, the amino acid profile shows increased tyrosine, phenylalanine, and methionine, reflecting impaired hepatic mitochondrial function and secondary disturbance of amino acid metabolism[155,156]. In neonates, tyrosine or phenylalanine elevation with normal succinylacetone levels can be detected on newborn screening[157]. Although not specific for mitochondrial disease, the recognition of persistent tyrosine elevation, particularly in combination with hepatopathy and other biochemical abnormalities, can provide an early clue toward MDS and support prioritization of mtDNA maintenance genes in the diagnostic work-up[156–158].

Urinary organic acid analysis may reveal accumulation of lactic acid, Krebs cycle intermediates (such as succinate, fumarate, and malate), dicarboxylic acids, ethylmalonic acid and 3-

methylglutaconic acid (3-MGA). These findings can provide supportive evidence of mitochondrial dysfunction but are generally nonspecific, as similar abnormalities may be seen in other metabolic conditions. Notably, 3-MGA is a recurrent yet heterogeneous finding in primary mitochondrial disorders: it may represent a discriminative feature in specific subtypes (e.g., Barth syndrome or DNAJC19 deficiency), but in most cases it occurs as a secondary byproduct of impaired mitochondrial energy metabolism. Thus, organic acid profiles are best interpreted as contextual biochemical signatures rather than disease-defining markers[145,146,159,159].

In recent years, novel serum-based biomarkers have emerged as valuable tools for diagnosis and disease monitoring. Two cytokine-like molecules, Fibroblast Growth Factor 21 (FGF21) and Growth Differentiation Factor 15 (GDF15), have gained increasing recognition as biomarkers of mitochondrial disease. FGF21 is induced in response to metabolic stress and is particularly elevated in mitochondrial myopathies outperforming lactate and CK but shows reduced sensitivity in predominantly neurological or hepatic presentations. Its serum levels correlate with the presence of COX-negative fibbers on muscle biopsy and are highest in patients with nDNA mutations, as shown in paediatric and adult cohorts. Nevertheless, FGF21 does not consistently reflect disease severity or stage, limiting its utility as a progression marker[160–162]. GDF15 has emerged as a more sensitive and less specific, marker of mitochondrial disease. It is elevated in various mitochondrial phenotypes, including those without overt myopathy, and may be particularly useful in neonates or in patients with hepatopathy[163]. Their combined use enhances diagnostic yield, may help guide investigation and has proven particularly valuable in differentiating mitochondrial hepatopathies from other paediatric liver diseases[163,164].

Neurofilament light chain (NF-L) is a neuronal cytoskeletal protein that reflects axonal injury. Recent data demonstrate elevated NF-L levels in mitochondrial encephalopathies and in CFS of patients with epilepsy or stroke-like episodes, such as MELAS or *POLG*-related. [165,166] Unlike FGF21 and GDF15, which are associated with muscle pathology, NF-L appears to correlate with central nervous system involvement and may serve as a biomarker of disease activity and neurodegeneration, measured in CSF and blood[165–167].

Taken together, biochemical markers provide valuable but heterogeneous diagnostic information in suspected mitochondrial disease and are summarized in Table 5.

***Table 5. Biochemical tests useful in the diagnosis of mitochondrial disorders***

| Essay (tissue) | Findings | Considerations |
|---|---|---|
| **Lactate (B, CSF)** | Elevated lactate | May be absent |
| **Pyruvate (B, CSF)** | Elevated pyruvate | Consider when lactate is high. Interpret with lactate and L/P ratio |
| **Lactate/pyruvate ratio (B, CSF)** | L/P ratio > 20 | Suggests OXPHOS dysfunction (if <10 suggests pyruvate dehydrogenase complex deficiency) |

| | | |
|---|---|---|
| **Alanine (P)** | Elevated (chronic presentations) | May be high even if lactate is normal. Normal values may argue against PMD in neonates |
| **Citrulline (P)** | ↓ (*MT-ATP6*) | Presumably, reflects ATP-dependent urea cycle impairment |
| **Tyrosine (P)** | ↑ (MDS) | Especially hepatocerebral forms, may be evident in newborn screening |
| **Organic acids (U)** | ↑ lactic acid, 3-MGA, Krebs intermediates (fumarate, succinate, malate) | Non-specific; 3-MGA may be a discriminant feature or an accompanying finding |
| **Ketonemia (B)** | ↑ βOHB/acetoacetate (>3) | May appear as paradoxical ketonemia<br>Seen during decompensation |
| **FGF21 (P)** | Elevated (esp. in mitochondrial myopathies) | Induced by metabolic stress; non-specific |
| **GDF15 (P)** | Elevated (broad MD phenotypes, incl. hepatopathy) | More sensitive; elevated even in non-myopathic MD |
| **Neurofilament light chain (NF-L) (P)** | Elevated (CNS involvement, MELAS, POLG syndromes) | Correlates with axonal damage, not MD-specific |
| **CSF amino acids/lactate/pyruvate** | ↑ lactate, pyruvate, alanine | More specific than serum; consider in encephalopathy |

*Abbreviations: B:blood;P: plasma; U:urine*

The presence of multiple abnormalities across these domains increases the likelihood of mitochondrial dysfunction, although none are diagnostic in isolation. Certain findings, such as hypocitrullinemia in *MT-ATP6*–related Leigh syndrome or persistent tyrosine elevation in hepatocerebral MDS, can serve as relatively specific indicators that help guide genetic prioritization[151,168].

Coenzyme Q10 (CoQ10) quantification has a particular role in mitochondrial medicine. Measurement in muscle remains the reference standard for documenting primary CoQ10 deficiency (less than 50% compared to controls), although its invasive nature has limited its use. In contrast, CoQ10 assessment in plasma, peripheral blood mononuclear cells or platelets is increasingly applied in the therapeutic setting. Plasma levels primarily reflect dietary intake and adherence to supplementation, while PBMCs and platelets may provide a more accurate estimate of intracellular CoQ10 status. Although informative for treatment monitoring, especially in patients under long-term supplementation, these measurements lack specificity for diagnosis[37,169,170].

### 3.5.4. Neuroimaging Features in Mitochondrial Disorders

Magnetic resonance imaging (MRI) is a key tool in the evaluation of neurological involvement in PMD. Although findings are heterogeneous and not pathognomonic, several patterns are highly suggestive of mitochondrial dysfunction, such as cerebral atrophy, cerebellar atrophy, white matter involvement, basal ganglia and brainstem involvement and stroke-like phenomenon[128,171]. Proton magnetic resonance spectroscopy ($^1$H MRS) usually shows lactate peak and/or reduced N-acetylaspartate (NAA)/Creatine (Cr) ratio[172].

The most frequently reported features include bilateral, symmetric hyperintensities of the basal ganglia, particularly the putamen and caudate nuclei, often seen in Leigh syndrome and other

early-onset encephalopathic presentations. These lesions are typically hyperintense on T2-weighted and FLAIR sequences and may show restricted diffusion during acute stages. The brainstem, thalami, and cerebellar dentate nuclei are also commonly affected, especially in disorders due to *SURF1*, *NDUFS1*, and *PDHA1* mutations[173,139].

Stroke-like lesions, which affect the cortex and subcortical white matter in a non-vascular distribution, are hallmark features of MELAS syndrome. These lesions are often migratory and associated with elevated lactate peaks on magnetic resonance spectroscopy (MRS), reflecting metabolic crises[174,128].

In some phenotypes, the predominant finding is extensive white matter involvement, which may mimic leukodystrophies, such as those caused by *POLG* mutations[175]. Patients with *EARS2* deficiency often display combined subcortical white matter involvement sparing periventricular white matter and deep grey matter abnormalities, including the basal ganglia and thalami[176,177].

Over time, MRI may reveal cavitation and atrophy of affected structures, particularly the basal ganglia in Leigh syndrome. However, transient or reversible lesions have also been reported, especially during early or fluctuating phases of disease[178].

To facilitate objective assessment of neuroimaging abnormalities in mitochondrial disease, semi-quantitative tools such as the Radboud Centre for Mitochondrial Medicine Paediatric MRI Score (RCMM-PMRIS) have been developed. This scoring system focuses on the six most characteristic MRI abnormalities, enabling reproducible evaluation of disease burden, longitudinal monitoring of progression, and potential use as an outcome measure in therapeutic trials[171]. More recently, imaging–genomic correlation approaches have highlighted how distinct radiological patterns can guide targeted genetic testing, with features such as cortical stroke-like lesions in MELAS, basal ganglia and thalamic involvement in *POLG*-related disorders, and posterior brainstem abnormalities in *SURF1* deficiency providing diagnostic clues[179]. Furthermore, large multicentre studies applying MRI pattern recognition in childhood basal ganglia disorders have shown that mitochondrial and other neurometabolic diseases cluster into reproducible imaging patterns, supporting the use of standardized radiological scoring systems and diagnostic algorithms to refine differential diagnosis and facilitate gene discovery[180].

### 3.5.5. Histological Analysis

The most characteristic histological finding is Ragged-Red Fibers (RRF), observed with Gomori trichrome staining, that represent abnormal accumulation of mitochondria within the muscle fibbers, giving them a distinct “ragged” appearance. This feature is a hallmark of many mitochondrial diseases, such as MERRF and KSS[181]. Despite not being frequent during childhood, their presence is considered more specific and cut-off are lower than for adults[182,183].

COX-negative fibbers are skeletal muscle fibbers that lack detectable cytochrome c oxidase (complex IV) enzymatic activity on histochemical staining, representing focal or generalized defects of the mitochondrial respiratory chain[184].

On electron microscopy, subsarcolemmal and intermyofibrillar accumulations of mitochondria are commonly observed, providing ultrastructural evidence of mitochondrial dysfunction[185].

### 3.5.6. Functional OXPHOS assessment

Functional assessment of mitochondrial respiratory chain function is critical for diagnosing mitochondrial diseases and is commonly performed in muscle and, less commonly in fibroblasts.

Functional assessment of the mitochondrial respiratory chain relies on spectrophotometric measurement of enzyme activities of complexes I–IV in skeletal muscle (and, less commonly, in fibroblasts). Complex I (NADH:ubiquinone oxidoreductase) activity is typically assayed by monitoring NADH-dependent reduction of ferricyanide; complex II (succinate dehydrogenase) by the reduction of 2,6-dichlorophenolindophenol (DCPIP); complex III (cytochrome bc1 complex) by assessing cytochrome c reduction–oxidation; and complex IV (cytochrome c oxidase, COX) by following the oxidation of reduced cytochrome C[186,187]. In addition to the individual complex activities, citrate synthase (CS) activity and as a tricarboxylic acid cycle enzyme restricted to the mitochondrial matrix, is routinely measured as a robust marker of mitochondrial content[186,187]. CS normalization improves diagnostic accuracy by accounting for differences in mitochondrial abundance between samples, as in reductions due to decreased mitochondrial content or muscle atrophy[188]. These enzymatic assays represent the current gold standard for functional evaluation of respiratory chain deficiencies. Residual activities below 40% of the control mean are generally considered abnormal, with values between 20–30% representing a borderline range and activities under 20% strongly indicative of a respiratory chain defect[182]. However, these thresholds require cautious interpretation and correlation with clinical and histological findings, and normal activities may still be observed in patients with a definite primary mitochondrial disorder[93,144,182].

ATP-generating capacity can be assessed by a luciferase-based bioluminescence assay, by measuring the emitted light during the conversion of ATP to ADP, which is proportional to the ATP concentration. A marked reduction in ATP production indicates impaired OXPHOS function[189].

Real-time measurements of oxygen consumption rates (OCR) provide dynamic insights into mitochondrial bioenergetics. Using analysers such as the Seahorse XF platform, basal respiration is recorded, followed by sequential addition of pharmacological inhibitors and uncouplers. This allows dissection of ATP-linked respiration, proton leak, maximal respiratory capacity, and spare respiratory capacity. Such profiling complements enzymatic assays by capturing the integrated respiratory performance of intact cells[190].

### 3.5.7. Quantification of mtDNA copy number

The diagnostic assessment of mtDNA copy number can be performed by Southern blot, dot blot, or quantitative real-time PCR, comparing the mtDNA content relative to nuclear DNA in clinically affected tissues[112,191,192]. mtDNA copy number can be measured by quantitative PCR (qPCR), most often by comparing mtDNA amplification to a nuclear single-copy gene[193]. Droplet digital PCR (ddPCR) allows absolute quantification with higher precision, particularly in samples with low template amounts[193]. Next-generation sequencing (NGS) read-depth analysis can also estimate mtDNA content[194].

mtDNA content is highly tissue and age-dependent; analyses should preferentially use the affected tissue and, regardless of platform, normalization to nuclear DNA and comparison with age-matched controls are essential to account for physiological variability[195,196]. Residual mtDNA levels below 30% of age-matched controls are generally considered consistent with depletion, although no universal consensus exists[192,196,197]. However, mtDNA copy number may be normal in unaffected tissues such as muscle, blood, or fibroblasts in some patients with documented *DGUOK* mutations, requiring a liver biopsy to confirm the diagnosis{Citation}.

## 4. Diagnostic approach of mitochondrial disorders

The diagnosis of mitochondrial disease requires a comprehensive and integrative strategy, combining clinical, biochemical, and molecular investigations. Initial evaluation should begin with a detailed clinical assessment. Based on this suspicion, first-line laboratory investigations may reveal patterns suggestive of mitochondrial dysfunction. However, these findings are often nonspecific and should be interpreted in context.

Ultimately, molecular genetic testing has become the cornerstone for establishing a definite diagnosis, but its interpretation still depends on correlation with clinical and biochemical data. In this way, a structured stepwise approach combining clinical, biochemical, imaging, histological, and genetic findings is essential to establish a definite diagnosis and initiate appropriate genetic counselling and management.

### 4.1. Clinical suspicion and red flags for mitochondrial disorders

Mitochondrial disorders are highly heterogeneous, and their diagnosis requires a high index of clinical suspicion. Early recognition is critical, as timely diagnosis can inform appropriate management and genetic counselling. Several key clinical, biochemical, and genetic features can serve as red flags for mitochondrial disease.

Consanguinity or shared geographic ancestry may indicate a higher likelihood of recessive nuclear-encoded mitochondrial disorders while a maternal inheritance pattern is strongly suggestive of an mtDNA-related disease.

The co-occurrence of neuromuscular manifestations (ptosis, progressive external ophthalmoplegia, myopathy, ataxia) with central nervous system features (developmental delay, seizures, encephalopathy, or stroke-like episodes not respecting vascular territories) is highly suggestive. Cardiac involvement (cardiomyopathy, arrhythmias), endocrine dysfunction (particularly diabetes), or the association of sensorineural hearing loss, optic atrophy, or pigmentary retinopathy with neurological disease should raise strong suspicion of a mitochondrial aetiology [93,198].

A progressive or stepwise course, often punctuated by episodes of metabolic decompensation triggered by increased energy demands, such as during periods of fasting, infections, fever, dehydration, or surgical procedures, represents a typical red flag. Drugs known to be mitochondrial toxic (e.g., valproate, aminoglycosides, linezolid) can exacerbate disease and should prompt caution. Recognizing these exacerbating factors is essential for both diagnosis and long-term management[198].

Persistent lactic acidosis in the absence of hypoxia remains a classical biochemical hallmark. 3-MGA aciduria when particularly elevated can be a discriminative feature for some genes[159]. Elevated FGF-21 and GDF-15 have emerged as robust serum biomarkers for mitochondrial myopathies.

Muscle biopsy findings such as ragged-red fibbers and COX-negative fibbers continue to be considered diagnostic red flags, particularly when genetic findings are inconclusive[107,198].

### 4.2. Diagnostic criteria

To standardize diagnostic approaches and stratify the likelihood of MD, several scoring systems have been proposed over the past three decades. Among the most influential are the frameworks developed by Walker for adults patients and later modified for paediatrics and the (Nijmegen) Mitochondrial Disease Criteria (MDC) and its subsequent updates[150,182,199–201].

The Modified Walker (or Bernier) criteria system is based on major and minor criteria across several domains: clinical presentation, neuroimaging, histopathology, respiratory chain enzymology, and molecular. For all there are major and minor criteria, except for genetic in which the identification of a nuclear or mtDNA mutation of undisputed pathogenicity is a major criterion. A definite diagnosis requires two major, or one major plus two minor criteria[182].

The original MDC proposal provided one of the earliest paediatric-focused diagnostic frameworks, combining general criteria (clinical, metabolic investigations and imaging, and histopathology can each contribute a maximum of four points) plus biochemical criteria (OCR, ATP production rate, and respiratory chain enzyme activity, that in combination confer a biochemical likelihood for a MD)[150].

Recognizing the need for a more standardized and quantifiable tool in children, a a bedside version based on a scoring system (0–12 points) was later proposed. It weighs data across three domains: clinical (muscular, CNS and multisystem disease), metabolic/neuroimaging findings abnormalities, and morphology from histopathologic studies. Each domain contributes independently (maximum 4 points each)[200].

The most recent revision, the Nijmegen Mitochondrial Disease Criteria, further adapts the latter score to the post-genomic era. This structured system assigns scores across three domains: clinical and metabolic & imaging with a total score of 8. A score ≥8 defines a definite mitochondrial disorder, 5–7 as probable, 3-4 as possible, and ≤2 as unlikely. Subsequent genetic findings could either confirm a diagnosis or elicit further evaluation through muscle biopsy and Respiratory chain complex deficiency or later functional assays[201].

A comparative summary of the major diagnostic frameworks for mitochondrial disease, highlighting their target populations, structure, scoring systems, and evolution over time, is presented in Table 6.

***Table 6. Comparison of diagnostic criteria for mitochondrial disease***

| | Bernier et al. (2002) | Wolf & Smeitink (2002) | Morava et al. (2006) | Witters et al. (2018) |
|---|---|---|---|---|
| **Target population** | Adults & children | Infants & children (0–16 y) | Paediatric | Paediatric & adult |
| **Domains** | Clinical<br>Neuroimaging,<br>Histopathology,<br>Respiratory chain enzymology<br>Molecular | General (clinical, metabolic/imaging, histopathology)<br>Biochemical | Clinical<br><br>Metabolic/imaging,<br>Morphology | Clinical<br>Metabolic/imaging |
| **Biochemical markers** | Not included | Not included | Lactate (B, CSF), alanine, L/P ratio<br>OAs: Krebs' cycle intermediates; Ethylmalonic, methylmalonic or 3-MGA aciduria | Lactate and Alanine (B, CSF) OAs: Krebs' cycle intermediates; Ethylmalonic, methylmalonic or 3-MGA |
| **Muscle biopsy** | Major/minor criteria (ragged-red fibbers, COX-negative fibbers) | Histopathology up to 4 points in "general" | Morphology (RRF, COX−, SDH/COX staining, EM) up to 4 points | Excluded from 0–8 score; used as supportive data |
| **Biochemical assays** | Respiratory chain enzyme activities, ATP synthesis (major/minor) | Biochemical block: $^{14}$C-substrate oxidation, ATP/PCr | Not included | Not included |

| | | | | |
|---|---|---|---|---|
| | | production, single enzyme assays | | |
| **Scoring** | Major (M)/minor (m) criteria | Combination of general and biochemical to form a matrix | Total score 12 (max 4 points each) | Total score 8 (max 4 points each) |
| **Classification** | Definite: ≥2 M or 1M+2m<br>Probable: 1M+ 1m or ≥3 m<br>Possible: 1 M or 2m | Matrix: Unlikely, Possible, Probable, Definite | Definite 8-12<br>Probable 5–7<br>Possible 2-4<br>Unlikely <2 | Definite ≥8<br>Probable 5–7<br>Possible 3-4<br>Unlikely <2 |
| **Validation** | Internally validated in the original cohort (n=118); no external/prospective validation | Initially tested in a paediatric cohort (n=30) with suspected respiratory chain disorders; limited internal validation. | Retrospectively validated in 44 children with genetically confirmed MD and 17 with non-mitochondrial genetic disorders; demonstrated diagnostic specificity. | Multicentre (n=136, genetically confirmed PMD); AUC (~0.91) showing high discriminative power for molecular confirmation |

Together, these frameworks offer complementary perspectives depending on the clinical setting and available resources. While pre-genomic systems emphasized muscle biopsy and metabolic testing, contemporary approaches increasingly incorporate molecular diagnostics while maintaining the value of functional assays and histological confirmation.

Currently, these provide a basis for stratifying clinical suspicion and guide further studies, rather than affirming a diagnosis.

### 4.3. Genetic and definite diagnosis of Mitochondrial Disease

The diagnosis of PMD has progressively evolved from a histopathology- and biochemistry-driven approach to one that is largely based on molecular genetics. A definite diagnosis is achieved when a pathogenic or likely pathogenic variant in a gene associated with mitochondrial disease is identified, in either mtDNA or nuclear DNA, with supportive clinical and/or biochemical evidence[93] . This transition has improved diagnostic precision, facilitated genetic counselling, and enabled individualized management plans.

Molecular confirmation relies on genetic studies, which may include:

- Complete mtDNA sequencing, typically performed on blood or muscle;
- Targeted nuclear gene panels associated with mitochondrial disorders or clinical exome sequencing when the phenotype is unclear;
- Screening for mtDNA deletions, preferably using skeletal muscle tissue for increased sensitivity;
- Segregation analysis and family-based testing, to clarify variant pathogenicity and inheritance.

Regardless of the genome involved, functional correlation with biochemical phenotypes remains essential, especially in cases with partial or atypical presentations.

Importantly, a negative or inconclusive genetic result does not exclude the diagnosis of a mitochondrial disease. In such cases, phenotype reassessment and consideration of secondary mitochondrial dysfunction and alternative diagnoses (mitochondrial mimics) should be pursued.

### 4.3.1. Diagnostic Yield of Next-Generation Sequencing strategies

NGS technologies, including targeted panels, whole-exome sequencing (WES), and whole-genome sequencing (WGS), have markedly improved the diagnostic yield in patients with suspected mitochondrial disorders. Several studies report yields ranging from 35% to 60%, depending on patient selection, inclusion of mtDNA sequencing, and the availability of trio-based analyses[49,202–206] . However, availability even in European countries is not universal and response times are frequently over 6 months[207].

Targeted panels focusing on known mitochondrial genes can achieve yields around 30–40%, with the advantage of faster turnaround times and easier variant interpretation[208]. WES allows broader coverage and the possibility of diagnosing mitochondrial mimics or discovering novel disease genes but often generates variants of uncertain significance and may miss intronic or structural variants. WGS further improves coverage and enables mtDNA variant calling from blood samples, but its higher cost and complexity limit routine use in many centres[100,209].

Combined sequencing of nuclear and mitochondrial genomes in critically ill children has been shown to increase diagnostic yield and expedite management decisions[202].

## 4.4. Muscle biopsy in the diagnosis of Mitochondrial Disorders

Once a first line exam for suspected MD, muscle biopsy remains a pivotal diagnostic tool in mitochondrial diseases, particularly when clinical presentations are nonspecific, and diagnosis is challenging.

Despite advances in genetic and biochemical diagnostics, muscle biopsy remains a critical component in diagnosing mitochondrial diseases. The combination of histological findings, such as RRF and COX-negative fibbers, with biochemical analysis provides a comprehensive understanding of mitochondrial dysfunction in skeletal muscle.

While needle biopsy and excisional biopsy are both valuable techniques for obtaining muscle samples in mitochondrial diseases, they differ in terms of invasiveness and diagnostic objectives. Needle biopsy, being less invasive, is often used for initial histological analysis and rapid biochemical testing, whereas excisional biopsy allows for the collection of larger tissue volumes,

making it more suitable for genetic evaluations and detailed functional studies of the mitochondrial respiratory chain. At our centre, we prefer to perform excisional muscle biopsy, where one portion of the sample is used for histological and immunohistochemical studies, while the other portion is utilized for functional analyses of the respiratory chain and genetic tests, such as mtDNA sequencing, rearrangement screening (deletions/duplications), and quantification of mtDNA copy number.

Muscle biopsy remains an essential tool for diagnosing mitochondrial diseases, especially in cases where other diagnostic tests, such as genetic sequencing or biochemical assays, do not provide conclusive results. Moreover, muscle biopsy helps distinguish between mitochondrial disorders and other conditions with overlapping symptoms, such as inclusion body myositis or Duchenne muscular dystrophy[185].

However, the presence of ragged-red fibbers is not exclusive to mitochondrial disorders and can also be observed in normal aging or in other myopathies[183]. Additionally, in some mitochondrial diseases, muscle biopsy may appear normal, especially in the early stages of the disease or in younger children[93,123] Respiratory chain deficiencies can also just appear later in MIDDS.

## 4.5. Challenges in diagnosing mitochondrial disorders

### 4.5.1. Functional and Biochemical Testing for undiagnosed patients

The detection of VUS is a frequent outcome of WES and WGS in mitochondrial disease diagnostics. In patients where NGS fails to identify a definitive molecular diagnosis, functional and biochemical studies remain essential. These include respiratory chain enzyme activity assays or OCR in muscle or fibroblasts, and metabolomic or proteomic studies. Quantification of mtDNA copy number and analysis of multiple deletions may also provide supportive evidence of mtDNA maintenance defects[100].

Advanced tools such as RNA sequencing and quantitative proteomics have enabled the functional validation of candidate variants, reclassification of VUS, and discovery of novel disease mechanisms. Their integration into the diagnostic workflow is expected to play a key role in the next decade[100,210].

### 4.5.2. Mitochondrial mimics

A growing proportion of patients with suspected mitochondrial disease are ultimately found to have alternative genetic conditions that phenotypically resemble mitochondrial disorders. These are often referred to as mitochondrial mimics or phenocopies[93,210].

As introduced in Section 1.2, mitochondrial dysfunction may result from pathogenic variants in genes not directly impairing oxidative phosphorylation or other essential mitochondrial pathways. These cases represent an etiological category distinct from PMD, referred to as mitochondrial mimics or MD phenocopies, in which mitochondrial dysfunction arises secondarily from pathogenic variants in genes whose primary function lies outside the canonical mitochondrial pathways. Although these genes are not directly involved in the biogenesis, structure, or catalytic activity of the respiratory chain complexes, their dysfunction leads to downstream mitochondrial impairment through disrupted metabolic crosstalk, abnormal organelle interactions, or accumulation of toxic intermediates.

Although the terms “mimic” and “phenocopy” are sometimes used interchangeably, phenocopy classically refers to a condition that replicates the phenotype of a defined genetic disorder without sharing its underlying molecular cause. This concept has since evolved to include genetic diseases that affect mitochondrial function indirectly or exhibit overlapping multisystem phenotypes. These conditions involve diverse cellular pathways, including peroxisomal metabolism, DNA replication, glycosylation, ion channel activity, and transcriptional regulation, yet can reproduce clinical and metabolic hallmarks of mitochondrial disease. Table 7 summarises selected examples of mitochondrial disease mimics, grouped by affected pathway and overlapping features with PMD.

***Table 7. Examples of mitochondrial disease mimics (phenocopies).***

| Category | Disease | Overlapping features | Ref. |
|---|---|---|---|
| **Disorders of vitamin and cofactor metabolism** | Disorders of intracellular cobalamin metabolism | Elevated lactate, basal ganglia lesions, complex deficiency | [12,38,39] |
| **Peroxisomal disorders** | Zellweger spectrum disorders | Myopathy, histological and ultra-structural findings | [211] |
| **Disorders of complex molecule degradation** | GM1 gangliosidosis, Niemann–Pick type C, Ceroid lipofuscinosis type 2 and 3 | Myopathy, developmental regression, basal ganglia lesions | [12,40–43] |
| **Congenital disorders of glycosylation (CDG)** | PMM2-CDG, ALG1-CDG | Cerebellar atrophy, multiorgan involvement, lactic acidosis, stroke-like episodes | [212] |
| **Channelopathies** | Epileptic encephalopathies, alternating hemiplegia of childhood, spinocerebellar ataxia | Stroke-like episodes, basal ganglia lesions, low coenzyme Q10 in muscle | [34,39–42,44–48,69] |
| **Neuromuscular disorders** | Spinal muscular atrophy, Charcot-Marie-Tooth, muscular dystrophies (e.g. Duchenne or desminopathy), congenital myasthenic syndromes, inclusion body myositis | Progressive or congenital muscle weakness, myopathic features, red-ragged fibbers and/or complex deficiency, mitochondrial DNA depletion | [34,39–42,44–48,213,46,33] |
| **Others / metabolic regulators** | *MORC2*, *HPDL*, *MECP2*, *PNPLA4*, *WFS1*, *WOOX*, *STXBP1* | Leigh-like MRI, elevated lactate, neurodegeneration | [39,35,42,214,49] |

Efforts to define distinguishing features between PMD and mimics - such as the absence of maternal inheritance, lack of RFF, or normal respiratory chain enzymology - have shown limited

diagnostic value. Many mimics present with elevated lactate, Leigh-like MRI lesions, or combined respiratory chain deficiencies in muscle biopsies[39,69]. With the increasing use of WES and WGS, an expanding number of patients with strong clinical suspicion for PMD are eventually diagnosed with non-mitochondrial genetic disorders. In large cohorts, 20–30% of such cases are reclassified as mitochondrial mimics[35,42,69].

The distinction between primary and secondary mitochondrial dysfunction is essential in clinical practice, as it influences genetic counselling, prognostic considerations, and therapeutic decisions[215,216]. Whole exome and genome sequencing (WES/WGS), complemented by functional and biochemical investigations, remain the most effective approaches for diagnostic resolution. Reanalysis of sequencing data, integration of multi-omics information, and the use of curated resources (e.g., MitoCarta, GENOMITexplorer) are increasingly relevant for clarifying undiagnosed cases[86,215,216].

While a confirmed pathogenic variant in a gene directly involved in mitochondrial function supports a diagnosis of PMD, recognition of secondary disfunction may redirect the diagnostic workup toward an alternative or potentially treatable aetiology. Misattributing secondary dysfunction to a primary mitochondrial disorder may lead to overdiagnosis, unnecessary invasive investigations, or ineffective treatments. Conversely, identifying reversible contributors to mitochondrial dysfunction can have direct implications for patient management, including metabolic correction, immunomodulation, or discontinuation of mitochondrial toxins[12,33,42].

Importantly, the distinction between PMD and mimics is not always binary. Some conditions initially classified as mimics may later be found to involve nuclear-encoded factors that indirectly modulate mitochondrial homeostasis, bioenergetics, or cofactor metabolism.

Among these mimics, disorders involving nuclear epigenetic regulation have gained attention. A notable example is *MORC2*-related disease, caused by mutations in the *MORC2* gene, which encodes a nuclear ATPase involved in chromatin remodelling and transcriptional repression. Although *MORC2* is not directly implicated in OXPHOS or mtDNA replication, its dysfunction has been associated with neurodevelopmental syndromes and axonal neuropathies that phenotypically overlap with mitochondrial disease. Functional studies demonstrate that *MORC2* mutations can disrupt the expression of nuclear-encoded mitochondrial genes and cause secondary mitochondrial bioenergetic impairment[119,217]. *MORC2*-related disease thus exemplifies epigenetically mediated mitochondrial dysfunction and highlights the need to consider non-canonical mechanisms in the diagnostic evaluation of suspected mitochondrial disorders.

# 5. Disease course and monitoring

## 5.1. Natural history

PMD exhibit a highly heterogeneous natural history, with significant variability in age at onset, rate of progression, and organ involvement. Most disorders manifest during childhood - approximately 80% before the age of 18 and 60% before 18 months – often with multisystemic phenotypes involving the central nervous system, muscles, heart, liver, gastrointestinal tract, and eyes. Elevated lactate is the most frequently reported biochemical abnormality, observed in over 70% of PMD entities[141].

Prognosis varies markedly across PMD subtypes, with early-onset presentation, hepatocerebral or cardiomyopathy involvement generally associated with poorer outcomes. Age of death is reported to be below 10 years in approximately 74% of disease entities, and below 5 years in more than half[141]. Earlier presentation is particularly important for some disorders such as LSMDS, *POLG* and *R2MMB*[77,218]. Survival is shortest in hepatocerebral syndromes and mitochondrial cardiomyopathies (median survival less than one year), and longest in phenotypes such as chronic progressive external ophthalmoplegia (CPEO) and SANDO (median above 20 years)[141,219,220].

Even within the same disease, genotype can also influence clinical course, with truncating genetic variants appear to predispose to worse prognosis[141]. Conversely, certain heteroplasmic mtDNA mutations with low tissue load may confer milder or organ-specific phenotypes. However, the same genetic variant may manifest with markedly different severity between individuals, reflecting the influence of heteroplasmy, nuclear modifiers, epigenetic factors, and environmental stressors.

Prognostic factors are summarized in Table 8, including some gene-specific prognostic factors such as anaemia and liver involvement in *POLG*[116].

***Table 8. Factors associated with worse prognosis in primary mitochondrial disease.***

| | Prognostic factor |
|---|---|
| **Age of onset** | Neonatal or infantile onset[141,218,221,220] |
| **Genotype** | Truncating variants[141] |
| | High heteroplasmy[82,222,223] |
| **Initial clinical features** | Multisystem involvement at diagnosis[141] |
| | Failure to thrive[224] |
| | Severe neurodevelopmental delay or regression[141] |
| | Intractable epilepsy or early seizure onset[218] |
| **Organ involvement** | Cardiomyopathy and arrythmia[141,219] |
| | Liver disease (including liver failure)[141,218,220] |
| | Anaemia[225] |
| | Renal involvement – chronic kidney disease (CKD) or acute kidney injury (AKI)[226] |
| | Respiratory insufficiency[224] |
| **Metabolic profile** | Persistent lactic acidosis and metabolic decompensations[224,227] |
| **Progression** | Rapid clinical deterioration after first symptoms[224,228] |

Given the variability and unpredictability of disease evolution, risk stratification should be individualized, considering both static (genotype, onset) and dynamic parameters (clinical progression, organ involvement). Multidisciplinary case review, integration of biochemical and neuroimaging data, and close follow-up are essential to adjust prognosis over time and support decision-making for patients and families.

Quality of life may be significantly diminished due to reduced autonomy, reliance on medical aids such as gastrostomy and ventilatory support, and an increased risk of mental health issues and hospitalization[141,229]. Morbidity is equally diverse, with frequent hospitalizations due to neurological decompensation, metabolic crises, infections, or cardiorespiratory complications). Respiratory failure and pulmonary infections are among the most frequent causes of death in mitochondrial patients[220,230].

PMD impose a substantial disease burden not only on affected individuals, who often experience progressive disability, multisystem complications, and reduced quality of life, but also on their families, who face significant psychosocial stress, financial strain, and caregiving responsibilities[231].

Comprehensive natural history studies are essential to define disease trajectories, establish genotype–phenotype correlations, and inform both clinical surveillance and therapeutic strategies. Measurable outcomes can serve as a baseline for evaluating efficacy in single-arm clinical trials[141]. However, current studies are often limited by retrospective designs, phenotypic and genetic heterogeneity, and underrepresentation of mildly affected or adult-onset cases.

Standardization of natural history data collection, including core clinical outcomes, biomarker trajectories, and patient-reported measures, is critical to improve trial readiness and comparability across cohorts[232,233].

### 5.2. Monitoring and clinical follow-up

Regular surveillance enables early detection of subclinical organ involvement, guides supportive interventions, and prevents irreversible complications. Although follow-up strategies should be tailored to the patient's genotype and clinical presentation, structured monitoring protocols have been proposed to ensure comprehensive care for any PMD[123,234]. The frequency of evaluations may need to be adapted according to disease severity, progression rate, and specific system involvement.

Multidisciplinary case management should be established early, particularly in patients with complex or multisystemic involvement. Coordination among neurologists, geneticists,

cardiologists, nephrologists, endocrinologists, and rehabilitation professionals enables personalized care and timely interventions, adapted to each patient's evolving needs.

Supportive care includes metabolic and nutritional optimization, coordination of specialist care, access to emergency guidelines, and genetic counselling. Patients and families should be provided with rare disease identification cards and emergency protocols to ensure adequate care in acute settings[234,235]. This ensures appropriate care in emergency settings and reduces the risk of iatrogenic harm, particularly during metabolic stress or anaesthesia.

Genetic counselling should address recurrence risk, inheritance patterns, reproductive options, and implications for family members, with particular attention for mtDNA-related diseases particularities[100,236]. Cascade testing should be sought for any relative at risk, including underage asymptomatic patients in whom disease onset is expected to be early onset and for whom surveillance would allow early intervention with disease-modifying treatments.

Supportive care plays a critical role in the long-term management of patients with mitochondrial disease, aiming to prevent metabolic decompensation, ensure coordinated multispecialty care, and support patients and families throughout the disease course.

## 6. Therapeutic Approaches for Mitochondrial Disorders

Mitochondrial disorders present significant therapeutic challenges due to their genetic heterogeneity and lack of universally effective treatments. Current management strategies focus on symptom relief, metabolic supplementation, and lifestyle interventions, while emerging therapies, including gene therapy and mitochondria-targeted pharmacological interventions, are undergoing clinical investigation.

### 6.1. Non-Pharmacological Interventions

#### 6.1.1. Nutritional interventions

The ketogenic diet (KD), characterised by high fat and low carbohydrate intake, promotes ketone body utilisation as an alternative energy source, while also modulating excitatory–inhibitory balance in the brain. KD has shown benefit in refractory epilepsy associated with mitochondrial disorders and in pyruvate dehydrogenase complex deficiency[237,238].

For patients with gastrointestinal dysmotility, a low-residue diet as shown benefits in reduction of stool consistency for those with diarrhoea and reductio of laxative use for those with constipation[239].

Other dietary manipulations, including high-protein, high-fat or low-carbohydrate regimens, have been proposed to adapt energy supply to specific enzymatic defects[240–242].

### 6.1.2. Exercise training

Exercise training represents a non-pharmacological but potent stimulator of mitochondrial biogenesis, through repeated activation of AMPK, SIRT1, and PGC-1α, leading to increased mitochondrial content and improved oxidative capacity. Controlled exercise regimens in mitochondrial disease patients have shown improvements in exercise tolerance and oxidative capacity, reinforcing the concept that pharmacological biogenesis stimulators aim to mimic. In patients with mitochondrial myopathies, aerobic training improves peak oxygen consumption ($VO_2$ peak), exercise tolerance, and muscle oxidative capacity without evidence of deleterious effects. Resistance training has additionally been shown to counteract muscle atrophy and improve quality of life[243–245].

Importantly, exercise interventions must be carefully tailored, as excessive exertion may exacerbate muscle fatigue or trigger metabolic decompensation in susceptible individuals.

### 6.1.3. Transplantation

Hematopoietic stem cell transplantation (HSCT) has been used in patients with MNGIE, with variable success depending on disease stage[246,247].

Organ transplantation has been considered for patients with severe organ-specific manifestations of PMD. Successful cases of liver transplantation have been reported in children with mitochondrial DNA depletion syndromes (e.g., *DGUOK*, *TK2*, *MPV17*) presenting with acute liver failure[248–250]. Similarly, kidney transplantation may be considered in mitochondrial nephropathies or syndromes with renal involvement[251,252], while heart transplantation has been performed in Barth syndrome (*TAZ*), with outcomes comparable to other forms of inherited cardiomyopathy[253] .

Outcomes are often compromised by multisystemic involvement and risk of perioperative metabolic decompensation. Current expert consensus suggests that transplantation should be reserved for selected patients with end-stage organ-predominant disease, considering other comorbidities[234,254].

## 6.2. Pharmacological interventions

### 6.2.1. Vitamins and cofactors

Coenzyme Q10 (ubiquinone) is the archetypal antioxidant therapy in mitochondrial medicine. As a lipid-soluble electron carrier in the respiratory chain and a major antioxidant, it is the treatment of choice in primary CoQ10 biosynthesis defects, where supplementation can markedly improve

neurological and muscular manifestations[255]. In non–CoQ10 deficiency mitochondrial disorders, results are more heterogeneous, with some studies reporting improved exercise tolerance or decreased lactate levels, while others found limited clinical benefit[207]. A meta-analysis of CoQ10 supplementation in mitochondrial myopathies found modest improvements in muscle function but no clear evidence of disease modification[256].

Idebenone, a short-chain synthetic analogue of $CoQ_{10}$, demonstrates superior bioavailability and has been approved in Europe for the treatment of Leber hereditary optic neuropathy (LHON). Controlled clinical trials showed improved visual recovery in subsets of patients[256].

Overall, while the biochemical rationale for the use of vitamins, cofactors, and antioxidants is strong, robust evidence of clinical efficacy remains scarce. These compounds continue to play an important role in symptomatic management, particularly given their safety profile, but they cannot yet be considered disease-modifying therapies.

Often, the cornerstone of therapy is the so-called “mitochondrial cocktail”, a combination of vitamins, cofactors, and antioxidants aimed at optimizing mitochondrial function and reducing oxidative stress. Among the most used compounds are Coenzyme Q10 (ubiquinone and ibedenone), riboflavin (vitamin B2), and L-carnitine, all of which contribute to ETC efficiency and fatty acid oxidation[207,257].

A global survey on vitamin and cofactor use in mitochondrial diseases found that 95% of specialists recommend supplementation, either universally or tailored to specific phenotypes. Despite widespread prescription, the study highlighted significant regional and specialty-specific differences in supplementation practices, underscoring the absence of standardized guidelines in this area. The findings emphasize the need for more rigorous clinical and preclinical investigations to evaluate the efficacy of these supplements. The observed variability in prescribing practices suggests a reliance on individual clinical judgment in the absence of robust evidence-based protocols[257].

Despite their widespread use, the efficacy of these interventions remains poorly substantiated by randomized controlled trials (RCTs) and lack control use of pharmaceutical formula and dosage[258]. A common clinical practice consists of administering a mitochondrial cocktail for a trial period of 3 to 6 months, followed by individualized assessment of clinical or functional response. This approach allows for the identification of potentially responsive patients, despite the absence of standardized biomarkers or universally validated outcome measures. Developing clear consensus statements and standardized guidelines is crucial to optimize therapeutic management of PMDs, ensuring treatments are grounded in validated clinical efficacy[257].

### 6.2.2. Anaplerotic and Substrate Therapies

Arginine and citrulline have been investigated in *MELAS syndrome*, where stroke-like episodes are linked to nitric oxide (NO) deficiency and impaired cerebral perfusion. Both compounds serve as NO precursors, and intravenous or oral arginine supplementation has been associated with reduced frequency and severity of stroke-like episodes in case series and small clinical studies Citrulline, being more efficiently converted to arginine, may provide a more sustained effect, but controlled trials are lacking.[259,260].

### 6.2.3. Redox Modulators and Antioxidants

EPI-743 (vatiquinone) is a para-benzoquinone that modulates cellular redox status via NAD(P)H:quinone oxidoreductase 1 (NQO1). Early-phase clinical trials in Leigh syndrome and other paediatric mitochondrial disorders suggested potential stabilisation of neurological decline[261,262].

Sonlicromanol (KH176) is a small molecule with redox-modulating properties and additional effects on redox-sensitive signalling. Phase II studies in adults with mitochondrial disease demonstrated safety and improvements in fatigue and neurocognitive function[263].

KL1333, a redox-active $NAD^+$ modulator, is designed to rebalance $NAD^+$/NADH ratios and indirectly enhance sirtuin-dependent pathways. Early clinical studies reported tolerability and positive effects on fatigue and larger trials are ongoing[264,265].

### 6.2.4. Mitochondria-Targeted Agents

A distinct therapeutic approach in mitochondrial medicine is the development of molecules that accumulate specifically at the inner mitochondrial membrane to stabilise bioenergetics and reduce oxidative stress. Elamipretide (SS-31) is the most advanced compound in this category. It is a cell-permeable tetrapeptide that selectively targets cardiolipin, stabilising the inner mitochondrial membrane, optimising electron transport, and reducing ROS production. Preclinical studies demonstrated improved mitochondrial respiration and ATP production across multiple models of mitochondrial dysfunction. Clinically, elamipretide has shown benefit in Barth syndrome, a disorder of cardiolipin biosynthesis, where it improved cardiac function and exercise capacity. Based on these data, it has received regulatory approval for Barth syndrome in the United States. Trials in primary mitochondrial myopathies and other mitochondrial disorders showed mixed outcomes, with improvements in some functional endpoints but failure to meet primary efficacy measures.[266,267,223]

### 6.2.5. Biogenesis Modulators

Several agents aim to enhance mitochondrial biogenesis and improve oxidative capacity by activating transcriptional and metabolic pathways. These strategies often converge on regulators such as PGC-1α, AMP-activated protein kinase (AMPK), and peroxisome proliferator-activated receptors (PPARs).

Bezafibrate, a pan-PPAR agonist, was one of the earliest compounds tested as a biogenesis stimulator. Preclinical studies demonstrated increased mitochondrial mass and improved oxidative phosphorylation in cytochrome c oxidase–deficient mice. However, a pilot trial in patients with mitochondrial myopathy showed significant worsening of biochemical endpoints [268,269].

Resveratrol, a polyphenol that activates SIRT1 and AMPK, induces PGC-1α–mediated biogenesis and antioxidant responses. While animal models suggested beneficial effects on mitochondrial function and endurance, its clinical translation is hampered by poor bioavailability and inconsistent efficacy in humans[270].

### 6.2.6. Oligonucleosides

Oligonucleosides are modified nucleic acids designed to interact with mitochondrial nucleic acid metabolism, either by stabilizing RNA or supporting mtDNA replication. Deoxynucleoside supplementation, particularly with deoxycytidine and deoxythymidine, has demonstrated significant clinical benefit in TK2-related mtDNA depletion syndromes. This strategy restores nucleotide pools, supports mtDNA replication, and has been associated with improved motor and respiratory outcomes[271,272]. It represents one of the few genotype-targeted therapies with clinical validation.

More recently, an open-label phase 2 trial reported that deoxycytidine/deoxythymidine supplementation was safe and associated with improvements in clinical scores, EEG, and biomarker stabilization in patients with *POLG*-related disorders, expanding the potential therapeutic scope of this strategy[273].

### 6.2.7. Gene and RNA-Based Therapies

Adeno-associated virus (AAV)-mediated gene replacement has reached advanced clinical stages for Leber's Hereditary Optic Neuropathy (LHON). AAV2-ND4 delivered via intravitreal injection demonstrated partial visual recovery in Phase III trials representing a major milestone in mitochondrial gene therapy[274,275].

### 6.2.8. Mitochondrial Transfer and Replacement

Mitochondrial augmentation therapy (MAT) refers to the transplantation of exogenous functional mitochondria into *ex-vivo* patient-derived cells. Early-phase clinical applications are being explored in Pearson syndrome, with demonstrated short-term safety and feasibility of MAT[276].

Additionally, mitochondrial replacement therapy (MRT), which includes techniques like spindle transfer and pronuclear transfer, has been employed to prevent the transmission of mtDNA mutations from mother to child. These methods have led to the birth of healthy offspring free from mitochondrial disease[277].

## 6.3. Current Clinical Trials in Mitochondrial Disease

In recent years, therapeutic development for PMD has advanced from empirical supplementation toward mechanism-based interventions, as summarized in Table 9. These ongoing clinical trials range from lifestyle interventions, metabolic modulation, redox-targeting agents, and molecularly precise therapies. They illustrate a paradigm shift from generic mitochondrial "cocktails" to targeted approaches that directly address defined pathogenic mechanisms.

***Table 9. Current Interventional Clinical Trials in Primary Mitochondrial Disorders.***

| Drug/Intervention | Phase | Mechanism of Action | Indication | Identifier |
|---|---|---|---|---|
| **Triheptanoin** | II/III | Anaplerotic substrate; replenishes TCA cycle intermediates | Pyruvate Dehydrogenase Complex Deficiency | NCT06340685 |
| **Nicotinamide (vitamin B3)** | | $NAD^+$ precursor; promotes mitochondrial biogenesis | Dominant Optic Atrophy *OPA1* | NCT06007391 |
| **KL1333** | I/II | $NAD^+$ modulator; enhances redox balance and energy metabolism | Mitochondrial myopathy due to mtDNA variants | NCT05650229 |
| **N-acetylcysteine** | II | Glutathione precursor; redox modulation | m.3243A>G Mutation and Low Brain Glutathione Levels | NCT05241262 |
| **Cysteamine–pantetheine disulfide (TTI-0102)** | I | Prodrug enhancing glutathione and CoA metabolism | MELAS (including m.3243A>G) | NCT06644534 |
| **Elamipretide (SS-31)** | III | Cardiolipin stabilization; improves ETC coupling | Primary mitochondrial myopathy | NCT04689360<br>NCT05162768 |
| **Sonlicromanol (KH176)** | II | Redox modulation; ROS scavenging; neurocognitive effects | mtDNA-related PMD | NCT06451757 |
| **Zagociguat (CY6463)** | IIb | Soluble guanylate cyclase (sGC) stimulator; enhances NO–cGMP signaling | MELAS due to mtDNA variants | NCT06402123 |
| **Deoxynucleosides** | II | Nucleotide precursor supplementation for mtDNA replication | TK2 deficiency | NCT06754098<br>NCT06590493<br>NCT04802707 |
| **PYC-001** | I | Peptide-antisense oligomer conjugate targeting mRNA. | Dominant Optic Atrophy *OPA1* | NCT06461286 |

| | | | | |
|---|---|---|---|---|
| **Gene therapy for LHON (*ND4*)** | III | AAV-mediated gene replacement | LHON (m.11778G>A) | NCT02064569<br>NCT04912843 |
| **Mitochondrial augmentation therapy (MNV-201)** | I/II | Autologous $CD34^+$ HSPCs enriched with allogeneic mitochondria | Pearson with or without progression to Kearns–Sayre syndrome | NCT06017869 |
| **Mesangioblasts transfusion** | | Autologous mesoangioblasts infusion | Mitochondrial myopathy due to m.3243A>G | NCT05962333 |
| **Structured exercise programs** | II | Lifestyle intervention; stimulates mitochondrial biogenesis and function | Mitochondrial myopathy | NCT05346627 |

At the same time, persistent challenges, including small sample sizes, heterogeneous phenotypes, and the lack of validated biomarkers, highlight the urgent need for harmonized outcome measures and adaptive trial designs to accelerate translation from bench to bedside.

Together, these studies reflect the growing therapeutic momentum in mitochondrial medicine and highlight the shift toward biomarker-informed, genotype-specific strategies.

## 6.4. Clinical Trial Design and Outcome Measures in Mitochondrial Disease

The recent expansion of therapeutic strategies for mitochondrial disease - ranging from metabolic support to targeted gene or enzyme modulation - has highlighted the limitations of traditional clinical trial designs. In particular, the heterogeneity of PMD genotypes, clinical presentations, and progression rates poses challenges for standardized endpoints and regulatory evaluation.

To address this, innovative trial designs are increasingly being adopted. Adaptive and basket trial frameworks, which allow multiple therapeutic agents to be tested across diverse molecular subtypes, offer a more flexible and efficient approach. Moreover, the integration of real-world evidence (RWE), patient-reported outcomes (PROs), and digital biomarkers is enhancing the sensitivity and relevance of efficacy assessments.

Initiatives such as the Mitochondrial Disease Clinical Registry (MITOREG), the United Mitochondrial Disease Foundation (UMDF) Clinical Trials Program, and the E-Rare Consortium are playing a critical role in facilitating patient recruitment, harmonizing data collection, and supporting global collaboration.

Judicious endpoint selection in PMD trials, considering the limitations of many currently used clinical scales is mandatory and should include patient-centred outcomes that reflect clinically meaningful domains such as functional capacity, fatigue, and quality of life[232]. Adequate and multicentre studies including deep-phenotyping and natural history will allow proper selection of primary and secondary endpoints for clinical trials.

Innovative clinical trial designs tailored to address the complexities inherent in PMDs call for the use of adaptive and basket trials and stress the integration of real-world evidence (RWE), digital biomarkers, and longitudinal datasets[278]. Clinical trials also benefit from international collaboration for biomarker validation, patient stratification, and harmonization of outcome measures[279,232]. Together, these perspectives provide a roadmap for optimizing trial design and accelerating therapeutic development in mitochondrial medicine.

## 7. Omics in Mitochondrial Disease

Omics technologies encompass large-scale, high-throughput analytical approaches that characterize the global molecular landscape of biological systems. These include genomics (DNA), transcriptomics (RNA), proteomics (proteins), epigenomics (epigenetic modifications), and metabolomics (metabolites). By capturing different layers of cellular information, omics enable the comprehensive profiling of gene-environment interactions and the identification of molecular pathways involved in health and disease.

In mitochondrial disorders, omics have revolutionized both diagnosis and mechanistic understanding by allowing researchers to investigate mitochondrial dysfunction from a systems biology perspective. Genomic sequencing has revealed the vast genetic heterogeneity of PMD, while transcriptomics and proteomics have elucidated tissue-specific responses to energetic stress. Among these, metabolomics has emerged as a uniquely informative tool, as it reflects the biochemical phenotype resulting from cumulative genomic, transcriptomic, and environmental influences.

Metabolomics is defined as the systematic study of small-molecule metabolites (<1,500 Da) within cells, tissues, or biofluids. As the final downstream products of cellular processes, metabolites provide a direct readout of physiological or pathological states. In the context of PMD, metabolomic profiling offers some advantages: it captures the systemic consequences of impaired oxidative metabolism, supports functional biomarker discovery, and enables dynamic assessment of treatment responses or disease progression.

### 7.1. Metabolomics in Mitochondrial Disease

Mitochondrial dysfunction alters key metabolic pathways, including the TCA cycle, fatty acid oxidation, amino acid catabolism, and redox homeostasis. Metabolomic profiling - whether untargeted or targeted - can therefore provide functional insight into the bioenergetic and biosynthetic derangements characteristic of PMD.

Metabolomics provides direct access to the biochemical phenotype of mitochondrial disease, capturing the downstream effects of impaired oxidative metabolism. In PMDs, metabolomic profiling - whether untargeted or targeted - has shown promise for the identification of disease-specific biomarkers, monitoring of therapeutic responses, and stratification of heterogeneous clinical presentations[280,281].

Emerging applications include the detection of characteristic metabolic abnormalities - such as 3-methylglutaconic aciduria, alterations in Krebs cycle intermediates, or amino acid imbalances[282,283] - as well as the use of specific metabolites as pharmacodynamic readouts to monitor interventions targeting mitochondrial function[284,285]. Furthermore, metabolomics has been integrated with genomic and transcriptomic data to model patient-specific biochemical adaptations and clarify variant pathogenicity[286].

For example, targeted metabolomics has been employed in clinical trials of elamipretide, a cardiolipin-stabilizing peptide, by quantifying plasma and muscle levels of Krebs cycle intermediates, acylcarnitines, and phosphate metabolites via $^{31}$P-MRS. Similarly, vatiquinone (EPI-743), a redox-modulating agent under investigation in MELAS and Leigh syndrome, has been associated with shifts in glutathione redox pairs and NADPH/NADP$^+$ ratios , although the clinical trial has been terminated without published results[262].

Together, these studies underscore the potential of metabolomics to generate dynamic, functional readouts of mitochondrial dysfunction and support the development of precision medicine approaches. However, limitations such as interindividual variability, analytical heterogeneity, and the lack of validated disease-specific signatures continue to hinder its clinical translation[287,288].

### 7.1.1. Strategies in Metabolomics Research

Metabolomics in mitochondrial disease is predominantly conducted using two analytical platforms: proton nuclear magnetic resonance ($^1$H NMR) spectroscopy and liquid chromatography coupled with mass spectrometry (LC-MS), each offering complementary strengths in sensitivity, quantification, and metabolome coverage[281,284].

$^1$H NMR is a robust, non-destructive, and highly reproducible technique requiring minimal sample preparation. It is particularly well-suited for untargeted analysis of biofluids such as urine, enabling detection of diverse low molecular weight metabolites - including organic acids, amino acids, and microbial co-metabolites[289,290]. Although less sensitive than MS-based approaches, its throughput and consistency make it especially valuable for exploratory studies and paediatric cohorts.

LC-MS, particularly when combined with tandem MS (LC-MS/MS), provides superior sensitivity and dynamic range, and is widely used in targeted metabolomics to quantify known metabolites

involved in mitochondrial energy metabolism - such as TCA cycle intermediates, acylcarnitines, and redox cofactors[284,285]. However, it requires meticulous optimization of chromatographic and ionization conditions and can be affected by matrix effects.

These platforms are increasingly viewed as complementary rather than competing. While NMR excels in hypothesis generation and metabolic fingerprinting, LC-MS offers high-resolution quantification of specific targets. Their integration may enhance biomarker discovery and improve diagnostic accuracy in heterogeneous mitochondrial phenotypes.

In rare diseases, metabolomic-genomic integration has been instrumental in elucidating pathogenicity in VUS, validating novel disease genes. Transcriptomic and epigenomic analyses further reveal mitochondrial-nuclear communication and tissue-specific adaptations to bioenergetic stress[291].

Despite advances, barriers to implementation persist - particularly in sample accessibility, data harmonization, and interpretation. Nevertheless, the continued development of multi-omics approaches is expanding the diagnostic and therapeutic potential of PMD research.

A critical methodological issue in PMD metabolomics studies is the selection of appropriate control groups. Most published studies compare patients with healthy individuals, a strategy that facilitates identification of broad metabolic deviations but may lack specificity for differential diagnosis. Comparisons with disease controls, such as individuals with phenotypically overlapping inborn errors of metabolism or non-genetic mitochondrial dysfunction, are less common but essential for evaluating the discriminative value of candidate biomarkers.This choice influences the interpretability and clinical relevance of the identified metabolic signatures and provides different outputs of the system (des)equilibrium[282].

### 7.1.2. Metabolomic Signatures in Primary Mitochondrial Disease

Several studies have demonstrated that metabolomic profiling can uncover disease-specific metabolic signatures in PMD, providing insights into altered biochemical pathways and potential diagnostic biomarkers. Pathway-level analyses of comprehensive multi-omics datasets have uncovered perturbations in metabolic networks, mitophagy, one-carbon metabolism, and inflammatory signalling [292,293,291].

An untargeted metabolomic profiling of plasma and urine samples in Leigh syndrome due to *LRPPRC* mutations, identified 45 metabolites that differentiated affected individuals from controls, including classical indicators of mitochondrial dysfunction such as elevated lactate and C14-18-acylcarnitines. Importantly, the study also revealed unexpected perturbations in amino acid catabolism, NADH/$NAD^+$ redox balance, and adipokine signalling, suggesting widespread systemic adaptations to defective oxidative phosphorylation[294].

Similarly, a multi-omics approach for a cohort of patients with the m.3243A>G mutation, identified 36 metabolites significantly altered in MELAS compared to healthy controls. The dysregulated metabolites spanned pathways involving Krebs cycle metabolism, glutathione metabolism, one-carbon metabolism, fatty acid beta-oxidation, and methylation. Among the most discriminative features were short-chain acylcarnitines (C2-5), various amino acids, and intermediates of the methionine cycle[295]. Notably, following work demonstrated that urinary metabolic signatures could distinguish between different phenotypes of the same mutation, correlating metabolic fingerprints with clinical phenotype and illustrating the potential of metabolomics to resolve intra-genotypic heterogeneity[296].

Additional metabolomic studies in PMDs have identified convergent biochemical patterns, including decreased TCA cycle intermediates (e.g., citrate, succinate), increased 3-methylglutaconic acid, disturbances in branched-chain and aromatic amino acid metabolism. These findings reflect mitochondrial dysfunction across multiple physiological axes - oxidative metabolism, redox balance, anaplerosis - and provide a systems-level perspective on disease mechanisms[297,262,298,299].

Together, these studies exemplify how metabolomic profiling can elucidate distinct biochemical derangements in PMDs, paving the way for biomarker discovery, phenotype refinement, and mechanistic investigation.

### 7.1.3. Biomarker Discovery and Clinical Translation

The identification of metabolomic biomarkers in PMDs holds considerable promise for improving diagnostic accuracy, monitoring disease progression, and evaluating therapeutic efficacy[262,300]. Metabolomics offers a functional readout of mitochondrial dysfunction that complements genetic and clinical data, potentially reducing diagnostic delay and enabling earlier intervention. However, such findings remain preliminary and require validation in prospective, longitudinal datasets.

Translating metabolomic insights into clinical utility remains challenging. Interindividual variability in metabolite levels, driven by factors such as age, sex, diet, circadian rhythm, microbiome composition, and comorbidities, can obscure disease-specific metabolic patterns, particularly in small or heterogeneous cohorts. Additionally, differences in analytical platforms ($^1$H NMR vs. LC-MS), sample types (plasma, serum, urine, cerebrospinal fluid), and pre-analytical conditions contribute to variability in results and hinder cross-study comparability.

# *Chapter II.* ***Aims and objectives***

To enhance the diagnostic accuracy, clinical characterization, and biomarker discovery in paediatric primary mitochondrial disorders (PMDs) through an integrated, multimethod approach combining clinical evaluation, genetic testing, functional studies, systematic literature review, and metabolomic profiling.

Specifically, this work aims to:

- Provide practical guidance for the diagnosis and management of suspected mitochondrial disease in children;
- Describe the clinical and genetic spectrum of cohort of patients with suspected mitochondrial disease, differentiating PMDs from clinically overlapping conditions ("mitochondrial mimics");
- Refine phenotype-genotype associations in mitochondrial disease, through the integration of clinical, imaging, histological, and genetic data, including the characterization of selected cases with novel or rare variants in mitochondria-related genes (mitochondrial or nuclear encoded);
- Characterize the urinary metabolic fingerprint of PMD using untargeted $^{1}$H-NMR metabolomics and explore their potential as diagnostic or monitoring biomarkers.

To achieve these aims, the thesis is organized around the following three thematic objectives:

**Objective I.** ***Characterize the spectrum of a cohort with suspected mitochondrial disease***

i. Describe the clinical presentations and patterns of organ involvement in children referred for suspected PMD.
ii. Analyse the diagnostic yield of next-generation sequencing (NGS) approaches, including targeted panels, mtDNA analysis, and whole exome sequencing (WES).
iii. Complete deep phenotypic characterization of genetically confirmed PMD cases.
iv. Identify and characterize non-mitochondrial diagnosis (mitochondrial mimics) and outline their phenotypic overlap with confirmed PMD.
v. Assess the diagnostic odyssey and diagnostic trajectory from symptom onset to final diagnosis.

Evaluate the concordance between initial clinical suspicion and genetic diagnostic outcomes.

***Objective II. Expanding the Phenotype Spectrum in Ultra-Rare Mitochondrial Disorders***

i. Provide detailed phenotypic and molecular characterization of ultra-rare nuclear gene disorders incorporating clinical, histological, and biochemical data, contributing to phenotype knowledge.

ii. Contribute new clinical and molecular evidence supporting disease–gene associations in mitochondrial medicine.

***Objective III. Explore metabolomic signatures as biomarkers in PMD***

i. Apply untargeted $^{1}$H NMR urine metabolomics to identify metabolic alterations in children with PMD, comparing urinary metabolomic profiles among PMD with healthy and disease control groups.
ii. Identify possible biomarkers of PMD with diagnostic or monitoring potential.
iii. Identify disease-specific metabolic patterns, including markers of renal involvement to putatively identify biomarker of kidney involvement in mitochondrial patients.

# *Chapter III.* ***Patients and Methods***

## 1. Study design

This cumulative PhD thesis consists of four peer-reviewed manuscripts and an additional thesis chapter, each addressing different aspects of primary mitochondrial disorders (PMDs). Given the heterogeneity of mitochondrial diseases, a multi-method approach was essential. This study integrates clinical research, metabolomics, genetic diagnostics, and systematic literature review to enhance our understanding of disease phenotypes, pathophysiology, diagnostic approaches, and mitochondrial mimics.

The overall methodological framework combines:

- A narrative clinical review aimed at developing institutional guidelines for the diagnosis, management, and follow-up of paediatric mitochondrial disorders (Manuscript 1).
- A single-centre cohort study of 178 paediatric patients with suspected PMD, combining deep phenotyping and comprehensive genetic evaluation (presented in the original thesis chapter and intended for journal submission).
- A detailed case report of a patient with ISD11 (*LYRM4*) deficiency, including biochemical and genetic characterization, reporting the second known affected family (Manuscript 2).
- The description of our most commonly affected PMD nuclear gene (*EARS2*), incorporating 3 cases from our cohort (2 not previously published) plus a systematic review of all published cases applying PRISMA methodology and meta-analysis to explore phenotype patterns (Manuscript 3).
- An experimental untargeted metabolomics study using proton nuclear magnetic resonance ($^{1}$H NMR) spectroscopy to identify urinary biomarkers in paediatric PMD comparing to healthy and disease control groups (chronic kidney disease (CKD) and suspected but not confirmed mitochondrial disorder) (Manuscript 4).

Each manuscript employed a tailored methodological strategy in line with its specific objectives, as summarized in Table 10.

***Table 10. Overview of Included Manuscripts and Methodological Approaches***

| Manuscript/Chapter | Main Research Focus | Study Design |
|---|---|---|
| **Manuscript 1 (Eur J Pediatr)** | Diagnosis, management, and follow-up of mitochondrial disorders in childhood | Narrative review, clinical guidelines development |
| **Chapter** | Genetic diagnosis in 178 patients with suspected PMD | Longitudinal NGS/WES-based genetic analysis |

| | | |
|---|---|---|
| **Manuscript 3 (JIMD Reports)** | ISD11 (*LYRM4*) deficiency: novel phenotype and biochemical/genetic characterization | Case report; biochemical, histological, and molecular and functional studies |
| **Manuscript 4 (Neurobiol Dis)** | *EARS2*-related mitochondrial disease: phenotypic spectrum and outcomes | Case series; systematic review (PRISMA) and meta-analysis |
| **Manuscript 5 (Metabolomics)** | $^{1}$H NMR urine metabolomics in PMDs | Experimental study, metabolomic profiling, healthy control and disease-control groups |

## 2. Patients

A total of 220 paediatric patients with clinical suspicion or confirmed diagnosis of PMD were recruited and 178 were included. All patients were followed at the Paediatric Metabolic Diseases Clinic of Centro Hospitalar Universitário de Santo António (Porto, Portugal).

The inclusion period extended from January 2018 to March 2025 and comprised both newly referred and previously followed patients, provided they remained under active follow-up during this timeframe. Some patients had been initially referred before 2018 but were reevaluated or monitored during the study period, allowing integration of updated diagnostic and molecular data.

The study had a longitudinal observational design, conducted in the context of routine clinical care at a specialized metabolic centre. All patients underwent systematic diagnostic reassessment and tailored investigation, based on their clinical evolution and previous diagnostic workup.

Several patients (14) included in this cohort had been previously reported[143,301,176,302–304]. These cases were re-included for longitudinal clinical follow-up, expanded phenotypic characterization, and integration into the comprehensive diagnostic framework developed in this study.

### *Inclusion criteria*

Patients were included if they were actively followed for suspected or genetically confirmed mitochondrial disease between January 2018 and March 2025. This cohort comprised both newly referred patients and individuals under longstanding follow-up, including retrospective cases whose initial referrals dated back to as early as 2006, provided they remained under regular surveillance during the study period.

Patient inclusion was based on a phenotype driven suspicion of mitochondrial disease, defined by the presence of characteristic clinical, imaging, or biochemical features, particularly when occurring in combination, with early onset, or in the absence of an alternative genetic diagnosis. Suspicion was particularly raised in the following settings:

- **Clinical features**: life-threatening events (e.g., sudden cardiorespiratory arrest, neonatal distress without sentinel obstetric event, or acute decompensation requiring intensive

care), neuroregression (especially following metabolic stress or infections), refractory epilepsy (including epileptic encephalopathies and myoclonic seizures), and early-onset movement disorders (e.g., dystonia, ataxia, or spasticity). Additional signs included generalized hypotonia, developmental delay, ptosis, ophthalmoplegia, and features suggestive of congenital myopathy (e.g., muscle weakness, exercise intolerance, recurrent rhabdomyolysis, or persistent hyperCKemia).

- **Neuroimaging**: bilateral symmetric lesions of the basal ganglia, thalamus, brainstem, or cerebellum; stroke-like episodes; cerebral or cerebellar atrophy; or presence of a lactate peak on proton magnetic resonance spectroscopy ($^{1}$H-MRS).
- **Biochemical findings**: recurrent or persistent hyperlactatemia (documented in at least two to three evaluations), elevated lactate-to-pyruvate ratio, hyperalaninemia, low plasma citrulline, increased urinary excretion of Krebs cycle intermediates or 3-methylglutaconic acid, or confirmed defects in mitochondrial respiratory chain complexes in muscle or fibroblasts.
- **Multisystem involvement**: sensorineural hearing loss, cardiomyopathy (hypertrophic, dilated, or noncompaction), unexplained liver dysfunction, chronic kidney disease or tubular dysfunction, and endocrine abnormalities such as non-autoimmune diabetes, hypothyroidism, adrenal insufficiency, or growth hormone deficiency.
- **Family history**: maternal inheritance patterns or multiple affected siblings were considered supportive.

Patients were excluded when the initial clinical suspicion was a different inborn error of metabolism or another non-mitochondrial genetic disorder, even when their phenotype met some of the above inclusion criteria.

In order to uniformize clinical practices and diagnostic classification a guideline for the diagnosis and follow-up of patients with suspected or confirmed mitochondrial disorders was created. In this diagnostic approach we characterize patients as having a possible (clinical evidence in 2 or more systems or suggestive features specially if neurological or muscular), probable (clinical plus biochemical and/or imaging), very probable (clinical, biochemical and functional criteria) and definite (genetic confirmation or mitochondrial DNA depletion, regardless of clinical, biochemical or functional criteria).

Several representative cases from this cohort were the subject of individual publications. Manuscript 2 described a patient with ISD11 (*LYRM4*) deficiency, identified through next-generation sequencing, representing the second reported family worldwide and confirming this gene as disease-causing. Manuscript 3 compiled the clinical and molecular findings of three

patients with *EARS2*-related leukoencephalopathy, all from our cohort, and included a systematic literature review and meta-analysis, conducted according to PRISMA guidelines.

For the metabolomics study (Manuscript 4), 23 patients were selected from the main cohort of suspected mitochondrial disease, including individuals with genetically confirmed primary mitochondrial disease (PMD, n = 13) and those with persistent clinical suspicion but no confirmed molecular diagnosis (n = 10). These were compared to three external control groups: (i) patients with chronic kidney disease (CKD, any stage, n = 29); and (ii) an opportunistic control group (n = 10) comprising otherwise healthy individuals followed in the Nephrology outpatient clinic, with no evidence of kidney or metabolic disease (e.g., mild vesicoureteral reflux or post-infectious follow-up), without age or sex matching between groups.

In children with chronic renal disease of unknown aetiology, mtDNA and exome sequencing were used to exclude primary mitochondrial disorders.

Urine samples were collected between February 2020 and July 2024. Due to pandemic-related constraints, a 2-year recruitment gap occurred between March 2020 and September 2022.

## 3. Phenotype assessment

Clinical and laboratory data were collected retrospectively from electronic medical records of all eligible patients followed at the Paediatric Metabolic Diseases Clinic of Centro Hospitalar Universitário de Santo António (Porto, Portugal), in whom a diagnosis of mitochondrial disease (MD) was considered.

Although data collection was primarily retrospective, a structured diagnostic protocol was progressively implemented and prospectively applied whenever feasible, enabling standardized follow-up and deep phenotyping in a substantial subset of patients.

The diagnostic workup varied according to age at onset, disease severity, clinical presentation, and availability of diagnostic tools at the time of evaluation. Consequently, not all patients underwent identical assessments, and data completeness was variable across domains.

Whenever possible, patients were systematically evaluated by a paediatric metabolic specialist. This included a comprehensive medical history, documentation of age at symptom onset and disease progression, and assessment of multisystem involvement. Family history was reviewed in all cases, with emphasis on consanguinity and inheritance patterns suggestive of mitochondrial disease (e.g., maternal transmission or multiple affected siblings).

Neurological and neuromuscular symptoms were carefully recorded, including encephalopathy, epilepsy (particularly myoclonic, focal, or drug-resistant forms), muscle weakness, hypotonia,

hypertonia, dystonia, ataxia, movement disorders, migraine, neuroregression, and exercise intolerance. Developmental history was reconstructed via documentation of psychomotor milestones, cognitive and language development, acquisition or loss of gait, and autonomy in activities of daily living (ADLs).

Systemic involvement was recorded based on subspecialty evaluations and laboratory findings, encompassing growth and development neurological assessment including brain MRI, cardiac, ophthalmologic and hearing assessments, liver, kidney, and endocrine profiles, hematologic and immunologic parameters and supportive care needs, such as requirement for enteral or parenteral nutrition, and non-invasive or invasive ventilation.

Metabolic investigations were guided by clinical suspicion and included plasma amino acids, urinary organic acids (GC-MS), acylcarnitine profiles (MS/MS), plasma and/or CSF lactate and pyruvate, and maximum serum creatine kinase (CK) levels. When both lactate and pyruvate were simultaneously measured, the lactate-to-pyruvate (L/P) ratio was calculated, with values >20 considered abnormal. Specific amino acid thresholds used for analysis included elevated alanine (>450 µmol/L), low citrulline (<10 µmol/L), and increased plasma lysine or proline. Abnormal urinary organic acid profiles were defined based on the presence of elevated Krebs cycle intermediates, 3-methylglutaconic acid, or other mitochondrial-related metabolites.

Neuroimaging evaluation was limited to brain MRI and proton magnetic resonance spectroscopy (MRS). MRI data were reviewed for the presence of characteristic abnormalities associated with mitochondrial disease, including T2/FLAIR hyperintensities in the basal ganglia, brainstem, thalami, or cerebellum; white matter signal abnormalities; cerebral or cerebellar atrophy; and stroke-like lesions. MRS findings were recorded when available, specifically the presence or absence of a lactate peak.

All available data were curated in a structured clinical research database and used for diagnostic classification and phenotype-genotype correlation. Missing data were acknowledged and considered in the interpretation of subgroup analyses.

For the systematic review (Manuscript 3), a structured search strategy was used in PubMed, Scopus, and Web of Science, following PRISMA guidelines (Page et al., 2021), with predefined inclusion criteria and dual reviewer validation. Data extraction followed standardized templates.

## 4. Diagnostic work-up

### 4.1. Muscle Biopsy and Functional Studies

Muscle biopsy was performed in a subset of patients (n = 48). Tissue samples were obtained from the deltoid muscle under sterile conditions, immediately preserved in saline-moistened gauze, and processed without delay.

Histological and histoenzymatic analyses were carried out according to standard procedures[185]. Stains included haematoxylin and eosin (H&E), modified Gomori trichrome, cytochrome c oxidase (COX), succinate dehydrogenase (SDH), sequential COX/SDH double staining, and NADH-tetrazolium reductase (NADH-TR) for fibber typing and assessment of oxidative activity. Spectrophotometric quantification of mitochondrial respiratory chain complex by enzyme activities and citrate synthase (CS) was performed in homogenates of frozen muscle samples, following established protocols[187]. Tissue specimens were stored at –80 °C until biochemical analysis. Deficiencies were classified as partial or significant, isolated or multiple, and interpreted in the context of age-adjusted reference ranges.

Biopsy results were integrated with genetic and clinical data to assess their diagnostic contribution. A biopsy was considered instrumental for the final diagnosis when histopathological findings directly supported a specific molecular diagnosis, when respiratory chain analysis revealed biochemical defects that enabled diagnostic confirmation, or when genetic studies conducted on muscle tissue yielded the definitive result.

### 4.2. Genetic Testing

Genetic analyses were conducted in certified diagnostic laboratories using a variety of platforms and strategies, reflecting evolving availability and clinical needs over the course of the study. The most commonly used first-tier approach was a targeted next-generation sequencing (NGS) panel encompassing 213 nuclear genes associated with mitochondrial disorders. Target enrichment was performed using the SureSelect QXT kit (Agilent Technologies), and sequencing was carried out on the Illumina MiSeq platform.

Mitochondrial DNA (mtDNA) analysis was performed in most patients; protocols ranged from targeted Sanger sequencing of common pathogenic mutations to full mtDNA sequencing by long-range PCR followed by NGS. DNA was extracted from blood or muscle, and libraries were prepared and sequenced according to standard protocols. Sequencing reads were aligned to the revised Cambridge Reference Sequence (rCRS) using SeqMan NGen (DNASTAR), and variant calling and annotation were performed in SeqMan Pro. Filtering criteria included variant type, population

frequency, heteroplasmy ≥10%, and predicted pathogenicity based on MITOMAP,and HmtVar. Variants of interest were further interpreted using APOGEE and MitoTIP for tRNA variants [305–308].

In selected cases, testing for large-scale mtDNA deletions and relative mtDNA copy number was also performed.

For those with complex multisystemic phenotypes or undiagnosed diagnoses following initial investigations – singleton or trio-based whole-exome sequencing (WES) was performed. Sequencing platforms and pipelines varied across diagnostic laboratories, according to the time and referral centre.

All initial variant classifications reported by the laboratories adhered to the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines[309]. Classification was based on multiple parameters, including variant type, predicted pathogenicity (SIFT, PolyPhen-2, MutationTaster), population allele frequencies (gnomAD, ExAC, 1000 Genomes), and segregation analysis, when applicable.

In a second stage, all variants of potential clinical relevance were independently reanalyzed by the study team using the Franklin® platform (Genoox, available at https://franklin.genoox.com), which incorporates ACMG-AMP criteria, curated internal and external databases (e.g., ClinVar, HGMD, OMIM), and *in silico* tools. This re-evaluation allowed harmonization of classification across laboratories and integration of updated evidence. Variant filtering prioritized pathogenicity, inheritance mode, and phenotypic concordance.

Candidate variants were reviewed in a multidisciplinary clinical board including paediatric metabolic specialists, geneticists, and neurologists. Sanger sequencing was used when necessary to confirm variants and perform segregation analysis in family members, according to inheritance patterns.

### 4.3. Changes in practice during the study period

Over the course of the study, diagnostic approaches evolved considerably, reflecting broader technological and institutional changes. Respiratory chain complex activity was predominantly measured in muscle biopsies. In selected cases, with only a few performed in peripheral blood leukocytes.

For mitochondrial DNA (mtDNA) analysis, earlier protocols relied on targeted Sanger sequencing of frequent point mutations (e.g., m.3243A>G, m.8993T>C/G). These assays were routinely performed at the National Institute of Health Dr. Ricardo Jorge (INSA), which provided nationwide access to molecular diagnostics for primary mitochondrial disorders. Full mtDNA sequencing was progressively introduced in the later phases of the study.

During the first half of the study period, few patients underwent whole-exome sequencing (WES), which was reserved for particularly complex or undiagnosed cases. However, in recent years, WES became a 1st tier approach for selected patients, especially for paediatric patients with multisystemic involvement.

These temporal variations in diagnostic access and methodology influenced the distribution of confirmed diagnoses, as well as the overall diagnostic yield across different time periods.

### 4.4. Diagnostic classification

To ensure consistency in clinical reasoning and facilitate comparative analysis, all patients were prospectively or retrospectively classified using a structured institutional four-tier diagnostic algorithm, based on the level of suspicion for PMD:

- Possible: Clinical suspicion involving two or more organ systems, without specific biochemical or imaging support;
- Probable: compatible clinical picture with supportive biochemical and/or neuroimaging findings;
- Very probable: evidence from clinical, biochemical, and functional studies (e.g., abnormal RCC); or evidence of mtDNA depletion.
- Definite: molecular confirmation of a pathogenic variant

This framework was developed and implemented institutionally to harmonize diagnostic pathways for patients with suspected or confirmed mitochondrial disease and was applied consistently across all sub-studies.

In parallel, the modified Nijmegen Mitochondrial Disease Criteria (MDC) were retrospectively applied to all patients to quantify the clinical likelihood of mitochondrial disease based on standardized scoring[201]. Based on the total score, patients are stratified into possible (score 2–4), probable (5–7), or definite (≥8) categories, calculated using the most comprehensive dataset available per patient; when data were missing, a conservative approach was used to prevent overclassification.

Molecular diagnoses were stratified into three levels of diagnostic certainty - definite, probable, or possible - based on an integrative clinical-genetic framework. This approach considered:

- The classification of the identified variant(s) according to ACMG and Association for Molecular Pathology (AMP) guidelines [309];
- The expected inheritance pattern (e.g., autosomal recessive, dominant, mitochondrial);
- Phenotypic concordance with the known clinical spectrum of the implicated gene(s).

For example, probable diagnoses were assigned in cases of compound heterozygosity involving one pathogenic/likely pathogenic variant and one variant of uncertain significance (VUS), when a strong phenotypic match existed.

The contribution of the molecular finding(s) to the clinical phenotype was further classified as: full when the identified variant(s) could explain the entirety of the clinical picture; or partial, when only a subset of the observed features could be attributed to the molecular defect.

Nuclear genes were considered to be involved in mitochondrial disease if they were listed in the PanelApp UK (version 9.41) expert-curated gene panel for mitochondrial disorders or had documented functional relevance to mitochondrial biology. Based on this integrative assessment, patients were allocated to one of three final diagnostic groups:

- PMD group: individuals with causative variants in genes directly involved in mitochondrial function (including both mtDNA and nuclear-encoded mitochondrial genes);
- Non-PMD group: individuals with causative variants in genes not primarily related to mitochondrial function but sufficient to explain the phenotype (genetic phenocopies or mitochondrial mimics);
- Unsolved group: individuals with no definitive or probable molecular diagnosis despite comprehensive genetic evaluation, including those with possible diagnoses based on inconclusive or insufficient evidence.

## 5. $^{1}$H NMR urine metabolomics

Urine metabolomics was performed by an $^{1}$H NMR experiment, as simplified in Table 11 and further detailed below.

***Table 11. Methods summary for the $^{1}$H NMR urine metabolomics experiment***

| | Procedure | Details |
|---|---|---|
| **Recruitment & Sample Collection** | Participant selection | Pediatric patients with PMD, SMD, CKD, and opportunistic controls; recruited Feb 2020–Jul 2024; informed consent obtained |
| **Sample collection** | Casual urine during routine visits; refrigeration and storage at −80 °C within 24 h | Exclusion of acute illness/infection. Selection of only 1 sample per patient excluding those during metabolic decompensation |
| **Sample Preparation** | Thawing at Room temperature<br>Centrifugation<br>Buffer and internal standard addition<br>pH adjustment<br>Transference to NMR tubes | At room temperature<br><br>0.2 M $KH_2PO_4$ in $D_2O$ and 0.1% TSP<br>to 7.40 ± 0.02<br>600 µL to 5mm tubes |
| **$^{1}$H NMR Acquisition** | Bruker AVANCE III, 500 MHz | Pulse sequence NOESY-1D<br>Calibration TSP at 0.00 ppm |

| | | |
|---|---|---|
| **Metabolite assignement** | Chenomx NMR Suite® and internal/public spectral libraries | Reviewed by an experienced metabolomics expert |
| **Data Processing & Statistical Analysis** | Spectral preprocessing<br>Alignment (Matlab R2014a) | Water/urea exclusion |
| **Normalization** | Probabilistic quotient normalization (PQN) and creatinine-normalized | |
| **Multivariate analysis** | PCA and PLS-DA (SIMCA-P 11.5); unit variance scaling; VIP-based loadings | $Q^2 \geq 0.5$ considered robust; cross-validation and permutation tests |
| **Peak integration** | Manual (Amix 3.9.5); PQN normalization (Matlab R2014a) | Selection of peaks without overlap |

*Abbreviations: PCA: Principal Component Analysis: Partial Least Squares Discriminant Analysis (PLS-DA)*

## 5.1. Recruitment and sample collection

Patients were selected by the metabolic and nephrology paediatric teams based on availability and willingness to participate, without age or sex matching between groups. As an observational study, no modifications to clinical management were made. This study analysed urinary samples collected from paediatric patients followed at our centre for suspected or confirmed mitochondrial or renal pathology, as well as healthy controls, between February 2020 and July 2024. A 2-year gap in recruitment occurred between March 2020 and September 2022 due to pandemic constraints. Inclusion and exclusion criteria are detailed in Table 12.

***Table 12. Detailed inclusion and exclusion criteria for urine metabolomics study.***

| Group | Inclusion Criteria | Exclusion Criteria |
|---|---|---|
| **Applicable to all** | Age >1M and <20 y | Active UTI |
| **Primary mitochondrial disorders (PMD)** | Genetically confirmed mitochondrial disease (nDNA or mtDNA) | Concomitant CKD<br>Acute intercurrent illness or metabolic decompensation at the time of sampling |
| **Secondary mitochondrial dysfunction (SMD)** | Suspected PMD based on clinical/biochemical presentation without genetic confirmation | Other diagnoses confirmed by genetic studies |
| **Chronic Kidney disease (CKD)** | eGFRcr 90 mL/min/1.73m$^2$* | Transplant recipient<br>CKD due to PMD |
| **Control** | Otherwise healthy patients with any mild condition (e.g. transitory creatinine elevation, CAKUT, or post-UTI follow-up) without evidence of CKD (eGFR; normal urinalysis, normal acid-base balance) | Other known organ/system involvement or development issues<br>Other suspected or confirmed (eg. genetically) conditions |

**Calculated by the creatinine-based Chronic Kidney Disease in Children (CKiD) U25 Equation except for patients younger than 12 months, where eGFR is based on the Schwartz formula, and for patients older than 18 years, where eGFR is based on CKD-EPI 2021 guidelines. Abbreviations: M: months; Y: years; CAKUT: Congenital Anomalies of the Kidney and Urinary Tract; eGFRcr: creatinine based estimated glomerular filtration rate; UTI: urinary tract infection.*

Patients were further classified according to the KDIGO 2024 guidelines, with glomerular filtration rate (GFR) estimated using the creatinine-based CKiD U25 Equation (or the average- creatinine-cystatin based U25 Equation when available), except for patients younger than 12 months, where estimated GFR (eGFR) was calculated using the Schwartz formula [310] , and for patients older than

18 years, where eGFR was based on CKD-EPI 2021 equation. CKD severity was staged according to the 2024 KDIGO guidelines [310] and patients were stratified into two subgroups: stages 1–2 (mild CKD) and stages 3–5 (moderate to advanced CKD).

Casual urine samples were collected during routine visits, excluding those obtained during acute illness or infection. Samples were refrigerated and stored at −80 °C within 24 h. For PMD patients with known abnormal urinary excretion patterns, multiple samples were collected to assess temporal variability; one representative sample per patient was selected for analysis.

## 5.2. Sample preparation

Frozen aliquots were thawed at room temperature and centrifuged to remove particulates. Supernatants were buffered with 0.2 M potassium phosphate ($KH_2PO_4$) in $D_2O$ containing 0.1% sodium of 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid (TSP). pH was adjusted to 7.40 ± 0.02 using potassium hydroxide (KOD) or hydrochloric acid (DCl) in $D_2O$. A total of 600 µL was transferred to 5 mm NMR tubes.

## 5.3. $^1$H NMR data acquisition

Spectra were acquired on a Bruker AVANCE III spectrometer operating at 500 MHz for proton, using a standard NOESY-1D pulse sequence. Chemical shifts were referenced to TSP at 0.00 ppm. For selected samples, 2D NMR (TOCSY, HSQC) was performed to support metabolite identification. Peak assignment was carried out using Chenomx NMR Suite® and internal/public spectral libraries.

Given the inclusion of patients with TMEM70 and TAZ-related disease, typically associated with 3-methylglutaconic (3MGA) aciduria, we specifically searched for $^1$H NMR signals corresponding to previously reported 3MGA resonances [311]. No compatible resonance pattern was detected in our cohort, suggesting that 3MGA was either absent, below detection threshold, or not consistently elevated in basal conditions.

## 5.4. Data preprocessing

Spectral region exclusion was applied to eliminate regions corresponding to the water peak and the highly variable urea signal, which could introduce artefacts in multivariate analysis. Alignment was performed in Matlab R2014a, followed by probabilistic quotient normalization (PQN) to account for dilution effects. For comparison, normalization to creatinine was also carried out, due to still frequent use of this metabolite for concentration ratioing in the clinic, although acknowledging the inadequacy of this metabolite as a reference.

Peak integration was performed after manual peak selection from the original spectra (Amix 3.9.5, Bruker BioSpin, Rheinstetten, Germany) and normalized using PQN. Statistical Analysis

For the untargeted metabolomics dataset, multivariate was performed to identify group-level differences and discriminant metabolic signatures through Principal component analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA), using SIMCA© (version 13.0) and MetaboAnalyst 5.0. PCA was employed for unsupervised clustering and outlier detection, while PLS-DA was used for supervised group discrimination. Model performance was evaluated based on cross-validated $R^2$ (explained variance) and $Q^2$ (predictive ability) metrics.

Univariate statistical analyses were conducted using IBM SPSS Statistics (version 30.0, IBM Corp., Armonk, NY, USA) and R software (version 4.3.2). Descriptive statistics were used to summarize clinical, biochemical, imaging, and genetic variables across the cohort. Categorical variables were reported as absolute counts and percentages, while continuous variables were described as median and interquartile range (IQR), given the non-normal distribution observed for most parameters.

Comparative analyses between diagnostic subgroups (PMD, non-PMD, undiagnosed) were performed using the chi-square ($\chi^2$) test or Fisher's exact test for categorical variables, and the Mann–Whitney U test or Kruskal–Wallis test for continuous variables, as appropriate. A $p$-value < 0.05 was considered statistically significant. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated to quantify the strength of associations.

Univariate comparisons of normalized metabolite levels across groups were performed using the Kruskal–Wallis test for multi-group comparisons and the Mann–Whitney U test for pairwise contrasts. Statistical significance was defined as $p < 0.05$. Additionally, variable importance in projection (VIP) scores, fold changes (FC), and Hedge's g effect sizes were calculated to prioritize metabolites. Metabolites were considered biologically relevant if they showed a FC ≤ 0.8 or ≥ 1.2 with $p < 0.05$, or a Hedge's $g \geq 0.5$ with statistical significance.

PLS-DA biplots for quantified peaks and corresponding VIP scores were generated using MetaboAnalyst 5.0. To investigate the diagnostic utility of urinary metabolites for PMD, receiver operating characteristic (ROC) curve analysis was conducted, with the area under the curve (AUC) calculated for each compound. Candidate metabolites were selected based on statistical significance from univariate tests, AUC criteria, and their importance in multivariate analyses. These metabolites subsequently served as the basis for constructing classification models, including logistic regression, PLS-DA, random forest, and linear support vector machines (SVM) to evaluate discriminative performance among diagnostic groups.

## 6. Standardization and Bias Minimization

To ensure methodological rigor and minimize bias across the different studies included in this thesis, several standardization strategies were implemented at both the clinical and analytical levels.

All patients were evaluated at a single tertiary centre (Centro Hospitalar Universitário de Santo António) by a dedicated team of metabolic and neuromuscular paediatricians, following a consistent institutional diagnostic framework. The same diagnostic classification criteria were applied across all clinical studies, enabling harmonization of diagnostic certainty levels. Despite some clinical discrepancy on follow-up and monitoring between clinicians, a general procedure was respected. Selected clinical cases of uncertain diagnosis were discussed in a multidisciplinary meeting, combining metabolic, genetic and neurologist specialists.

Biochemical and histological assays (e.g., mitochondrial respiratory chain enzyme measurements and muscle histochemistry) were performed in certified and experienced laboratories according to established protocols[185,188].

Genetic testing was performed at accredited national and international laboratories, using validated panels and exome platforms and variant interpretation followed ACMG/AMP guidelines[309].

For Manuscript 3, the literature review followed PRISMA guidelines with dual screening and data extraction by two independent reviewers[312].

For the metabolomics study (Manuscript 4), metabolite annotation followed the Metabolomics Standards Initiative (MSI), using in-house libraries, CHEMOMX library (Chenomx NMR Suite (version 9.0, Chenomx Inc., Edmonton, Canada) and the Human Metabolome Database (HMDB), and metabolite assignment was reviewed by an experienced metabolomics expert.

## 7. Ethical Considerations

All studies included in this thesis were conducted in accordance with the ethical principles laid out in the Declaration of Helsinki (1964 and its subsequent amendments) and in compliance with European and national data protection legislation, including the General Data Protection Regulation (GDPR, Regulation EU 2016/679) and Law no. 58/2019, which implements the GDPR in Portugal.

Approval was obtained from the Institutional Ethics Committee of Centro Hospitalar Universitário de Santo António for all studies involving human subjects, including retrospective data collection, genetic analysis, and biological sample processing (References 2016.194(164-DEFI/153-CES) and

82018.162(139-DEFI/138-CES)) and written informed consent was obtained from all participants or their legal guardians, in accordance with the Declaration of Helsinki. The metabolomics study was additionally conducted in compliance with Good Clinical Practice (GCP) guidelines.

Publication consent was specifically obtained for patients with confirmed or suspected molecular diagnoses, except in cases involving deceased individuals or those already published, where inclusion was permitted under applicable ethical frameworks.

All retrospective data were fully anonymized, and no personal identifiers were retained or processed during data extraction and analysis. Data handling strictly followed the principles of data minimization, integrity, and confidentiality, with appropriate technical and organizational safeguards. Given the extended duration of follow-up and the presentation of only de-identified data (e.g., age at onset, last known age), individual reidentification is not possible.

The full metabolomics dataset generated for Manuscript 4 has been deposited in the Metabolomics Workbench under Study ID ST003924 (DOI: 10.21228/M81Z64) and will be made publicly available upon publication, in accordance with FAIR principles and ethical standards for open data in biomedical research.

# *Chapter IV.* **Results**

To reflect the diversity of methodological approaches and the cumulative nature of scientific inquiry throughout this doctoral project, the results are organized into two thematic sections, each corresponding to a major research axis. This structure is conceptual rather than chronological, integrating clinical, genetic, and metabolomic data to address key diagnostic challenges in paediatric mitochondrial disease.

### *Section I: Establishing a Diagnosis of Mitochondrial Disorder in Childhood*

This section examines the evolving strategies for the diagnosis of primary mitochondrial disease (PMD) in children, combining clinical algorithms, cohort-level analyses, and illustrative case studies that span the spectrum of diagnostic resolution.

It begins with the first manuscript, which proposes and validates a structured diagnostic algorithm for paediatric PMD. This algorithm aligns clinical decision-making with contemporary international recommendations and incorporates local multidisciplinary expertise, aiming to optimize diagnostic efficiency and accuracy. This manuscript stems from the theoretical and methodological groundwork necessary to conduct the subsequent clinical and translational investigations.

The second component presents a comprehensive retrospective analysis of the cohort of children followed at our centre with suspected mitochondrial disease. This study investigates the diagnostic yield of genomic testing, the frequency of genetic mimics and secondary mitochondrial dysfunction, and the trajectory of undiagnosed cases, offering valuable real-world insights into the diagnostic process.

### *Section II: Expanding the Phenotype Spectrum in Ultra-Rare Mitochondrial Disorders*

The second section details this thesis' contribution to the phenotypic expansion of ultra-rare primary mitochondrial disorders. It includes:

*i*) a case report describing the identification and functional validation of compound heterozygous variants in *LYRM4*, encoding ISD11, in a child from the cohort. As the second family and third reported case worldwide, this study broadens the clinical and molecular spectrum of ISD11 deficiency and reinforces the pathogenicity of *LYRM4*;

*ii*) a case series of *EARS2*-related leukoencephalopathy, characterized by early-onset neurodevelopmental regression and neuroimaging features consistent with LTBL (leukoencephalopathy with thalamus and brainstem involvement). This work is

complemented by a systematic literature review and meta-analysis, providing an updated phenotypic landscape and discussing diagnostic and prognostic implications.

### *Section III: Metabolomic Insights into Mitochondrial Dysfunction*

This section explores the application of untargeted $^{1}$H-NMR urinary metabolomics as a diagnostic and stratification tool in paediatric patients with suspected mitochondrial disease. Through the development of multivariate classification models, the study demonstrates the ability of metabolic signatures to discriminate PMD from phenocopies and genetically undiagnosed cases. Furthermore, it proposes novel urinary biomarkers and discusses the potential of metabolomics to complement genomic data, particularly in functionally ambiguous or negative exome results.

For manuscripts already published or accepted for publication, a synthesis of key findings is presented in each section. The full text of each article is included in the Appendices section at the end of the thesis.

Chapter IV. Results

# Section I. Establishing a diagnosis of mitochondrial disorder in childhood

Diagnostic evaluation of primary mitochondrial disease (PMD) in children remains challenging due to the clinical heterogeneity, evolving phenotypes, and overlap with other neurometabolic disorders. Traditional criteria, while useful, often fail to capture the nuances of early presentations or to provide practical guidance in settings without ready access to complementary gene-specific functional assays for variant validation.

## 1. Diagnostic Approach

At the outset of this project, the absence of a unified and pragmatic diagnostic approach for suspected mitochondrial disease in children posed a significant barrier not only to clinical decision-making, but also to the systematic inclusion of patients in longitudinal studies, molecular testing, and metabolomic profiling.

To address this, we developed a structured, tiered diagnostic framework, adapted to the paediatric and local context, aiming to improve diagnostic acuity and standardize clinical assessment. This approach, presented in Figure 6, integrates clinical features, biochemical findings, neuroimaging, histopathology, respiratory chain enzyme activity and genetic confirmation, leading to a three-tier classification (possible, probable or definite diagnosis).

A possible diagnosis can be made if the presentation is suggestive (particularly with neurological and/or muscular involvement). A probable diagnosis is considered when clinical presentation is combined with biochemical abnormalities and/or neuroimaging findings and/or immunohistochemical changes, or when functional assays demonstrate reduced activity of one or more respiratory chain complexes. A definite diagnosis requires molecular confirmation (mtDNA or nuclear gene variants) or, in the original algorithm, the presence of a primary mitochondrial DNA depletion syndrome (>70% depletion).

Considering the evolving definition of a definite diagnosis for PMD and better patient stratification, we have later classified cases with mtDNA depletion without molecular confirmation separately as “very probable” rather than definite, to reflect current diagnostic practices. Accordingly, the results of the cohort study presented in the following section follow this revised classification.

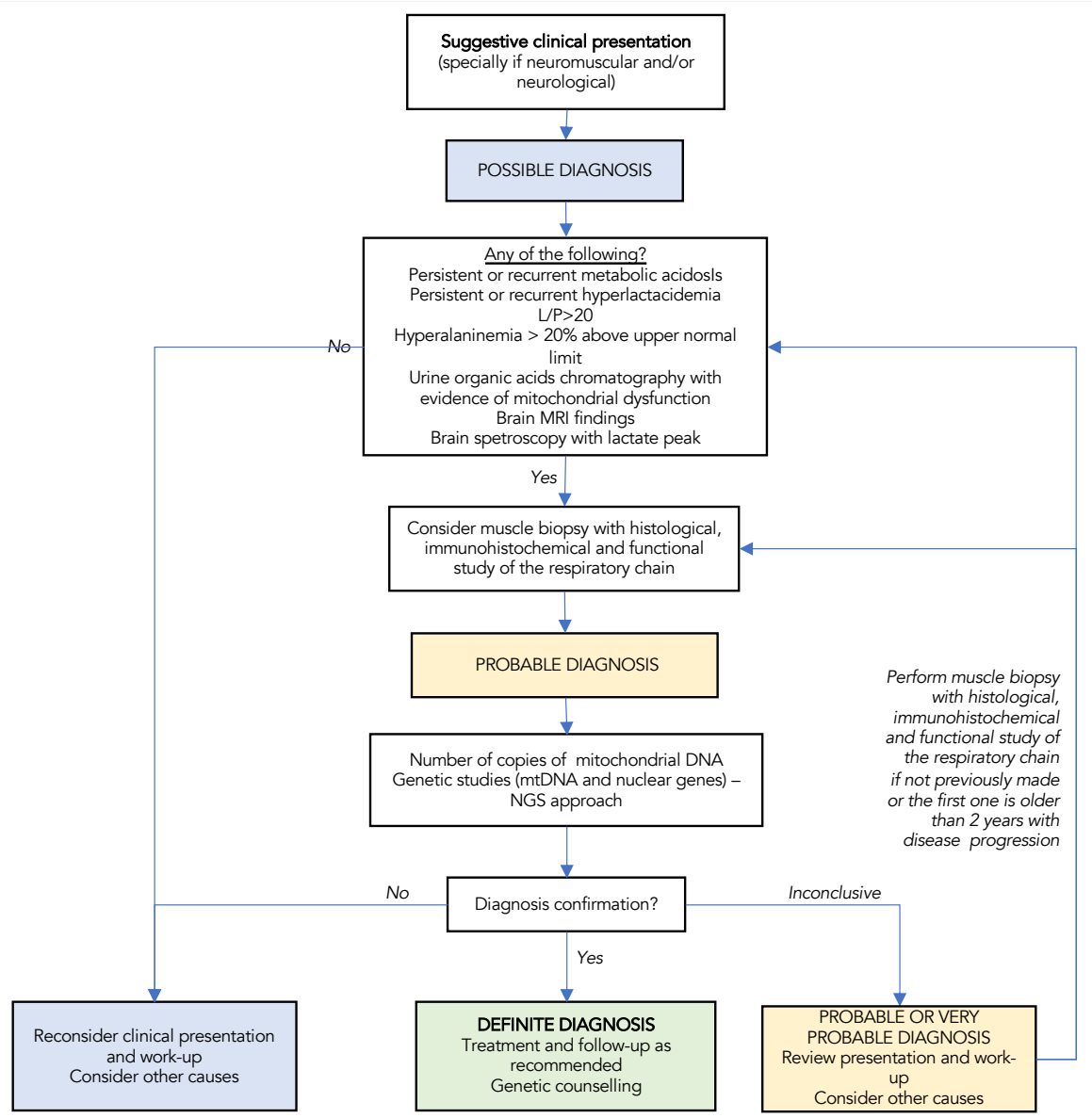


***Figure 6. Diagnostic algorithm for suspected mitochondrial disease.*** *Proposed workflow integrating clinical presentation, biochemical findings, neuroimaging, muscle biopsy, and molecular testing for the diagnosis of mitochondrial disease in children. Diagnostic certainty is stratified as possible, probable, or definite, depending on the convergence of clinical, biochemical, histological, and molecular data. Reproduced with permission from Springer Nature*[81].

If genetic studies are inconclusive, the clinical presentation, biochemical profile, and alternative diagnoses should be reconsidered. Establishing the likelihood of mitochondrial disease is helpful in determining which patients should undergo targeted or untargeted investigations.

In addition, by integrating clinical, biochemical, imaging, functional, and molecular findings, it offers a pragmatic framework for evaluating suspected PMD in paediatric patients, supporting rational clinical decision-making and guide prioritization for genetic testing. Moreover, its implementation in our centre has improved diagnostic consistency across clinicians and enabled

consistent data collection, phenotypic stratification, and prioritization of genomic and functional studies throughout the cohort.

Given the multisystemic and unpredictable nature of PMD, longitudinal follow-up should follow a common structure for all mitochondrial patients, as detailed in Table 13[123,234]. The primary aim of such a comprehensive follow-up is to avoid overlooking comorbidities and to enable detailed characterization of disease progression.

***Table 13. Recommended follow-up for mitochondrial disorders.***

| Work-up | Notes |
|---|---|
| **At every visit** | |
| Anthropometry, blood pressure, heart rate, general physical exam, nutritional status and development milestones | Include quality of life evaluation<br>Reassess symptoms by organ/system |
| **Annual** | |
| Venous blood gas analysis, blood count, glucose, urea, creatinine, cystatin-C, AST, ALT, ALP, GGT, CK, sodium, potassium, chloride, total calcium, magnesium, phosphorus, iron, vitamin D, HbA1c, ACTH, cortisol, IGF1, PTH, FT4, TSH) | Adjust to previous findings or complains |
| Urinalysis, proteinuria, albumin to creatinine ratio, (microalbuminuria), alpha-1-microglobulin, beta-2-microglobulin, creatinine, phosphorus, glycose, sodium, calcium) | Include urinary amino acids if proximal tubulopathy is suspected<br>If abnormalities are detected, refer the patient to a nephrologist and perform a renal ultrasound |
| If renal disease suspected or confirmed: Include in follow-ups | Consider nephrological referral if abnormalities persist |
| ECG and echocardiogram | Refer to Cardiology if abnormalities present |
| Neurological clinical evaluation | EEG if epilepsy (or suspected) |
| Referral to Neurology if needed | Neurological referral for evaluation of epilepsy, movement disorders, cognitive dysfunction |
| **Every 2 years** | |
| Hearing evaluation | Refer to an otorhinolaryngologist if needed, (*earlier if new complaints*) |
| Ophthalmologic evaluation (visual acuity, visual fields, eye movements, ptosis and fundoscopy | Referral to Ophthalmology if necessary |
| Vitamin D, phospho-calcium metabolism<br>Bone densitometry | Especially if non-ambulatory; Repeat earlier if there are fractures or metabolic bone disease concerns |
| Scoliosis, contractures, luxations, limb deformities | Refer to Orthopaedics if significant musculoskeletal issues are present |
| **Every 5 years** | |
| Brain MRI (with spectroscopy if not previously performed) | Monitor brain changes associated with mitochondrial disease<br>Earlier if acute neurological symptoms or development regression |
| Formal developmental or cognitive assessment | Earlier if cognitive changes arise |
| Neuropsychological evaluation | Evaluate for cognitive decline and psychiatric symptoms.<br>(earlier if new complaints) |
| **As needed** | |
| Respiratory assessment (polysomnography) | if sleep apnoea, snoring, or restless sleep are present; Referral to sleep medicine if sleep-related symptoms are present |
| Pulmonary function tests if respiratory insufficiency is suspected | Assess lung function in patients with suspected respiratory failure, particularly in patients with respiratory insufficiency, feeding difficulties, or malnutrition |

| | |
|---|---|
| Nutritional and/or Gastroenterology evaluation | Consider nutritional support for weight issues, feeding difficulties, or malnutrition |
| Immunological assessment if recurrent or severe infections are present | Monitor immunoglobulin levels and vaccine response in cases of recurrent infections |

A personalized follow-up profile can and should be further tailored to the patient's phenotype, genotype and current knowledge of disease's natural history.

This approach is equally valuable in undiagnosed patients with a probable diagnosis, as it enables continuous phenotypic characterization and can guide the re-evaluation of the etiological investigation over time. For the latter, consider performing regular chromatography of urinary organic acids and plasma amino acids for example, every 2 years.

## 2. A single-centre cohort study

A total of 220 paediatric patients with suspected mitochondrial disease were evaluated during the study period. Of these, 42 were excluded due to insufficient clinical data or follow-up (n=23) or adult age at study entry (n=18), or negative family screening (n=1). The final study cohort comprised 178 patients (70 females, 108 males) who underwent comprehensive clinical, biochemical, and genetic assessment.

As summarized in Figure 7, a definite diagnosis was achieved in 38.2% of patients, including 2 patients (1.1%) found to have non-genetic aetiologies follow multidisciplinary evaluation: one case with neonatal distress due to congenital severe anaemia (Hb 3.6g/dl) and subsequent multisystem repercussion, and one case of prenatal valproate exposure.

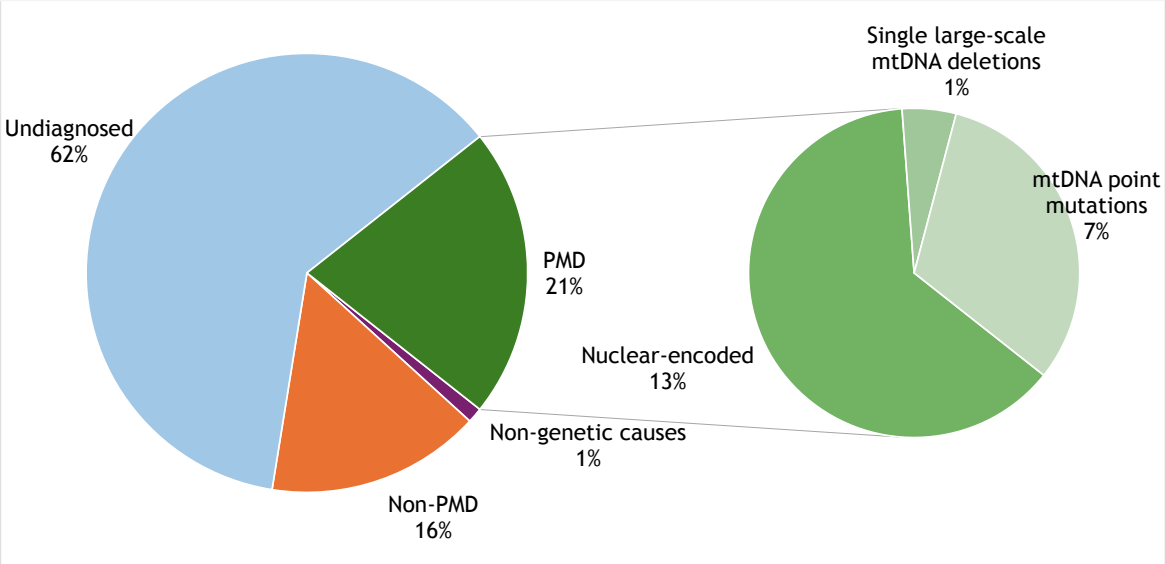


***Figure 7. Diagnosis in our study cohort.*** *Among the 178 patients with suspected mitochondrial disease, 38 (21%) have primary mitochondrial disease (PMD), 28 (16%) were found to have a genetic diagnosis unrelated to mitochondrial dysfunction (non-PMD), 2 (1%) had a non-genetic aetiology, and 110 (62%) remained undiagnosed. Within the PMD group, 13% had pathogenic variants in nuclear-encoded mitochondrial genes, 7% harboured mtDNA point mutations, and 1% had single large-scale mtDNA deletions.*

Among the 66 patients with a confirmed molecular diagnosis, 38 (57.6%) were classified as having PMD (20 females and 18 males) and 28 (42.4%) with an alternative genetic condition (non-PMD, 7 females and 21 males). In the PMD group, 24 patients had a diagnosis in in nuclear-encoded mitochondrial genes, 12 patients had with confirmed pathogenic point mutations in mtDNA, and 2 patients had with single large-scale mtDNA deletions.

Of the 178 patients included in the cohort, 110 individuals (62.0%) remained without a confirmed genetic diagnosis at the time of data cutoff. Among these 110 patients, 5 patients exhibited significant spontaneous clinical improvement, leading to discontinuation of the diagnostic work-up and 5 patients were lost to follow-up.

## 2.1. Phenotype spectrum

We next analysed the clinical spectrum of genetically confirmed patients to delineate distinguishing phenotypic features between primary mitochondrial disease (PMD) and alternative genetic diagnoses (non-PMD). Both groups presented with early-onset, multisystem disorders; however, their clinical trajectories and organ involvement patterns showed distinct distributions. Patients with confirmed PMD exhibited an early symptom onset (mean 1.7 years), frequent multisystem involvement, and high rates of biochemical and imaging abnormalities consistent with mitochondrial dysfunction. The non-PMD subgroup included patients with alternative genetic diagnoses mimicking mitochondrial disease, characterized by overlapping neurological phenotypes but lower frequency of metabolic derangements and mitochondrial imaging findings.

The median age at symptom onset was 1.3 years, and a slight male predominance was observed (57 males, 53 females). The most frequent clinical features were developmental delay (60%), hypotonia (42%), epilepsy (35%), myopathy (30%), and cerebellar signs (28%), largely overlapping with the phenotype of genetically confirmed PMD cases.

Results are presented for major diagnostic groups (PMD, non-PMD and undiagnosed) regarding onset, clinical course, outcomes, clinical presentation, biochemical, neuroradiological and muscle biopsy findings.

### 2.1.1. Onset, Clinical Course and Outcomes

The mean age at symptom onset was earlier in PMD (1.7 years) than in non-PMD (2.4 years). Neonatal onset was observed in 21.1% of PMD versus 14.3% of non-PMD, and 11.8% of undiagnosed patients. Most cases (above 80%) in all groups began before 5 years of age.

More than one third of patients experienced either an acute or sub-acute clinical course. Regression was more frequent in PMD (18.4%) than in non-PMD (10.7%) or undiagnosed (9.1%). Acute metabolic decompensations occurred in all groups: 31.6% of PMD, 21.4% of non-PMD, and 29.1% of undiagnosed patients. The most common acute decompensation was episodic metabolic acidosis and/or ketosis (10.5% PMD vs. 7.1% non-PMD vs. 1.8% undiagnosed), followed by acute encephalopathy (7.9% PMD) and hyperammonaemia (5.3% PMD). Hypoglycaemia was not a common finding in PMD (5.3%).

At the time of last follow-up, mean patient age was 13.5 years in the PMD group and 13.9 years in non-PMD. Dependence for daily activities (considered for those who last known age was at least 5 years of age) affected 61.3% of PMD and 60.0% of non-PMD patients, contrasting with only 2.7% of the undiagnosed group. Similarly, 21.1% of PMD required enteral feeding support and 13.2% needed non-invasive ventilation, compared to lower proportions in non-PMD (7.1% and 0.0%, respectively) and undiagnosed (5.5% and 2.7%). The absence of independent ambulation was

observed in 22.6% of PMD patients, compared to 16.0% among non-PMD patients and 7.3% in undiagnosed individuals.

Overall survival was markedly reduced in the PMD group, with a mortality rate of 26.3%, substantially higher than in non-PMD (7.1%) and undiagnosed patients (8.2%). The mean age at death was also lowest among PMD cases (10.2 years), compared to 6.9 years in non-PMD and 6.6 years in the undiagnosed group. A summary of these findings is presented in Table 14.

***Table 14. Onset, clinical course and outcome in our cohort by group of diagnosis.***

| | PMD (n=38) | non-PMD (n=28) | Undiagnosed (n=110) |
|---|---|---|---|
| | n (%) | n (%) | n (%) |
| **Onset** | | | |
| **Mean age at symptom onset** | 1.7 | 2.4 | 2.4 |
| **Neonatal** | 8 (21.1%) | 4 (14.3%) | 35 (31.8%) |
| **<1 year** | 13 (34.2%) | 7 (25.0%) | 24 (21.8%) |
| **1–4 years** | 13 (34.2%) | 12 (42.9%) | 27 (%) |
| **5–10 years** | 3 (7.9%) | 4 (14.3%) | 16 (%) |
| **>10 years** | 1 (2.6%) | 1(3.5%) | 8 (%) |
| **Clinical course** | | | |
| **Regression** | 7 (18.4%) | 3 (10.7%) | 10 (9.1%) |
| **Metabolic crisis, any** | 12 (31.6%) | 6 (21.4%) | 32 (29.1%) |
| Neonatal distress | 1 (2.6%) | 1(3.6%) | 7 (6.4%) |
| Hypoglycemia | 2 (5.3%) | 2(7.1%) | 9 (8.2%) |
| Episodic ketosis and/or metabolic acidosis | 4 (10.5%) | 2(7.1%) | 2 (1.8%) |
| Hyperammonemia | 2 (5.3%) | 0(0.0%) | 0 (0.0%) |
| Acute encephalopathy | 3 (7.9%) | 2(7.1%) | 2(1.8%) |
| Acute movement disorder/ataxia | 0 (0.0%) | 0(0.0%) | 3(2.7%) |
| Cardiorrespiratory arrest | 1 (2.6%) | 0(0.0%) | 1 (0.9%) |
| **Worsening triggered by infections** | 5 (13.2%) | 3(10.7%) | 7(6.4%) |
| **Outcome** | | | |
| **Deceased** | 10 (26.3%) | 2 (7.1%) | 9(8.2%) |
| **Mean age of death (years)** | 10.2 | 9.5 | 6.9 |
| **Survival (years)** | 13.7 | 14.7 | 14.4 |
| **Follow-up (years)** | 9.1 | 10.0 | 10.2 |
| **Daily activities dependence*** | 19 (61.3%) | 15 (60.0%) | 3 (2.7%) |
| **No independent walking*** | 7 (22.6%) | 4 (16.0%) | 7 (7.3%) |
| **Non-invasive ventilation support** | 5 (13.2%) | 0 (0.0%) | 3 (2.7%) |
| **Feeding trough PEG/nasogastric tube** | 8 (21.1%) | 2 (7.1%) | 6 (5.5%) |

*Demographic features, age at symptom onset, presentation type (e.g., neonatal onset, regression, metabolic crises), and clinical outcomes in patients with primary mitochondrial disease (PMD), alternative genetic diagnoses (non-PMD), and undiagnosed cases. Data are expressed as absolute counts and percentages within each diagnostic group, unless otherwise indicated. *Calculated for those with a last known age of at least 5 years.*

### 2.1.2. Clinical presentation

The PMD subgroup exhibited more frequent multisystem involvement, notably affecting the central nervous system, skeletal muscle, endocrine axis, and gastrointestinal tract. Conversely, non-PMD patients presented predominantly with isolated or dual-system involvement, frequently confined

to the nervous system. Single organ disease was uncommon (12.4%), in one PMD with early onset proximal tubulopathy, one non-PMD with early onset epileptic encephalopathy and 20 undiagnosed mainly neurologic or muscular symptoms).

The phenotype spectrum observed in patients within our cohort is summarized in Table 15, highlighting the absolute and relative frequency of key clinical features across groups.

***Table 15. Clinical features across diagnostic groups.***

| | PMD (n=38) n (%) | non-PMD (n=28) n (%) | Undiagnosed (n=110) n (%) |
|---|---|---|---|
| **Neurological** | | | |
| Epilepsy (any) | 19 (50%) | 9 (32.1%) | 37 (33.6%) |
| Myoclonic epilepsy | 3 (7.9%) | 1 (3.6%) | 4 (3.6%) |
| Pyramidal signs | 9 (23.7%) | 7 (25%) | 7 (6.4%) |
| Extrapyramidal signs | 15 (39.5%) | 14 (50%) | 25 (22.7%) |
| Spasticity | 4 (10.5%) | 1 (3.6%) | 4 (3.6%) |
| Dystonia | 8 (21.1%) | 6 (21.4%) | 10 (9.1%) |
| Chorea/Choreoathetosis | 2 (5.3%) | 2 (7.1%) | 1 (0.9%) |
| Tremor | 2 (5.3%) | 0 (0%) | 2 (1.8%) |
| Dysarthria | 5 (13.2%) | 1 (3.6%) | 2 (1.8%) |
| Bradykinesia | 0 (0%) | 2 (7.1%) | (0%) |
| Cerebellar involvement* | 17 (44.7%) | 12 (42.9%) | 21 (19.1%) |
| Cerebellar Ataxia | 15 (39.5%) | 12 (42.9%) | 17 (15.5%) |
| Neuropathy, any | 3 (7.9%) | 1 (3.6%) | 2 (1.8%) |
| Headaches, any | 12 (31.6%) | 2 (7.1%) | 4 (3.6%) |
| Migraine | 0 (0%) | 1 (3.6%) | 1 (0.9%) |
| Central hypoventilation | 3 (7.9%) | 0 (0%) | 2 (1.8%) |
| **Development** | | | |
| Development delay | 28 (73.7%) | 17 (60.7%) | 37 (33.6%) |
| Cognitive impairment** (any) | 21 (70%) | 18 (65.4%) | 23 (22.5%) |
| Moderate-to-severe ID | 5 (16.7%) | 5 (19.2%) | 2 (2%) |
| Autism Spectrum Disorder | 1 (2.6%) | 3 (10.7%) | 4 (3.6%) |
| Isolated language delay | 3 (9.1%) | 2 (7.1%) | 5 (4.8%) |
| Absent language (never acquired) | 10 (30.3%) | 2 (7.1%) | 4 (3.8%) |
| **Muscular** | | | |
| Myopathy, any | 24 (63.2%) | 15 (53.6%) | 28 (25.5%) |
| Muscle weakness | 4 (10.5%) | 2 (7.1%) | 4 (3.6%) |
| Exercise intolerance | 1 (2.6%) | 2 (7.1%) | 2 (1.8%) |
| Myositis/Rhabdomyolysis | 2 (5.3%) | 1 (3.6%) | 2 (1.8%) |
| HyperCKemia (persistent or intermittent) | 8 (21.1%) | 7 (25%) | 7 (6.4%) |
| **Cardiac** | | | |
| Left ventricular non-compaction | 1 (2.6%) | 0 (0%) | 2 (1.8%) |
| Dilated Cardiomyopathy | 2 (5.3%) | 1 (3.6%) | 9 (8.2%) |
| Hypertrophic Cardiomyopathy | 5 (13.2%) | 1 (3.6%) | 4 (3.6%) |
| LV dysfunction | 0 (0%) | 0 (0%) | (0%) |
| AV block | 2 (5.3%) | 2 (7.1%) | 2 (1.8%) |
| Wolf-Parkinson-White | 2 (5.3%) | 0 (0%) | 1 (0.9%) |
| **Oculomotor** | | | |
| Nystagmus | 2 (5.3%) | 2 (7.1%) | 6 (5.5%) |
| Strabismus | 4 (10.5%) | 3 (10.7%) | 1 (0.9%) |
| Ptosis | 3 (7.9%) | 1 (3.6%) | 1 (0.9%) |
| Ophthalmoparesis | 3 (7.9%) | 1 (3.6%) | 7 (6.4%) |

| | | | |
|---|---|---|---|
| **Ophthalmological** | | | |
| *Retinitis pigmentosa* | 2 (5.3%) | 0 (0%) | 1 (0.9%) |
| Optic atrophy | 2 (5.3%) | 2 (7.1%) | 4 (3.6%) |
| Cataracts | 0 (0%) | 1 (3.6%) | 3 (2.7%) |
| Hearing | (0%) | (0%) | (0%) |
| Sensorineural hearing loss | 8 (21.1%) | 2 (7.1%) | 0 (0%) |
| **Gastrointestinal** | | | |
| Elevated liver enzymes | 12 (31.6%) | 2 (7.1%) | 4 (3.6%) |
| Liver failure | 3 (7.9%) | 0 (0%) | 1 (0.9%) |
| Hepatomegaly | 2 (5.3%) | 2 (7.1%) | 3 (2.7%) |
| Feeding difficulties | 9 (23.7%) | 3 (10.7%) | 4 (3.6%) |
| Recurrent vomiting | 5 (13.2%) | 1 (3.6%) | 7 (6.4%) |
| **Growth** | | | |
| Failure to thrive | 18 (47.4%) | 7 (25%) | 25 (22.7%) |
| Short stature | 11 (28.9%) | 6 (21.4%) | 12 (10.9%) |
| Microcephaly | 3 (7.9%) | 2 (7.1%) | 9 (8.2%) |
| **Endocrine** | | | |
| IGF1/GH deficiency | 5 (13.2%) | 1 (3.6%) | 2 (1.8%) |
| Hypothyroidism | 2 (5.3%) | 2 (7.1%) | 4 (3.6%) |
| Hypogonadism | 1 (2.6%) | 0 (0%) | 0 (0%) |
| Diabetes mellitus | 2 (5.3%) | 0 (0%) | 2 (1.8%) |
| Diabetes insipidus | 0 (0%) | 0 (0%) | 1 (0.9%) |
| **Kidney** | | | |
| Any renal involvement | 8 (21.1%) | 2 (7.1%) | 11 (10%) |
| Glomerular filtration rate < 90mL/min/1.73 $m^2$ | 4 (10.5%) | 0 (0%) | 1 (0.9%) |
| Requiring dialysis | 3 (7.9%) | 0 (0%) | 0 (0%) |
| Proteinuria | 7 (18.4%) | 1 (3.6%) | 4 (3.6%) |
| Microalbuminuria | 5 (13.2%) | 1 (3.6%) | 3 (2.7%) |
| Nephrotic syndrome | 1 (2.6%) | 0 (0%) | 0 (0%) |
| Tubulopathy, any | 2 (5.3%) | 2 (7.1%) | 5 (4.5%) |
| Proximal tubulopathy | 2 (5.3%) | 0 (0%) | 2 (1.8%) |
| Renal cysts | 1 (2.6%) | 0 (0%) | 1 (0.9%) |
| **Haematology** | | | |
| Normocytic anaemia | 7 (18.4%) | 0 (0%) | 6 (5.5%) |
| Macrocytic anaemia | 0 (0%) | 0 (0%) | 1 (0.9%) |
| Pancytopenia | 1 (2.6%) | 0 (0%) | 1 (0.9%) |
| Neutropenia | 2 (5.3%) | 0 (0%) | 1 (0.9%) |
| Thrombocytopenia | 3 (7.9%) | 0 (0%) | 3 (2.7%) |
| **Immune** | | | |
| IgG deficiency | 4 (10.5%) | 0 (0%) | 1 (0.9%) |

*Data are shown as absolutes counts and percentages for each clinical sign or symptom within diagnostic subgroup (confirmed PMD, non-PMD diagnoses, and undiagnosed). *Except isolated strabismus; **Cognitive impairment: considering patients alive after 2 years of age. Abbreviations: AV: auriculoventricular.*

Developmental and neurological involvement were predominant in genetically diagnosed patients. Cognitive impairment was observed in 70.0% of PMD and 65.4% of non-PMD cases, compared to 22.5% in the undiagnosed group. Language acquisition was absent in nearly one-third of PMD patients (30.3%), but in only 7.1% and 3.8% of non-PMD and undiagnosed individuals, respectively. Moderate-to-severe intellectual disability was also more common in diagnosed groups (16.7–19.2%) than in undiagnosed (2.0%).

Epilepsy affected half of the PMD patients (50.0%) with lower frequencies in non-PMD (32.1%) and undiagnosed patients (33.6%). Myoclonic seizures were commonly although not exclusive associated to PMD (3 cases versus 1 case in non-PMD). Pyramidal signs occurred in 23.7% of diagnosed patients and only 6.4% of undiagnosed, while extrapyramidal signs (e.g., dystonia, tremor) were seen in 39.5% of PMD and 50.0% of non-PMD, but only 22.7% of undiagnosed individuals. Cerebellar features, particularly ataxia, were present in 40% of genetically diagnosed cases, contrasting with 19.1% in undiagnosed. Central hypoventilation, a proxy of brainstem dysfunction, was limited to PMD (7.9%) and rare in other groups. Neuropathy was uncommon across all cohorts.

Myopathic features were prominent in PMD (63.2%) and non-PMD (53.6%), but much less so in undiagnosed patients (25.5%). HyperCKemia and rhabdomyolysis were more frequent in genetically confirmed cases, consistent with muscle membrane instability or metabolic dysfunction.

Cardiac involvement was more frequent in PMD, particularly hypertrophic cardiomyopathy (13.2%) and rhythm abnormalities such as AV block and WPW syndrome. These findings were rare in the other groups, suggesting greater diagnostic relevance in PMD.

Oculomotor and visual manifestations (e.g., ptosis, ophthalmoparesis, optic atrophy) were modestly more frequent in PMD and non-PMD. Retinitis pigmentosa was almost exclusive to PMD. Cataracts were observed sporadically across all groups, with limited specificity.

Renal dysfunction was notably more prevalent in PMD (21.1%), particularly proteinuria and reduced eGFR. Tubulopathies and dialysis dependency occurred only in diagnosed individuals, reinforcing the importance of renal monitoring in mitochondrial disease.

Gastrointestinal and growth abnormalities such as elevated liver enzymes, feeding difficulties, and failure to thrive (FTT) were most frequent in PMD. Short stature and microcephaly followed a similar trend.

Endocrine involvement, including IGF-1/GH deficiency and hypothyroidism, was more frequent in PMD, albeit with overlap across groups.

Hematologic and immune findings were largely restricted to PMD, with normocytic anaemia as the most common, presenting in 18.4% of PMD patients. No patient with macrocytic anaemia was diagnosed with PMD in our cohort. IgG deficiency was found in 10% of patients (although not universally screened).

Classical phenotypes were not commonly identified, except for 14 cases in LSS, and one with Kearns-Sayre.

### 2.1.3. Biochemical findings

In addition to clinical manifestations, we evaluated biochemical parameters that reflect mitochondrial energy dysfunction. Distinct patterns of hyperlactacidemia, amino acid alterations (e.g., alanine, citrulline), and urinary organic acids (e.g., Krebs intermediates, 3-methylglutaconic, acid,) were observed across the diagnostic groups, as presented in Table 16.

***Table 16. Biochemical findings across diagnostic groups.***

| Parameters | PMD (n=38) n (%) | non-PMD (n=28) n (%) | Undiagnosed (n=110) n (%) |
|---|---|---|---|
| **Elevated lactate (>2.2 umol/L)** | 34 (89.5%) | 17 (60.7%) | 41 (37.3%) |
| **Metabolic acidosis** | 10 (26.3%) | 0 (0.0%) | 4 (3.6%) |
| **High L/P (>20)** | 15 (39.5%) | 6 (21.4%) | 5 (4.5%) |
| **Amino acids** | | | |
| **Elevated Alanine[1]** | 12 (31.6%) | 6 (21.4%) | 17(15.5%) |
| **Elevated Lysine[1]** | 4 (10.5%) | 1 (3.6%) | 4(3.6%) |
| **Low citrulline (<10 µmol/L)** | 4 (10.5%) | 0 (0.0%) | 3(2.7%) |
| **Organic acids** | | | |
| **Ketones** | 7 (18.4%) | 3 (10.7%) | 7(6.4%) |
| **Krebs metabolites** | 4 (10.5%) | 2 (7.1%) | 18(16.4%) |
| **Dicarboxylic acids** | 7 (18.4%) | 3 (10.7%) | 12(10.9%) |
| **3-methylglutaconic acid (3-MGA)** | 5 (13.2%) | 12 (42.9%) | 6(5.5%) |
| **3-methylglutaric aciduria** | 0 (0.0%) | 0 (0.0%) | 3(2.7%) |
| **Glutaric and/or 3-hydroxyglutaric acids** | 1 (2.6%) | 1 (3.6%) | 4(3.6%) |
| **Ethylmalonic acid** | 2 (5.3%) | 0 (0.0%) | 3(2.7%) |
| **Methylmalonic acid** | 1 (2.6%) | 1 (3.6%) | 2(1.8%) |
| **4-OH-Phenyl-pyruvic or -lactic acids** | 4 (10.5%) | 2 (7.1%) | 3(2.7%) |

*Frequencies of relevant abnormalities are presented for the PMD, non-PMD, and undiagnosed groups. Results reflect routine metabolic investigations performed during diagnostic evaluation. [1]High lysine and alanine were considered when values were higher than 20% upper limit normal range.*

Hyperlactacidemia, either persistent or intermittent, was the most frequent biochemical abnormality in PMD, affecting 89.5% of patients, compared to 60.7% in non-PMD and 37.3% in undiagnosed individuals. This finding highlights its diagnostic value, albeit with limited specificity in distinguishing mitochondrial from non-mitochondrial aetiologies.

Metabolic abnormalities were predominantly observed in patients with genetically confirmed PMD but also occurred in a subset of undiagnosed cases. Among PMD patients, 33.3% (10/30) exhibited elevated lactate (isolated or combined with hyperalaninaemia), increased creatine kinase, plus specific urinary organic acid (uOA) abnormalities. Interestingly, similar metabolic alterations were also detected in 17 undiagnosed patients, including isolated hyperlactatemia (n=6) and various combinations involving alanine, CK, and uOA (n=11). In contrast, no metabolic abnormalities were found in more than half of the patients with non-mitochondrial diagnoses (n=17).

Among plasma amino acid alterations, elevated alanine was observed in 31.6% of PMD and 21.4% of non-PMD patients, while remaining less frequent in the undiagnosed group (15.5%). Other changes included increased lysine (10.5% in PMD) and low citrulline (10.5% in PMD), both rare in other groups, suggesting secondary disruption of urea cycle or amino acid metabolism in mitochondrial dysfunction.

Urinary organic acid profiles were similar between groups. Dicarboxylic aciduria (18.4%), ketonuria (18.4%), and increased Krebs cycle intermediates (10.5%) were more prevalent in PMD, supporting impaired oxidative metabolism and mitochondrial energy failure. 3-Methylglutaconic (3-MGA) aciduria, however, was disproportionately frequent in non-PMD patients (42.9%). Findings such as ethylmalonic aciduria, methylmalonic acid, and 4-hydroxyphenylpyruvate or lactate derivatives were infrequent and variably distributed across groups.

A significant association was observed between diagnostic classification and metabolic findings ($\chi^2(6) = 18.2$, $p = 0.006$). Patients with confirmed PMD were more likely to exhibit both elevated lactate/alanine and abnormal organic acid profiles, while isolated abnormalities in organic acids were exclusive to the non-PMD group. Conversely, a considerable proportion of undiagnosed patients had only mild or no metabolic findings. The observed linear trend ($p = 0.003$) further supports the discriminative value of combined metabolic alterations for distinguishing between diagnostic classes.

Low plasma citrulline levels (<10 µmol/L) were observed exclusively in patients with PMD, with 4/22 PMD patients (18.2%) affected versus none in the non-PMD group (0/11). Despite this apparent trend, the association did not reach statistical significance (Fisher's exact test, $p = 0.276$), probably due to low occurrence. However, these finding was also observed in 2 undiagnosed patients with acute ketosis with or without hypoglycaemia, with citrulline <10 µmol/L. These findings suggest that while low citrulline might be specific of PMD, potentially reflecting secondary urea cycle involvement, but its presence is not pathognomonic and may also occur in the context of metabolic decompensation.

### 2.1.4. Neuroradiological features

In patients with PMD, cerebral white matter abnormalities were as common as basal ganglia involvement or cerebellar involvement (55.2%), including both T2 hyperintensities and/or atrophy. Brainstem involvement was also notable in this group (31.0%), particularly affecting the pons (24.1%) and midbrain (20.7%). Stroke-like was observed in only one PMD patient (m.3946A>G) presenting with Leigh syndrome. Figure 8 presents representative examples of brain MRI findings in our patient cohort.

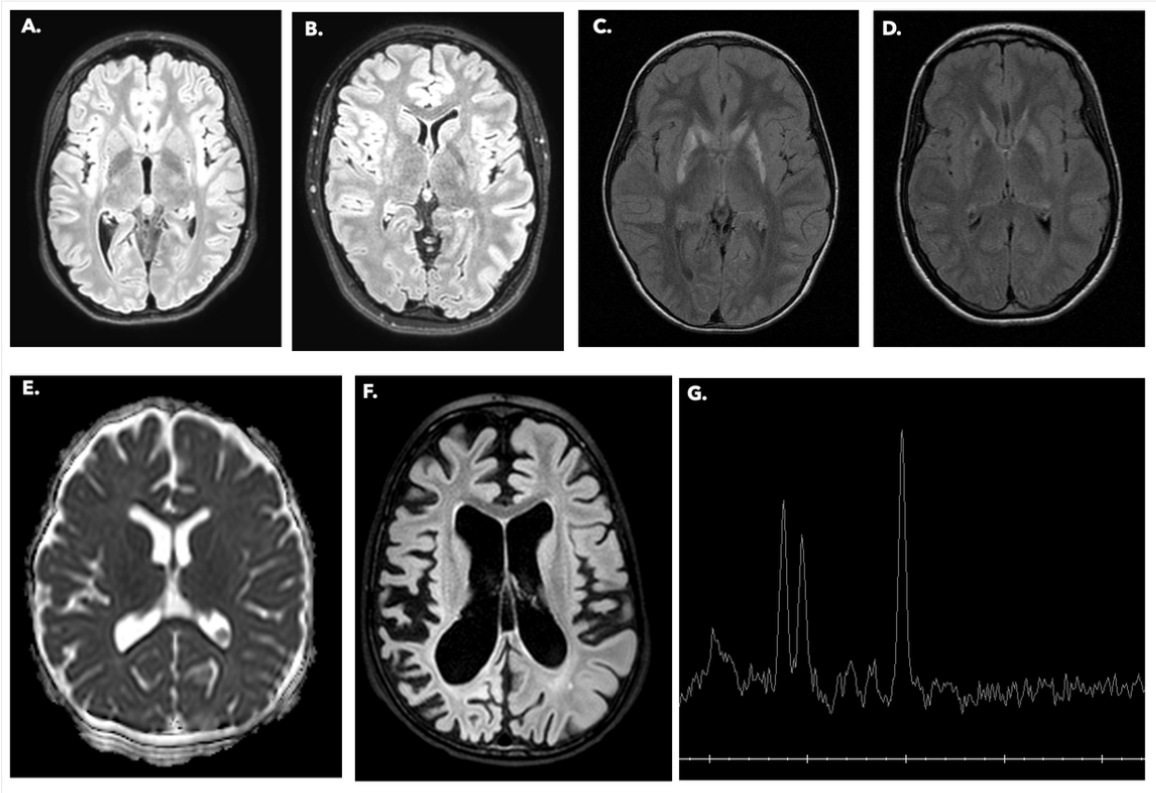


***Figure 8. Brain MRI in mitochondrial disorders in our cohort.*** *(A–D) Axial T2 FLAIR-weightd images showing bilateral basal ganglia hyperintensities, in four patients with Leigh syndrome. (E) Restricted diffusion in the peri-rolandic cortex bilaterally in a patient with POLG disease. (F) Severe cerebral atrophy (both supratentorial and infratentorial), basal ganglia in a patient with FASTKD2 disease. (G) Single voxel MR spectroscopy (MRS) showing the classical inverted lactate doublet at 1.33 ppm.*

In the non-PMD group (n=17), MRI abnormalities were less frequent and less specific. Cerebellar changes were the most common (41.2%), followed by cerebral white matter involvement (23.5%) and basal ganglia abnormalities (17.6%). Brainstem alterations were rare (11.8%), and none of the patients exhibited a lactate peak on MRS.

Among undiagnosed patients (n=44), MRI changes were generally less prominent, with white matter abnormalities (13.6%), cerebral atrophy (13.6%), and basal ganglia involvement (20.5%) being the most common. Only 9.1% had cerebellar changes, and a lactate peak was detected in a single case (7.7%). Importantly, one child exhibited bilateral symmetric lesions in the basal ganglia and brainstem, with magnetic resonance spectroscopy (MRS) demonstrating a lactate peak - a pattern highly suggestive of Leigh syndrome. Despite this, no pathogenic variants were identified on exome or mtDNA sequencing.

The distribution of brain MRI abnormalities across the three diagnostic groups is summarized in Table 17.

***Table 17. Brain MRI findings by group.***

| | PMD n=29 | non-PMD n=17 | Undiagnosed n=44 |
|---|---|---|---|
| **Agenesis/hypogenesis of the corpus callosum** | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) |
| **Delayed myelination** | 0 (0.0%) | 1 (5.9%) | 1 (2.3%) |
| **Optic nerve atrophy** | 1 (3.4%) | 0 (0.0%) | 1 (2.3%) |
| **Cerebral white-matter** | | | |
| **Cerebral White matter, any** | 16 (55.2%) | 4 (23.5%) | 6 (13.6%) |
| **Periventricular WM** | 2 (6.9%) | 0 (0.0%) | 4 (9.1%) |
| **Cerebral atrophy** | 5 (17.2%) | 3 (17.6%) | 6 (13.6%) |
| **Stroke-like lesions** | 1 (3.4%) | 0 (0.0%) | 1 (2.3%) |
| **Cavitated lesions** | 1 (3.4%) | 0 (0.0%) | 1 (2.3%) |
| **Cerebral grey-matter** | | | |
| **Thalamus and hypothalamus** | 8 (27.6%) | 1 (5.9%) | 4 (9.1%) |
| **Basal ganglia, any** | 16 (55.2%) | 3 (17.6%) | 9 (20.5%) |
| **Lenticular nucleus (putamen and globus pallidus)** | 13 (44.8%) | 2 (11.8%) | 7 (15.9%) |
| **Striatum (lenticular plus caudate nucleus)** | 13(44.8%) | 3 (17.6%) | 8 (18.2%) |
| **Restricted diffusion (DWI/ADC)** | 4 (13.8%) | 0 (0.0%) | 2 (4.5%) |
| **Basal ganglia atrophy or cavitated lesions** | 6 (20.7%) | 2 (11.8%) | 1 (2.3%) |
| **Brainstem** | | | |
| **Brainstem, any** | 9 (31.0%) | 2 (11.8%) | 2 (4.5%) |
| **Medulla oblongata** | 3 (10.3%) | 0 (0.0%) | 0 (0.0%) |
| **Pons** | 7 (24.1%) | 1 (5.9%) | 0 (0.0%) |
| **Midbrain** | 6 (20.7%) | 1 (5.9%) | 0 (0.0%) |
| **Brainstem atrophy** | 2 (6.9%) | 0 (0.0%) | 1 (2.3%) |
| **Cavitated lesions** | 1 (3.4%) | 0 (0.0%) | 0 (0.0%) |
| **Cerebellum** | | | |
| **Cerebellum, any** | 16 (55.2%) | 7 (41.2%) | 4 (9.1%) |
| **White matter** | 9 (31.0%) | 1 (5.9%) | 2 (4.5%) |
| **Dentate nuclei** | 2 (6.9%) | 1 (5.9%) | 4 (9.1%) |
| **Cerebellar atrophy** | 11 (37.9%) | 4 (23.5%) | 3 (6.8%) |
| **Cavitated lesions in WM** | 1 (3.4%) | 0 (0.0%) | 0 (0.0%) |
| **MRS** | | | |
| **Lactate peak** | 10 (40.0%) | 0 (0.0%) | 1 (7.7%) |

*Values are expressed as number of patients (percentage within each group who underwent MRI). Unless otherwise specified, abnormalities refer to T2/FLAIR hyperintensities. Abbreviations: WM, white matter; MRS, magnetic ressonance spectroscopy.*

Compared to PMD patients, non-PMD individuals had higher odds of presenting with putaminal lesions, although the association did not reach statistical significance (OR = 0.164, $p$ = 0.104). The direction of the effect suggests that putaminal involvement may be more common among patients with non-mitochondrial genetic disorders, potentially reflecting the broader heterogeneity of phenocopies in this group. In contrast, T2/FLAIR hyperintensities in the basal ganglia (any location) were significantly associated with a diagnosis of primary mitochondrial disorder, being observed in 53.3% of PMD patients versus 17.6% of non-PMD (OR = 0.174, p = 0.018, Fisher's exact test). T2/FLAIR hyperintensities in the white matter were also more prevalent in the PMD group compared to non-PMD (53.3% vs. 23.5%, respectively), with a statistically significant association (OR = 0.25, p = 0.042).

Cerebellar atrophy was more frequently observed in patients with genetically confirmed PMD (37.9%) compared to non-PMD cases (23.5%) and was rare among undiagnosed individuals (7.3%). The overall distribution across the three diagnostic categories was statistically significant (Fisher-Freeman-Halton exact $p = 0.006$). However, when the analysis was restricted to patients with a confirmed genetic diagnosis (PMD vs non-PMD), this difference did not reach statistical significance (Fisher's exact $p = 0.352$). These findings suggest that cerebellar atrophy may be more specific to PMD compared to undiagnosed cases, but not sufficiently discriminative between PMD and genetic mimics.

A lactate peak on brain magnetic resonance spectroscopy (MRS) was exclusively observed in PMD cases (33.3%) and absent in non-PMD patients, showing a significant association with mitochondrial disease status (Fisher's exact p = 0.016).

### 2.1.5. Mitochondrial Disease Score

A standardized MDC-based scoring approach was applied to all genetically diagnosed patients in the cohort, enabling structured comparison of clinical, biochemical, and imaging features between individuals with confirmed PMD and those with alternative genetic diagnoses (non-PMD). As illustrated in Figure 10, patients with PMD exhibited higher cumulative clinical scores, particularly within the neurological and multisystem domains.

Using the Modified MDC classification, 39% of PMD patients fulfilled criteria for "Definite" mitochondrial disease (≥8 points), while none of the non-PMD cases met this threshold. Conversely, 71% of non-PMD patients were classified as "Possible" (2–4 points), reflecting the presence of mild or nonspecific features commonly shared with mitochondrial phenotypes. These findings underscore the discriminative value of metabolic and neuroimaging domains in identifying true PMD cases and highlight the limitations of relying on clinical features alone in the presence of genetic mimics.

The greater clinical, metabolic, and radiological burden observed in PMD cases, and the utility of cumulative scoring to distinguish mitochondrial disease from genetic mimics is illustrated in Figure 10. This combined scoring approach reveals a clear separation between PMD and non-PMD cases, particularly in laboratory domains and final diagnostic categorization.

| | score | PMD | non-PMD |
|---|---|---|---|
| **Muscular (max.2)** | 0 | 8 | 9 |
| | 1 | 22 | 11 |
| | 2 | 8 | 8 |
| **Neurological (max.2)** | 0 | 1 | 3 |
| | 1 | 7 | 8 |
| | 2 | 30 | 17 |
| **Multisystem (max. 3)** | 0 | 14 | 12 |
| | 1 | 7 | 12 |
| | 2 | 8 | 4 |
| | 3 | 9 | 0 |
| **Total Clinical (max.4)** | 0 | 0 | 0 |
| | 1 | 0 | 1 |
| | 2 | 7 | 7 |
| | 3 | 7 | 9 |
| | 4 | 24 | 11 |
| **Metabolic** | 0 | 4 | 6 |
| | 1 | 0 | 3 |
| | 2 | 19 | 14 |
| | 3 | 11 | 2 |
| | 4 | 4 | 0 |
| **Imaging** | 0 | 5 | 0 |
| | 1 | 0 | 0 |
| | 2 | 11 | 2 |
| | 3 | 7 | 0 |
| | 4 | 1 | 0 |
| **Total Metabolic &MRI** | 0 | 4 | 8 |
| | 1 | 0 | 3 |
| | 2 | 6 | 13 |
| | 3 | 7 | 2 |
| | 4 | 21 | 2 |
| **Diagnostic suspicion** (max.8) | 2 to 4 | 6 | 20 |
| | 5 to 7 | 17 | 8 |
| | >= 8 | 15 | 0 |
| **Plus biopsy** (max 12) | 2 to 4 | 6 | 14 |
| | 5 to 7 | 16 | 12 |
| | >= 8 | 16 | 2 |

***Figure 9. Mitochondrial Disease Criteria (MDC) in genetically confirmed cases.*** *Bar plots illustrate the distribution of clinical, metabolic, and imaging MDC scores for PMD (left) and non-PMD (right) patients. The bottom panels display overall diagnostic suspicion based on the Modified MDC scale (range 2–12), with and without inclusion of muscle biopsy data. Color shades represent levels of diagnostic likelihood: possible (score 2–4), probable (5–7), and definite (≥8).*

A total clinical score of 4 (maximum) was observed in 63% of PMD cases, compared to only 28% of non-PMD cases. Most PMD patients fulfilled high MDC scores, with 63.2% classified as 'definite' according to the Modified MDC scale, and a majority harbouring nuclear or mtDNA pathogenic variants. Considering the two patients with patients with the lowest score, one was referred during family screening of a mitochondrial variant (P41) and the other was found to have m.8993T>C variant in exome-based testing with mitochondrial variants screening (P04) and only presents with level 3 autism spectrum disorders and epilepsy (2 crisis, well-controlled with antiepileptics). Although many non-PMD patients met moderate MDC scores, only a minority reached the 'definite' category, underscoring the importance of genomic testing in differentiating mitochondrial mimics.

Metabolic criteria contributed significantly to the diagnostic stratification: 66% of PMD patients scored ≥2 in the metabolic domain, while this was observed in only 36% of non-PMD patients. Brain MRI findings were also more frequently abnormal in PMD, with 58% scoring ≥2, whereas none of the non-PMD group reached a score above 2, and the majority scored 0. When combining metabolic and imaging findings, the total MDC laboratory score (max. 4) reached the maximum in 55% of PMD cases, compared to just 7% in the non-PMD group, while 64% of non-PMD patients scored ≤1.

A strong association was observed between final diagnostic classification and the level of clinical suspicion based on the modified MDC score ($\chi^2(4) = 60.1$, $p < 0.001$). Most undiagnosed patients had only a "possible" score, reflecting the clinical uncertainty in this group. In contrast, the majority of confirmed PMD cases had "probable" or "definite" scores, supporting the diagnostic utility of MDC scoring in this cohort. Non-PMD patients were evenly distributed across "possible" and "probable" categories, highlighting potential phenotypic overlap with mitochondrial disorders.

The total MDC score (without histology)) differed significantly across diagnostic classes (Kruskal–Wallis $p < 0.001$). Post hoc pairwise comparisons (Bonferroni-adjusted) showed that patients with confirmed PMD had significantly higher scores than both undiagnosed (adjusted $p < 0.001$) and non-PMD patients (adjusted $p < 0.001$). No significant difference was found between non-PMD and undiagnosed groups (adjusted $p = 0.318$).

For undiagnosed patients, MDC scores ranged from 2 to 9. Six patients scored ≥8 (definite mitochondrial disease), and 14 additional cases fell within the "probable" range (scores 5–7). Among those with high scores, one patient reached a maximum score of 9 and had mtDNA depletion documented in muscle tissue, strongly suggesting a primary mitochondrial DNA maintenance defect. However, exhaustive genetic testing - including WES and mtDNA copy number analysis - failed to identify a causative variant, highlighting the current limitations of diagnostic resolution.

## 2.2. Genotype

### 2.2.1. PMD patients

In the present cohort, all cases were considered *PMD* according to the Genomics England PanelApp *Mitochondrial disorders* panel, which defines genes with established or emerging evidence for mitochondrial pathogenicity. Diagnosis are presented in Table 18, classified accordingly to the International Classification of Inherited Metabolic Disease (ICIMD)[121]. This hierarchical, pathway-oriented framework groups inherited metabolic diseases according to

shared biochemical and pathogenic mechanisms, thereby facilitating a mechanistic understanding of functionally related conditions.

Because the ICIMD system classifies disorders according to their predominant biochemical mechanism, several conditions considered PMD in our cohort (as defined by the Genomics England PanelApp Mitochondrial disorders panel) were distributed across other ICIMD categories, such as such as defects in *PDHX*, *DLD*, *TAZ*, *PANK2*, or *SLC19A3* assigned to energy substrate, cofactor, or organelle biogenesis disorders. This discrepancy reflects the different purposes of both frameworks: ICIMD groups conditions by biochemical pathway, whereas PanelApp relies on expert curation to define which genes are included within the mitochondrial disease spectrum. Overall, the 38 patients with confirmed PMD harboured pathogenic variants in both mitochondrial DNA (mtDNA) and nuclear-encoded genes, encompassing a wide range of molecular mechanisms.

***Table 18. Primary mitochondrial disorders diagnosed in our cohort[1]***

| Category | Subcategory | Genes | n |
|---|---|---|---|
| **Mitochondrial disorders** | | | 32 |
| **mtDNA-related disorders** | mtDNA-encoded OXPHOS proteins | | |
| | Complex I subunits | *MT-ND1* | 1 |
| | | *MT-ND3* | 1 |
| | | *MT-ND5* | 2 |
| | ATP synthase subunits | *MT-ATP6* | 5 |
| | mtDNA-encoded tRNAs and rRNAs | *MT-TL1* | 2 |
| | | *MT-TE* | 1 |
| | Single Large-Scale mtDNA Deletions | | 2 |
| **Nuclear-encoded disorders of oxidative phosphorylation** | Complex I subunits and assembly factors | *NDUFS1* | 1 |
| | | *NDUFV2* | 2 |
| | Complex III subunits and assembly factors | *BCS1L* | 1 |
| | Complex IV subunits and assembly factors | *FASTKD2* | 1 |
| | ATP synthase subunits and assembly factors | *TMEM70* | 1 |
| **Disorders of mitochondrial cofactor biosynthesis** | Coenzyme Q10 biosynthesis | *COQ8A* | 1 |
| | Lipoic acid and iron-sulfur cluster metabolism | *LYRM4* | 1 |
| **Disorders of mtDNA maintenance and replication** | Nucleotide pool maintenance | *ABAT*[2] | 1 |
| | | *DGUOK* | 1 |
| | mtDNA replication and maintenance | *POLG* | 1 |
| | | *RRM2B* | 1 |
| **Disorders of mitochondrial gene expression** | Mitochondrial transcript processing and modification | *HSD17B10* | 1 |
| | | *PNPT1* | 1 |
| | Aminoacyl-tRNA synthetases | *EARS2* | 3 |
| | | *VARS2* | 1 |
| | | *NARS2* | 1 |
| **Others mitochondrial disorders classified in other groups by ICIEM** | | | 6 |
| **Disorders of energy substrate metabolism** | Pyruvate metabolism | *PDHX* | 2 |
| | | *DLD* | 1 |
| **Disorders of organelle biogenesis, dynamics and interactions** | Mitochondrial membrane biogenesis and remodelling | *TAZ* | 1 |
| **Disorders of vitamin and cofactor metabolism** | Pantothenate and CoA metabolism | *PANK2* | 1 |
| | Thiamine metabolism | *SLC19A3* | 1 |

*[1]Diagnosis are classified according to the International Classification of Inherited Metabolic Disease (ICIMD). [2]Also classified as Gamma-aminobutyric acid neurotransmitter disorder.*

mtDNA mutations included classical point mutations (e.g., *MT-ATP6*, *MT-ND3*, *MT-ND5*, *MT-TL1*, *MT-TK*, *MT-CYB*) and large-scale deletions, collectively accounting for multiple cases typically associated with Leigh syndrome, MELAS spectrum, or NARP. Nuclear gene defects involved diverse pathways such as mitochondrial translation - particularly affecting aminoacyl-tRNA synthetases (aaRS) like *EARS2*, *VARS2*, and *NARS2* (n=5) - and mtDNA maintenance and replication genes (e.g., *POLG*, *DGUOK*, *RRM2B*; n=3). Mutations in genes encoding nuclear-encoded OXPHOS subunits (e.g., *NDUFS1*, *NDUFV2*), enzymes of pyruvate metabolism (*PDHX*, *DLD*), and components involved in OXPHOS assembly or cofactor synthesis (e.g., *TMEM70*, *COQ8A*, *TAZ*) were also observed.

Additional aetiologies included defects in mitochondrial RNA processing (*PNPT1*), mitochondrial biogenesis and assembly factors (*BCS1L*, *LYRM4*), and phenocopies or noncanonical mitochondrial disorders (e.g., *ABAT*, *SLC19A3*, *PANK2*, *HSD17B10*). Several patients carried rare or previously unreported genotypes, highlighting the expanding genetic heterogeneity of PMD. This functional classification underscores the molecular complexity of mitochondrial disease and reinforces the utility of pathway-based categorization in clinical diagnostics and research.

### 2.2.2. Mitochondrial mimics

Despite initial suspicion of mitochondrial pathology, these patients presented with phenotypes attributable to other neurodevelopmental, neurometabolic, or syndromic conditions. Common clinical features included global developmental delay (93%), axial hypotonia (75%), epilepsy (54%), movement disorders such as dystonia or ataxia (61%), and FTT (50%).

Biochemical investigations frequently revealed elevated plasma lactate (24/28; 86%) and mild alanine elevation (7/28; 25%), findings that may have contributed to the initial diagnostic hypothesis.

Molecular diagnoses were confirmed by exome or genome sequencing and included syndromic forms of developmental and epileptic encephalopathy (e.g., *STXBP1*, *CDKL5*, *HCN1*), congenital myopathies and muscular dystrophies (e.g., *DMD*, *CHKB*, *LMNA*), and rare neurodevelopmental syndromes (e.g., *CRELD1*, *NSD2*, *ANKRD11*) as presented in Table S2 (Supplementary Material).

These findings highlight the importance of considering a broad differential diagnosis in children with overlapping mitochondrial phenotypes and support the role of genomic analysis in clarifying aetiologies beyond primary mitochondrial disorders.

### 2.2.3. Undiagnosed

Among the 110 patients without a confirmed genetic diagnosis, 30 individuals (27%) were found to harbour rare variants of potential interest in genes previously associated with mitochondrial or

neurogenetic disease. Although insufficient to establish a definitive diagnosis at the time of data cutoff, these findings were considered of potential clinical relevance and were thus categorized into four subgroups, according to diagnostic likelihood and stage of investigation: *i*) Strong candidate variants; *ii*) Candidate variants; *iii*) VUS of low clinical relevance and *iv*) Non-contributory variants. Monoallelic pathogenic or likely pathogenic variants in autosomal recessive genes were, by default, considered non-contributory (carrier state).

Strong candidate variants were found in two patients, in whom we considered to have a probable molecular diagnosis, based on strong phenotypic concordance and the identification of likely pathogenic variants in genes consistent with their clinical presentation and mode of inheritance supported by compatible segregation studies, although confirmatory functional assays were not available: one male patient carried a *de novo* likely pathogenic variant in *MECP2* and a deceased patient with early-onset progressive dilated cardiomyopathy harboured four VUS in *TTN* (AD).

Candidate variants were observed in six patients, who carried either biallelic VUS in autosomal recessive disease genes, variants in mitochondrial genes, or structural variants of uncertain significance. These findings were suggestive but lacked sufficient evidence for diagnostic confirmation and remain with as an uncertain diagnosis:

- One patient with biallelic VUS in *PHKB*, associated with glycogen storage disease type IXb;
- One patient with biallelic VUS in *VARS2*, a nuclear-encoded mitochondrial gene associated with recessive encephalomyopathy;
- One patient with a structural VUS involving a duplication at Xq13.1.
- One patient was lost before investigation could be completed with recurrent acute liver failure (RALF) and concomitant 3-MGA-uria who carried a novel mtDNA variant (m.3264T>C, 50% heteroplasmy) but was lost to follow-up before further analysis.
- Two cases with monoallelic pathogenic or likely pathogenic variants in genes with AR inheritance with high phenotypic concordance together with supportive evidence but negative search for a second allele, disease-specific biomarkers, or preliminary functional concordance) (*DARS2, ETFDH)*.

VUS of low clinical relevance were identified in 22 patients, in whom rare variants in genes of potential interest were detected but considered non-contributory to the clinical presentation. Categorization was based on inadequate segregation or findings inherited in *cis*, absence of a second pathogenic allele in recessive genes, or insufficient functional or phenotypic correlation. Two patients presented mitochondrial DNA variants of uncertain significance: one with a homoplasmic m.9331T>C variant and one with low-level heteroplasmy for m.7695T>C (<20%). In all cases, the available evidence was deemed insufficient to support a confirmed molecular diagnosis, due to the absence of functional validation, incomplete segregation analysis,

or lack of consistent phenotypic correlation. Nevertheless, these findings represent potentially diagnosis and warrant further investigation.

A summary of these results is provided in Table 19.

***Table 19. Summary of variants of potential clinical relevance in undiagnosed patients.***

| Diagnostic likelihood | Genetic findings | n | Gene or mitochondrial variant |
|---|---|---|---|
| **Strong candidate variants (probable diagnosis)** | VUS/LP variants with phenotype and mode of inheritance concordance and compatible segregation studies | 2 | *MECP2, TTN* |
| **Candidate variants (uncertain diagnosis)** | Biallelic VUS in AR genes | 2 | *PHKB, VARS2* |
| | Structural variant of uncertain significance | 1 | dupXq13.1 |
| | mtDNA VUS with incomplete investigation | 1 | m.3264T>C (50% heteroplasmy) |
| | Monoallelic LP variants with phenotype concordance in genes AR | 2 | *DARS2, ETFDH* |
| **VUS of low clinical relevance** | Monoallelic P/LP variants in AR genes without strong phenotypic concordance (carrier state) | 5 | *TRMU, CNTNAP1, NDUFS1, PYGM* |
| | Monoallelic VUS in genes with AD | 5 | *ITPR1, SLC25A3, SRPX2, KDM6B, RYR1* |
| | Monoallelic (or two i*n cis*) VUS in AD genes segregating from a healthy parent | 3 | *POLG* (n=2), *DNM1L* |
| | Monoallelic (or two i*n cis*) VUS in AR genes with insufficient clinical support | 6 | *PLA2G6 (in cis), NSD2, TTN, COL13A1, STIM1* |
| | mtDNA variants of uncertain significance | 3 | m.9331T>C (homoplasmy), m.7695T>C (<20% heteroplasmy) |

*AD, autosomal dominant; AR, autosomal recessive; LP, likely pathogenic; mtDNA, mitochondrial DNA; VUS, variant of uncertain significance.*

Taken together, these 31 patients illustrate the complexity of variant interpretation in the context of suspected mitochondrial disease, underscoring the limitations of exome-based diagnostics and the challenges posed by variants of uncertain or emerging significance.

Seven patients were still under investigation at the time of analysis, pending segregation studies, reverse phenotyping, or functional validation. Three of these patients carried variants in nuclear-encoded mitochondrial genes:

- One patient with a *de novo* missense VUS in *ATAD3A* (c.1145A>G), predicted deleterious and consistent with autosomal dominant transmission.
- One patient with hypertrophic olivary degeneration and a small intragenic duplication in *LRPPRC*, classified as a VUS.
- One patient with ethylmalonic aciduria and two VUS in *ACADS*, associated with short-chain acyl-CoA dehydrogenase deficiency.

Four additional patients carry variants in non-mitochondrial genes:

- One patient with a compound heterozygous combination of a likely pathogenic variant and a VUS in *CNTNAP1*, with partial phenotypic concordance.
- One patient with a predicted deleterious VUS in *DES*, compatible with the observed phenotype, and two additional VUS in *VPS13D*.
- One patient with two heterozygous VUS in *KCNMA1*, segregating from each parent, a gene associated with both autosomal dominant and recessive inheritance.
- One patient carrying a likely pathogenic variant in *TBCD* (AR), under further genetic and functional reassessment.

A summary of these genetic findings in undiagnosed patients included in the cohort is illustrated in Figure 10.

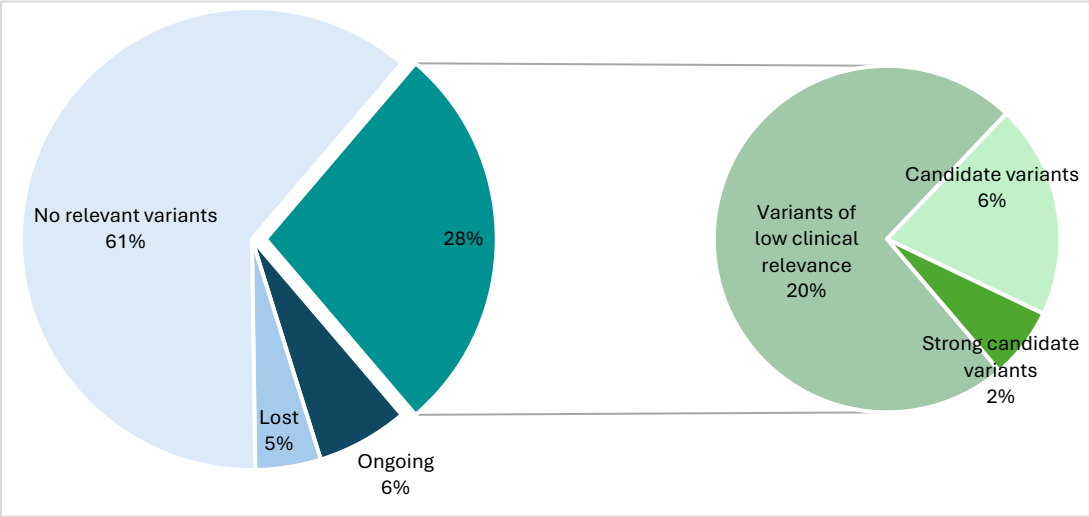


***Figure 10. Genetic findings in in the undiagnosed group.*** *Less than a third of the patients (28%) had genetic variants that warranted further assessment. These were classified according to relevance to diagnosis.*

Noteworthy, one patient has a Leigh spectrum syndrome without genetic diagnosis and another had development delay with progressive acquisitions, mild cognitive impairment, mild cardiomyopathy, whose MRI was normal but MRS revealed a lactate peak in voxel centred in the basal ganglia (even without associated involvement) and severe mtDNA depletion in muscle (11% copy number of mtDNA), strongly suggestive of a mitochondrial maintenance defect, yet no definitive molecular cause was found. At recent follow-up, ptosis was observed for the first time.

## 3. Diagnostic Odyssey

All patients in the cohort underwent at least one form of genetic investigation. Genetic testing was performed using multiple modalities, including targeted nuclear gene panels, mtDNA analyses, whole exome sequencing (WES), chromosomal microarray, methylation studies, and Sanger sequencing.

## 3.1. Testing strategies

For patients with pathogenic point mutations in mtDNA (n=12), the majority (n=11) were diagnosed through mtDNA sequencing as a first-line or in parallel test. In one case, diagnosis was achieved after a WES-based nuclear gene with mtDNA screening for common mutations, followed by confirmatory testing that revealed the pathogenic m.3434A>G in 56% in blood.

Among the two patients with single large-scale mtDNA deletions, one had previously undergone targeted testing for common point mutations, while the other had a more extended evaluation: sequencing of nuclear mitochondrial genes and full mtDNA sequencing were performed before the muscle biopsy, where a large-scale simple deletion was found.

For the 24 patients with pathogenic or likely pathogenic variants in nuclear-encoded mitochondrial genes, diagnostic strategies were heterogeneous:

- 15 patients were diagnosed via targeted NGS panels, used either as first-line tests or in parallel with mtDNA analysis;
- 5 patients received their diagnosis through WES, one as the initial test (neonatal presentation);
- 1 patient was diagnosed via Sanger sequencing, prompted by a strong clinical hypothesis (GRACILE phenotype with *BCS1L* pathogenic variant);
- 1 patient was diagnosed through a non-mitochondrial gene panel targeting ataxia-related genes (*COQ8A*).

Among the 28 patients ultimately diagnosed with alternative genetic disorders, diagnostic trajectories were similarly diverse, often involving multiple rounds of testing. In only 4/28 (14.3%) the diagnosis was obtained through first-line molecular studies: one with neonatal presentation that had WES performed as a first-line investigation, in patients with muscular dystrophies for whom muscle biopsy was performed early and genetic confirmation was performed directly by MLPA ( 2 patients with Duchenne muscular dystrophy one patient whose father had a diagnosis of mitochondrial myopathy, and the biopsy of the child elicited the diagnosis of Emery-Dreifuss dystrophy, confirmed by Sanger, in both the child and the affected father).

In the remaining 24 patients, the diagnosis was reached through second-line investigations, all following nondiagnostic results in initial mitochondrial work-up (mtDNA testing, either targeted analysis of common mutations or complete mtDNA sequencing and mitochondrial NGS gene panel) and 8 chromosomal microarrays (arrayCGH), which was nondiagnostic. VUS were reported in 6 patients (1 mitochondrial, 4 nuclear, 1 copy number variant (as previously presented).

Twelve patients were diagnosed through WES-based testing, 10 at after initial report and in two cases with variants initially as VUS, subsequently considered disease-causing following

multidisciplinary re-evaluation, segregation analysis, clinical reassessment, and review of evolving evidence.

Prior to a definite diagnosis, seven patients were found to have variants in other genes that elicited further work-up for eventual (re-)classification: *CPT2*, *DNM1L*, *NDUFS1*, *PMM2*, *NOTCH3*, m.14912T>G and a duplication dup16p13.2, encompassing the genes *METTL22* and *ABAT* (≈81 kb).

## 3.2. Diagnostic yield

A total of 88 patients (49.4%) underwent mtDNA analysis with overall diagnostic yield of 13.6%. Screening for common mtDNA mutations was obtained in 22, resulting in 7 molecular diagnoses (diagnostic yield 38.9%). Full mtDNA sequencing allowed further diagnosis in 5 of 67 tested patients (diagnostic yield 7.5%) and detection of 4 VUS. The choice between full sequencing and targeted mutation screening was largely influenced by historical testing availability and clinical prioritization. In addition, mtDNA deletion and copy number analysis was conducted in 13 patients, identifying 2 single large-scale deletions and 1 case of multiple deletions associated with variants in *RRM2B* gene.

Targeted NGS panels for mitochondrial disorders were performed in 99 patients, with a diagnostic yield of 19.2%. An additional 22 patients underwent other targeted NGS panels (not WES-based), focusing on specific differential diagnoses, with a diagnostic rate of 36.4%. WES or WES-based gene panels were performed in 50 patients. A molecular diagnosis was established in 19 cases (diagnostic yield 38.0%), including 6 diagnoses from WES-based panels (n=11; yield: 54.5%) and 12 from standard WES (n=39; yield: 30.8%). In addition, 14 VUS were reported (2 from targeted, 12 from non-targeted WES). Fifteen additional patients had ongoing or pending WES analysis at the time of data cutoff.

Test type and corresponding results are summarized in Figure 11.

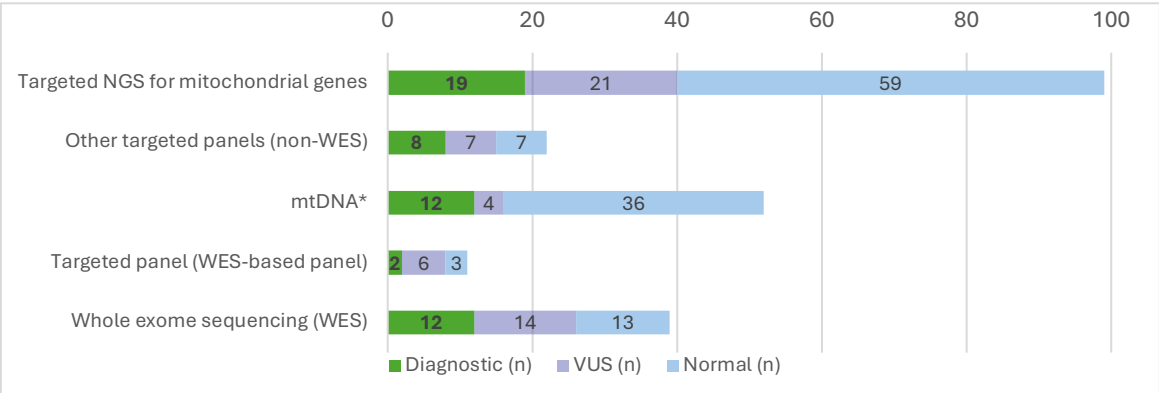


***Figure 11. Distribution of genetic tests applied in our cohort and corresponding results.*** *Stacked bar chart showing the number of patients (n) undergoing each genetic testing approach, categorized by result: diagnostic (green), variant of uncertain significance (VUS; purple), and nondiagnostic (light blue) *includes mtDNA testing for most common mutations and/or complete sequencing.*

Several classes were diagnosed through alternative genetic testing approaches, such as MLPA used to confirm diagnosis elicited by muscle biopsy: in the cases of Duchenne muscular dystrophy and Sanger sequencing in Emery-Dreifuss muscular dystrophy. Chromosomal microarray (array CGH) identified pathogenic findings in two patients and VUS in two others - one of whom was later diagnosed with an alternative genetic disorder.

Methylation studies led to two additional diagnoses. In one case, methylation-specific MLPA revealed a heterozygous deletion and hypomethylation at 15q11-q13, consistent with loss of the maternal allele. In the second case, methylation analysis identified hypomethylation of the imprinting control region 1 (ICR1) at 11p15.5. In one additional case, a high-resolution exonic CNV array (XON) identified a variant of uncertain significance in a clinically relevant gene, although no definitive diagnosis could be established.

## 3.3. Contribution of muscle biopsy for the diagnostic process

Muscle tissue study remains a valuable diagnostic tool in the evaluation of suspected mitochondrial disease, particularly in settings where genetic testing is inconclusive or unavailable. In this cohort, muscle biopsy was performed in a substantial proportion of patients with confirmed PMD (n = 23) and non-PMD diagnoses (n = 25).

Histological and immunohistochemical findings ranged from normal to markedly abnormal, including RRF, COX-deficient fibbers, and fibre-type atrophy. One non-PMD case (diagnosed with Emery-Dreifuss dystrophy) even showed RRF in histological studies. In a minority of cases, the biopsy findings directly contributed to the final diagnosis, either through characteristic histological features or by enabling genetic or biochemical confirmation.

Respiratory chain activity analysis, performed in nearly all biopsied patients, provided additional functional insights, with some cases revealing isolated or multiple complex deficiencies. Table 20 summarizes the key histological and biochemical findings observed in both diagnostic groups.

***Table 20. Muscle biopsy findings in primary mitochondrial disease and alternative diagnosis.***

| | PMD (n=38) | non-PMD (n=28) |
|---|---|---|
| **Histology and immunohistochemistry** | 23 | 25 |
| **Normal** | 12 | 9 |
| **Type 2 fibber atrophy** | 5 | 4 |
| **Type 1 fibber atrophy** | 0 | 2 |
| **Nonspecific findings** | 3 | 6 |
| **RRF (isolated or in combination)** | 1 | 1 |
| **COX-negative fibbers (isolated or in combination)** | 1 | 1 |

| Respiratory chain activity | 23 | 22 |
|---|---|---|
| **Performed** | 23 | 22 |
| **Normal** | 12 | 10 |
| **Partial isolated deficiencies (<40%)** | 4 | 4 |
| **Significant isolated deficiencies (<30%)** | 2 | 3 |
| **Multiple deficiency (≥ 2 complexes)** | 4 | 5 |
| **Significant contribution for final diagnosis** | 4 | 4 |

Muscle biopsy findings were classified as instrumental for the final diagnosis when they contributed directly and decisively to disease confirmation in 10.5% of PMD and 14.3% of non-PMD patients. This included cases where diagnostic histological or immunohistochemical features (e.g., dystrophic patterns, presence of ragged-red or COX-deficient fibbers with multiple mtDNA deletions) pointed to a specific genetic condition; where mitochondrial DNA deletions or pathogenic variants were detected in muscle-derived DNA; or where enzymatic analysis in muscle revealed multiple respiratory chain deficiencies that supported a suspected molecular diagnosis. Such cases demonstrate the continued diagnostic value of muscle biopsy, especially in complex or genetically ambiguous presentations.

## 3.4. Time to diagnosis and diagnostic intervals

Table 21 summarizes the key milestones and intervals across the diagnostic trajectory for patients with confirmed PMD and those with alternative genetic diagnoses (non-PMD).

***Table 21. Diagnostic odyssey for mitochondrial and non-mitochondrial patients.***

| | PMD (n=38) mean [min–max]) | Non-PMD (n=28) mean [min–max] |
|---|---|---|
| **Age at symptom onset** | 1.7 [0.00–10.00] | 2.4 [0.00–14.00] |
| **Age at first metabolic consult** | 4.6 [0.00–17.00] | 3.9 [0.00–14.00] |
| **Age at diagnosis** | 7.7 [0.08–21.00] | 10.7 [0.50–23.00] |
| **Time from onset to first consult**[1] | 3.2 [0.00–14.75] | 1.5 [0.00–11.00] |
| **Time from onset to diagnosis**[1] | 6.4 [0.01–20.75] | 8.3 [0.20–21.00] |
| **Time from first consult to diagnosis**[1] | 2.9 [0.08–17.80] | 6.9 [0.30–19.00] |
| **Follow-up duration (y)** | 9.1 [0.00–25.00] | 10.0 [1.90–25.00] |

*Data are presented in years. [1]One patient referred presymptomatic for familial screening was excluded.*

Age at symptom onset was earlier in the PMD group (1.7 years) than in the non-PMD group (mean 2.4 years). However, time from symptom onset to first metabolic consultation was longer among PMD patients compared to non-PMD (3.2 vs. 1.5 years), indicating a delay in referral despite earlier clinical signs. In contrast, age at diagnosis was also lower in the PMD group than non-PMD (7.7 vs. 10.7 years), with corresponding lower time from first metabolic consultation to diagnosis (2.9 vs. 6.9 years). Follow-up duration was broadly similar between groups (9.1 vs. 10.0 years). In two cases, diagnosis was only achieved post-mortem following neonatal fulminant presentation (*NARS2* and *HSD17B10*).

The introduction of NGS (in our setting around 2017) significantly shortened the diagnostic trajectory in both PMD and non-PMD groups., Time between first metabolic evaluation and diagnosis was reduced from 4.2 to 0.9 years in PMD patients and from 8.2 to 2.1 years in non-PMD, highlighting the critical role of NGS in resolving complex phenotypes. This evolution highlights the impact of technology access in shortening diagnostic delays.

Chapter IV. Results

# Section II. Expanding the Phenotype Spectrum in Ultra-Rare Mitochondrial Disorders

The application of a structured diagnostic algorithm across a longitudinal paediatric cohort enabled the identification of ultra-rare mitochondrial disorders.

## 1. ISD11-deficiency (*LYRM4*)

Mitochondrial disorders are usually progressive and multisystemic. Nevertheless, in children presentation may be acute, fatal or episodic and recurrent. Predisposing factors for an acute event are catabolic states such as infectious intercurrence, surgical procedures or use of drugs that impair mitochondrial function. Work-up during symptom-free intervals may be normal.

We reported a case of a girl born from non-consanguineous healthy parents, with an episode of cardiorespiratory arrest at 2 years of age following a respiratory viral illness, being admitted in a pediatric intensive care unit (UCI). Work-up showed persistent hyperlactacidemia and the identification of respiratory syncytial virus (RSV) in respiratory secretions and Herpes 6 in cerebrospinal fluid (CSF). She had normal ECG, echocardiogram and brain MRI. She showed a positive evolution without evidence of neurological sequelae and was discharged pending metabolic results, later being lost in follow-up. Growth and development were reported as normal.

At 3 years of age, after a cardiac arrest following acute respiratory insufficiency, she was again admitted to UCI (this time at our center). Parents reported nasal discharge, cough, fever and reduced appetite for the previous 4 days, with a progressive decrease of activity. The day of the admission, she became progressively lethargic and showed difficulty to breath prior to the cardiorespiratory arrest. Physical exam was normal. Initial work-up showed normal blood counts, negative C-reactive protein, transaminases 2 times the normal upper limit and creatine kinase 3-4 times, persistently high lactate (2.3-4.1 mmol/L), respiratory secretions positive for RSV, normal CSF counts, positive PCR for Enterovirus. Cardiac evaluation was again normal, and immunodeficiency was excluded. Initially elevated liver enzymes and creatine-kinase rapidly returned to normal values.

Plasma amino acids were irrelevant and acylcarnitines had low free carnitine (6.5 μM, normal range, NR 19-48 μM) with normal acethylcarnitine. Urinary organic acids on admission showed ketonuria and the moderate excretion of 3-methylglutaconic acid (3-MGA) 154 μmol/mmol creatinine (NR <19 μmol/mmol creatinine) and mild excretion of 3-hydroxy-isovaleric acid (3-OH-IVA) 85 μmol/mmol creatinine (NR 10.4-67.0 μmol/mmol creatinine) and 3-hydroxy-methylglutaric (3-HMG) 93 μmol/mmol creatinine (NR 6.2-49.7 μmol/mmol creatinine), as shown in Figure 12 showed massive excretion of 3-methylglutaconic acid (3-MGA) accompanied by mild excretion of 3-hydroxy-isovaleric and 3-hydroxymethylglutharic acids (Figure 12). These findings were similar to the work-up performed during the previous episode. After recovery, lactate and organic acids

rapidly normalized. After 5 days she was weaned off ventilation support, with a very positive recuperation was discharged two weeks later, with oral carnitine supplementation.

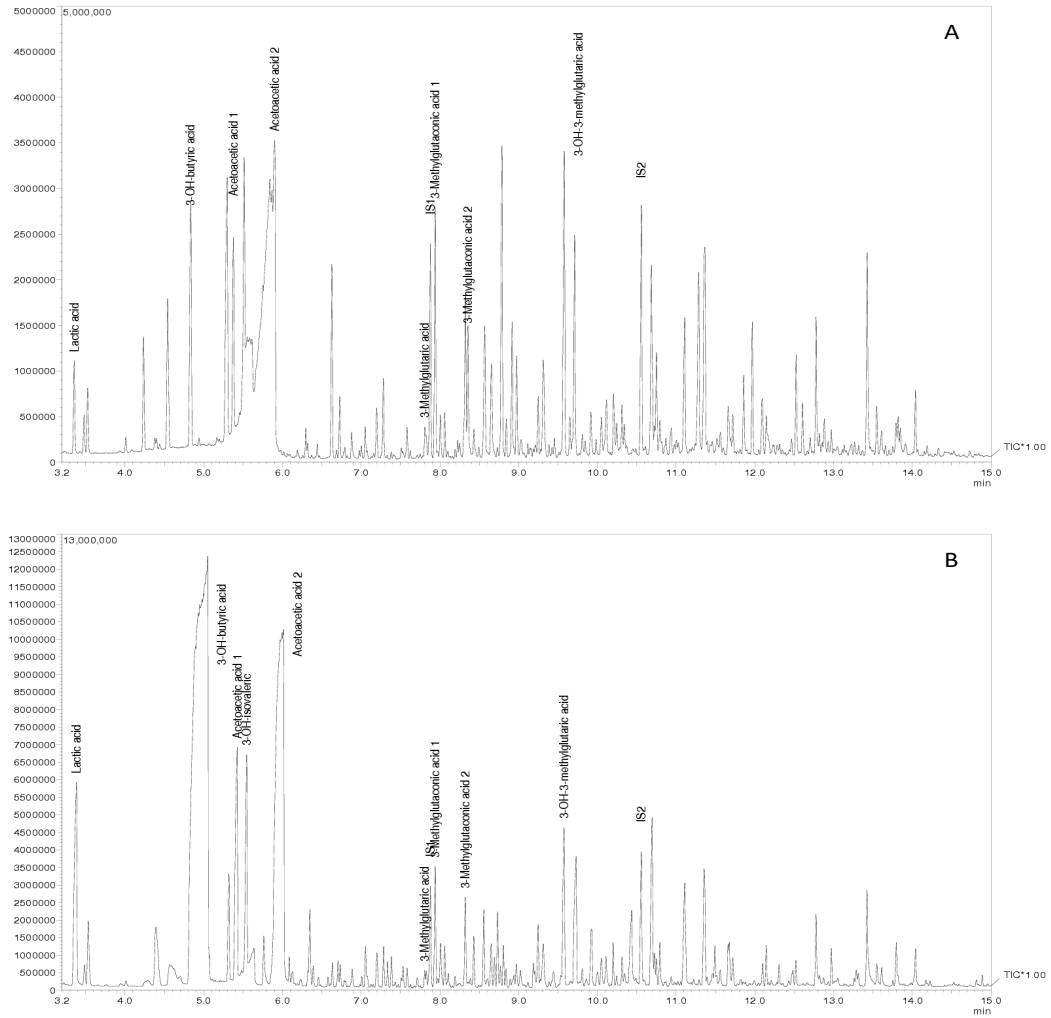


***Figure 12. Excretion of 3-methylglutaconic during acute crises.*** *Chromatograms of urinary organic acids:(A) cardiorespiratory arrest, (B) during febrile mild illness without clinical deterioration. IS1: internal standard 1 (3-phenylbutiric acid); IS2: internal standard 2 (3-tricarballylic acid).*

Later work-up during intercurrent illness showed excretion of recurrent moderate to massive 3-MGA-uria. During an intercritical and asymptomatic interval, metabolic re-evaluation had no hyperlactacidemia, ketosis, elevated CK or altered organic acid excretion.

Targeted next generation sequence for mitochondrial disorders reveled a novel homozygous probably pathogenic novel variant in the *LYRM4* gene (c.92A>G, p.(Tyr31Cys)), inherited from both parents. The pathogenicity was predicted bioinformatically by SIFT, PolyPhen-2 and MutationTaster as probably pathogenic variant. The p.Tyr31Cys variant affects an amino acid that is highly conserved in different species through evolution, was absent in 100 alleles from Portuguese subjects and is not reported in public SNP databases, including dbSNP, the Exome Variant Server and gnomAD (without any reported homozygote). From the publication of this report

in 2019 until now, combined deleterious prediction scores and population data have remained consistent.

This gene encodes form ISD11 codes for a small protein (11 kDa) a key component of the mitochondrial iron-sulfur cluster (ISC) assembly machinery. Iron-sulfur clusters are crucial as they serve as co-factors of the oxidative phosphorylation. Alterations in ISD11 were previously described in two cousins, leading to a combined oxidative phosphorylation deficiency and acute presentation in the first years of life.

Muscle histology showed occasional atrophy of muscle fibbers, frequent accumulation of lipid droplets, without other changes such as RRF or COX negative fibbers. Functional assay of the respiratory chain showed elevated citrate synthetase activity (354,3 nmol/min/mg, NR 85,0-180 nmol/min/mg) multiple complex deficiency (I 40%, II 23% and IV 31%).

She is now 11 years old without any further metabolic crisis, with age-appropriate development (in fact, a merit student) and growth. She performs regular and intense physical activity, with occasional periods of fatigue usually ameliorated by carnitine supplementation, titrated by plasma levels. In the last year she showed mild but persistent thrombocytopenia, with no evidence of peripheral destruction, most likely of medullary origin.

This diagnosis established the patient as the third genetically confirmed case of ISD11 deficiency worldwide, and the second affected family, confirming *LYRM4* as disease-causing gene and a new cause for combined mitochondrial deficiency[313]. Within these three patients, rapid clinical status deterioration with respiratory failure and arrest during an infectious intercurrence appear to be a common feature. When surviving these acute crises, apparently asymptomatic periods follow.

The consistent and significant (>40 μmol/mmol creatinine) excretion of 3-MGA only during acute events was not previously reported in patients with any Fe-S cluster deficiency and extends the differential diagnosis of 3-methylglutaconic aciduria as a presenting feature[159]. This case reinforces the recommendation that *LYRM4* be included in gene panels for metabolic crises, especially in early years of life and mitochondrial disease.

It also represents an example of how life-threatening events disproportional to symptoms and medical history should elicit the possibility of energy related inherited metabolic disorder. Work-up must be performed as soon as possible, while the child is acutely ill, as it may be a unique opportunity to diagnose a critical condition, since biochemical abnormalities may be transient.

## 2. Phenotypic Spectrum of *EARS2*-Deficiency

Mitochondrial aminoacyl-tRNA synthetase deficiencies are an increasingly recognized subgroup of primary mitochondrial disorders, frequently associated with early-onset encephalopathy and heterogeneous outcomes. Among these, biallelic pathogenic variants in *EARS2*, encoding mitochondrial glutamyl-tRNA synthetase, have been classically linked to a distinct neuroimaging phenotype termed LTBL (leukoencephalopathy with thalamus and brainstem involvement and high lactate). However, expanding clinical data, including our own findings, challenge the notion of a uniform presentation and suggest that *EARS2* deficiency represents a broader phenotypic spectrum.

Within our cohort, we identified three unrelated patients with biallelic *EARS2* variants, accounting for approximately 6% of all published cases globally at the time (n = 43 at the time of publication). Their markedly divergent clinical and radiological features illustrate the continuum of severity seen in *EARS2*-related disease:

- 34-day-old male, transferred to our center with vomiting, feeding refusal, hypotonia and poor suction and FTT, associated with lactic acidosis (pH 7.21; lactate 6.6 mmol/L) and elevated liver enzymes (2,5x the upper normal limit). Subsequent evaluations showed persistent lactic acidosis (7.8-9.54 mmol/L), ketonuria, increased liver enzymes and unspecific markers of mitochondrial dysfunction (amino acids and urinary organic acids). Brain MRI revealed corpus callosum agenesia, T1 hypointensity in brainstem (hypomyelination) with hyperintensity in T2 affecting the cerebellum (including dentate nuclei) and T2 hyperintensities in cerebral white matter (subcortical parieto-occipital, anterior temporal, and frontal regions). MRS showed lactate, alanine, and pyruvate peaks. He died at 60 days of age following progressive respiratory failure and severe lactic acidosis.
- 10-month-old boy born with transient icterus, hepatomegaly and feeding difficulties with an apparent loss of functions, including smile and speech, feeding difficulties and spasticity. During his first months of life, he had axial hypotonia, pyramidal syndrome, and nephrolithiasis (transient) and recurring metabolic acidosis and hypoglycemia, persistent hyperlactatemia and elevated liver enzymes. Brain MRI depicted bilateral symmetrical hyperintensities, predominantly affecting subcortical and periventricular white matter, with notable involvement of the corpus callosum, putaminal nuclei, thalami, mesencephalon, periaqueductal gray matter, dentate nuclei, and the posterior brainstem and MRS exhibited a lactate peak in cerebral cortex. Follow-up (total follow-period of 14 years) revealed global significant global delay, slowly progressive deterioration with epilepsy, chronic obstructive pulmonary disease and obstructive sleep apnea,

constipation and neurogenic bladder dysfunction, while maintaining recurrent metabolic derangements.

- 15-month-old female, delivered at full term following an uneventful pregnancy and labor with Apgar score of 10/10 and normal birth weight. She was referred to our clinic due to axial hypotonia first noted at 6 months and MRI performed at 7 months suggestive of MD. MRI findings revealed signal abnormalities in both infra and supratentorial structures, presenting hyperintensity on T2 and FLAIR sequences, with a confluent pattern in the semioval centers and corona radiata, while sparing the U fibers and the periventricular white matter. She had no history of regression nor epilepsy. She had moderate global developmental delay with generalized muscle weakness and increased deep tendon reflexes. Laboratory investigations were unremarkable (including liver enzymes and creatine kinase), except for consistently elevated serum lactate levels ranging from 2.8 to 5.4 mmol/L, high serum alanine and intermittent mild urinary excretion of glutaric acid. There was no indication of peripheral neuropathy, cardiopathy, or organomegaly. She had normal growth and no feeding difficulties. MRI at 2,5 years demonstrated extensive hyperintensities in T2 and FLAIR in the periventricular and subcortical white matter of the cerebral hemispheres, particularly in the posterior periventricular regions, bilateral involvement of basal ganglia, thalami, pons, and midbrain (especially peri-aqueductal gray matter). Areas of demyelination in the supratentorial white matter were also observed. MRS was normal. Follow-up was unremarkable, with mild learning disability and attention deficit disorder. She showed static mild cerebellar syndrome, with ataxia, changes in balance and fine motor skills difficulties.

This inter-individual variability prompted a systematic phenotype clustering approach, developed through pooled analysis of published cases. Meta-analysis of clinical data, brain MRI, and metabolic parameters allowed us to propose a classification into mild, intermediate, and severe phenotypes. This classification is based on the following clinical features at presentation: regression or acute deterioration, absent or severe developmental delay, encephalopathy and/or irritability, epilepsy, liver failure, FTT, feeding difficulties, and/or metabolic or lactic acidosis (not isolated hyperlactacidemia). Patients were classified as mild when none of these features were present, moderate when one feature was present, and severe when two or more features were observed at initial presentation.

Distribution of clinical features based on this classification allowed to identify some clusters of presentation. EARS2 deficiency more commonly manifests as severe phenotype (53%), as a combination of regression or absent or severe developmental delay, metabolic acidosis, FTT and/or feeding difficulties. Movement disorders were restricted to the severe phenotype and epilepsy was more common. These patients presented earlier mainly (80%) in the first 3 months of

life and the remaining during the first year of life. Prognosis was poor, with 40% of patients dying within the first year of life and a mean survival of 3,52 years. A subset of cases had a catastrophic clinical course, characterized by severe, rapidly progressive onset requiring hospital admission. All presented during infancy (half of them as neonates) and 72% died within six months, with a median age at death of 3,4 months. The occurrence of metabolic acidosis may signify a more severe clinical presentation and is associated with an unfavourable prognosis.

Moderate phenotype was observed more commonly with infancy to childhood onset (median age of 5,2 months). Muscle tone changes (67%), development delay (44%) and regression (22%) were the most common findings. Two thirds had clinical improvement, while 22% progressed. All milder cases presented with development delay with or without hypotonia (83%) at median age of 9,5 months. All patients showed clinical improvement, with initial hypotonia often progressing to spasticity (33%).

Phenotype clustering was associated with abnormalities of the corpus callosum, with 55% of individuals in the catastrophic group exhibiting either complete or partial agenesis. In cases classified as moderate or mild phenotypes, corpus callosum abnormalities typically presented as a thin posterior segment. The distribution of these clusters and their clinical trajectories is summarized in Figure 13.

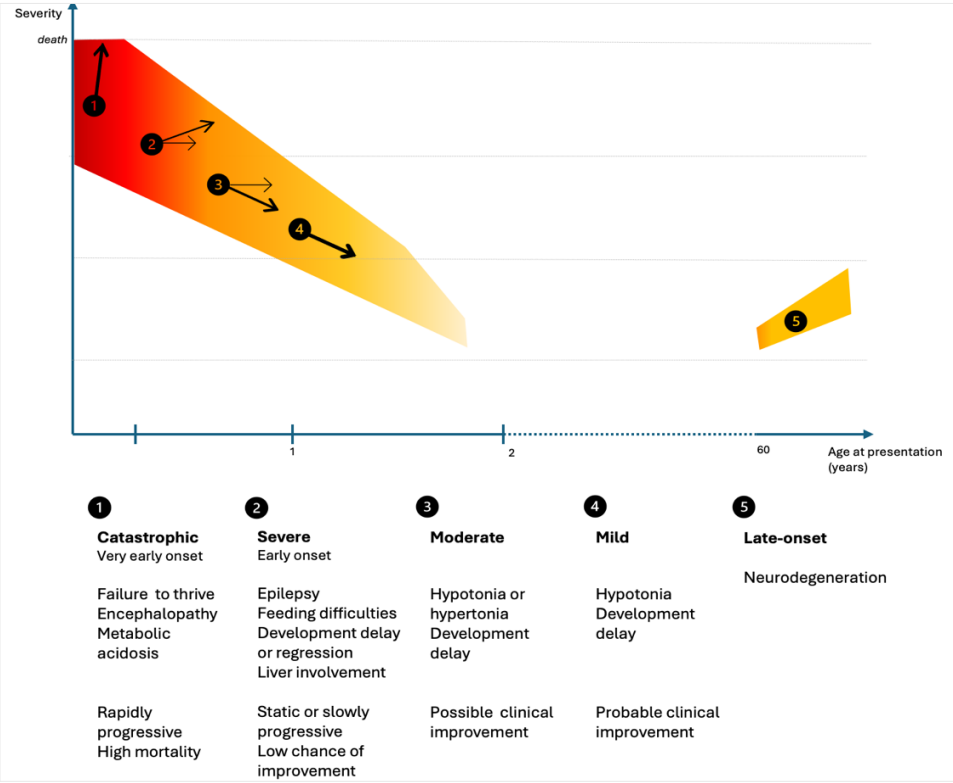


***Figure 13. Phenotypic spectrum of EARS2-related disease.*** *Patients were stratified into five categories (catastrophic, severe, moderate, mild, and late onset, according to clinical features at presentation and subsequent disease course. Severity (y-axis) is plotted against age at presentation (x-axis). Arrows illustrate the range of trajectories observed within each category.*

We suggest that specific features at presentation appear to closely associate with the course and outcomes of the disease. Thus, this classification irrespective of age at onset, initial outcomes, or subsequent follow-up, suggest a potential prognostic value of early clinical features, such as encephalopathy, regression, and liver involvement. It illustrates the continuum spectrum of the disease, ranging from rapidly progressive neonatal disease with high mortality to milder phenotypes with developmental delay and partial recovery, as well as rare late-onset neurodegeneration.

The distribution of all reported pathogenic variants in EARS2 is shown in Figure 14, mapped onto the genomic structure and protein domains. No consistent genotype–phenotype correlations were observed. Most variants cluster within the catalytic domain (exons 2–4), particularly around codons 96–110, have been described with variable severity. Variants affecting the initiation codon (p.Met1?) and early residues (p.Ile62Phe, p.Lys65Glu, p.Arg55His/Leu) were often associated with catastrophic or severe early-onset disease. Overall, no single mutational hotspot fully accounts for the phenotypic spectrum, but recurrent variants in specific residues highlight critical regions for EARS2 function.

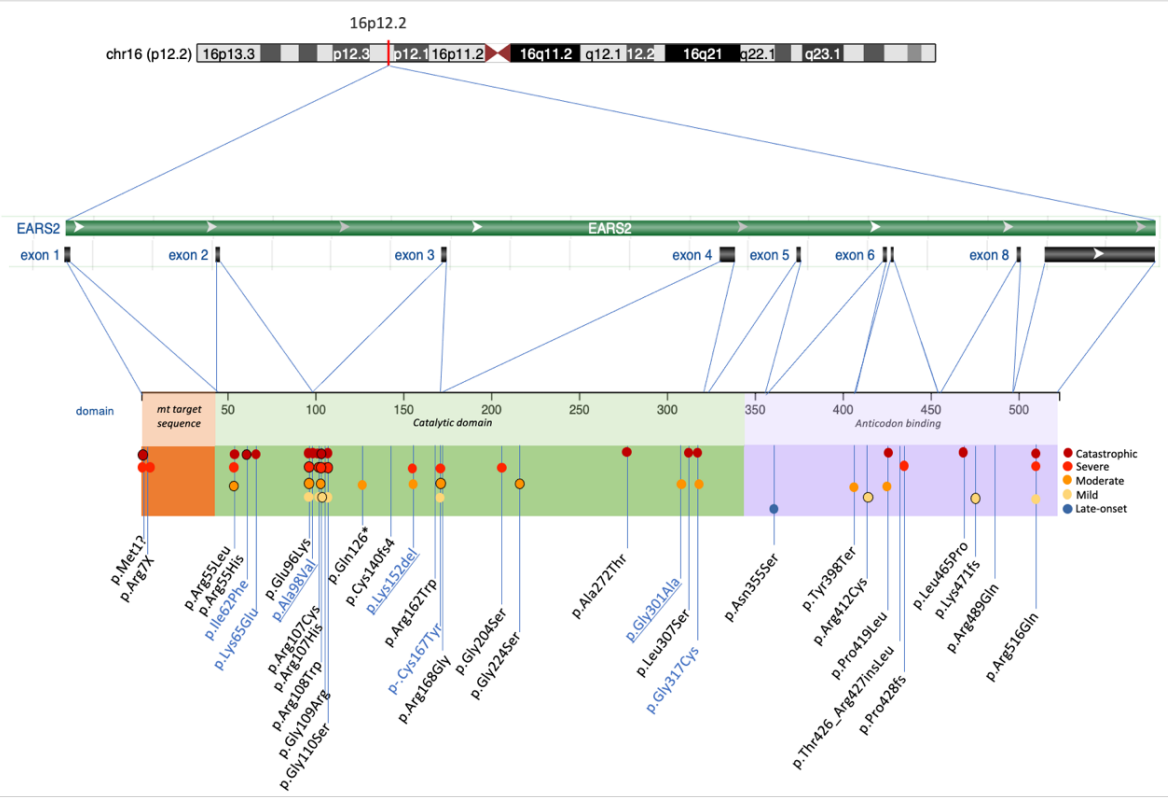


***Figure 14. Genotype–phenotype correlations in EARS2-related disease.*** *Schematic representation of the EARS2 gene (chromosome 16p12.2) and protein with functional domains (mitochondrial targeting sequence, catalytic domain, anticodon-binding domain). Reported pathogenic variants are mapped according to their protein position. The clinical severity associated with each variant is indicated by colour: catastrophic (red), severe (orange), moderate (yellow), mild (green), and late-onset (blue). Homozygous variants are in blue, underlined if describe only in homozygosity. Intronic variants are not represented (one case). Phenotypes with more than one case described for the same variant are outlined in black.*

Six patients with *EARS2*-related disease of Portuguese origin have been reported in the literature; their clinical phenotypes and genotypes are presented in Table 22[143,176]. The recurrent variant p.Met1? was found in four individuals all from Portuguese ancestry, combined with different variants in the second allele. Reported disease courses severe but slowly progressive disease to early fatal outcomes in infancy. The p.Ile62Phe variant has been reported in three patients, two of them of confirmed Portuguese origin, each presenting with early onset and a rapidly progressive course.

***Table 22. Reported Portuguese patients with EARS2-related disease.***

| P. | Variant | Onset | Phenotype | Progression | Outcome |
|---|---|---|---|---|---|
| **8*** | c.1A>G, p.Met1?<br>c.320G>A, p.Arg107His | Early infancy | Severe | Slow Progression | Alive (14y) |
| **26** | c.184A>T, p.Ile62Phe<br>c.1A>G, p.Met1? | Early infancy | Catastrophic | Rapidly progressive and fatal | Dead (5M) |
| **29** | c.1A>G, p.Met1?<br>c.286G>A, p.Glu96Lys | Unknown | Moderate | Unknown | Alive (6y) |
| **30** | c.1A>G, p.Met1?<br>c.286G>A, p.Glu96Lys | Early infancy | Catastrophic | Probable rapidly progressive and fatal | Dead (2M) |
| **42*** | c.1235G>A, p.Arg412His<br>c.1413del, p.Lys471fs | Infancy | Mild | Clinical improvement<br>Radiological progression | Alive (8y) |
| **43*** | c.184A>T, p.Ile62Phe<br>c.920T>C, p.Leu307Ser | Early infancy | Catastrophic | Rapidly progressive and fatal | Dead (2M) |

**Patients from our cohort (P8, P42 and P43). Variants are described according to NM_018196.4. Abbreviations: y, years; M, months.*

This work contributed to the redefinition of *EARS2* deficiency as a spectrum disorder and demonstrated that, contrary to earlier descriptions, the classical LTBL phenotype is not universal, and the designation may in fact obscure the recognition of broader clinical spectrum.

Our data suggest that early phenotype-based stratification may offer a practical tool for prognosis, particularly in the absence of reliable genotype-based predictions. Beyond its phenotypic insights, this study underscores the value of longitudinal clinical follow-up, systematic reanalysis, and international data aggregation for ultra-rare mitochondrial conditions.

Conducting natural history studies will enhance our understanding and may provide validation for the proposed scoring system.

Chapter IV. Results

# Section III. Metabolomic Insights into Mitochondrial Dysfunction

## 1. Urinary Metabolomic Profiling in Paediatric Primary Mitochondrial Disease

Renal involvement is a well-documented and prevalent characteristic of PMD, presenting either as an initial symptom or manifesting during the progression of the disease[126,226,299,314,315]. The most identified renal phenotypes in PMD are proximal tubulopathy, nephrotic syndrome, focal segmental glomerulosclerosis, and end-stage chronic kidney disease (CKD). Renal involvement may also be present in disorders characterized by secondary mitochondrial dysfunction, even when kidney impairment is not a typical feature of their phenotype, such as in Prader-Willi syndrome[316].

Variations in mitochondrial and nuclear-encoded mitochondrial genes are known risk factors for kidney disease and CKD progression[317]. Non-mitochondrial CKD and acute kidney injury are also linked to mitochondrial metabolism, reflecting a close interplay between mitochondrial function and renal homeostasis[290,318–323].

Urinary metabolite profiling, particularly through techniques such as $^{1}$H NMR or mass spectrometry, has become an interesting non-invasive method for assessing mitochondrial dysfunction[132]. Several metabolomics studies in CKD patients, irrespective of its aetiologies, revealed higher Krebs cycle metabolites and lower mitochondrial function and biogenesis, correlating with CKD stage and progression[280,324–326,284,327,288,328].However, only a few studies focused on the metabolomic profile of PMD patients, some without genetic confirmation, with most focusing on targeted organic acid analyses or plasma-based metabolomics[294]. Additionally some studies lacked genetic confirmation limiting its impact [294,329,330]. Most often, design studies included healthy controls and less frequently pre-symptomatic carriers, disease control groups (myopathy) or clinical controls (suspected but unconfirmed disease) [46,282,299,330,331].

Metabolomics enables functional characterization of mitochondrial dysfunction by identifying disease-specific biochemical alterations. Distinct metabolic signatures have been reported in various PMD subtypes, including MELAS, Leigh syndrome, and mitochondrial myopathies. For instance, untargeted metabolomic analyses in MELAS revealed a panel of 36 discriminatory metabolites associated with redox imbalance, one-carbon metabolism, and amino acid perturbations[295]. In monogenic Leigh syndrome (*LRPPRC*), 45 metabolites were identified, many of which mapped to energy metabolism and amino acid catabolism[294]. The urine metabolome of adult mitochondrial patients with the m.3243A>G variant showed significant differences from healthy controls, without renal involvement or heteroplasmy correlation, showing impaired redox balance, β-oxidation, one-carbon metabolism, trans-sulfuration pathway and methylation cycle [295,296,299].

To date, no studies have directly compared the urinary metabolomes of PMD and CKD patients, despite the growing evidence that renal dysfunction in mitochondrial disorders can mimic and coexist with CKD.

A subset of patients from our cohort was enrolled in prospective metabolomic study using untargeted $^{1}$H NMR spectroscopy to analyse urinary samples. In this study, we applied untargeted $^{1}$H NMR analysis to urine samples from paediatric patients with genetically confirmed PMD, suspected mitochondrial disease (SMD), CKD, and controls, aiming to identify shared and distinct metabolic patterns among these groups. In this context, untargeted $^{1}$H NMR metabolomics of urine may offer novel insight into disease-specific metabolic pathways and early renal involvement in PMD.

Recruitment process is illustrated in Figure 15. A total of 58 paediatric participants were included, distributed into four groups: primary mitochondrial disease (PMD; n=13), suspected mitochondrial disease without confirmed genetic diagnosis (SMD; *n* = 10), CKD (n=26). Healthy controls (n=10) were recruited among individuals attending nephrology outpatient follow-up for non-metabolic conditions and with normal kidney function. CKD patients were classified according to KDIGO 2024 staging into mild (stages 1–2) and moderate to advanced (stages 3–5) disease. No samples collected during acute decompensation or intercurrent infection were included.

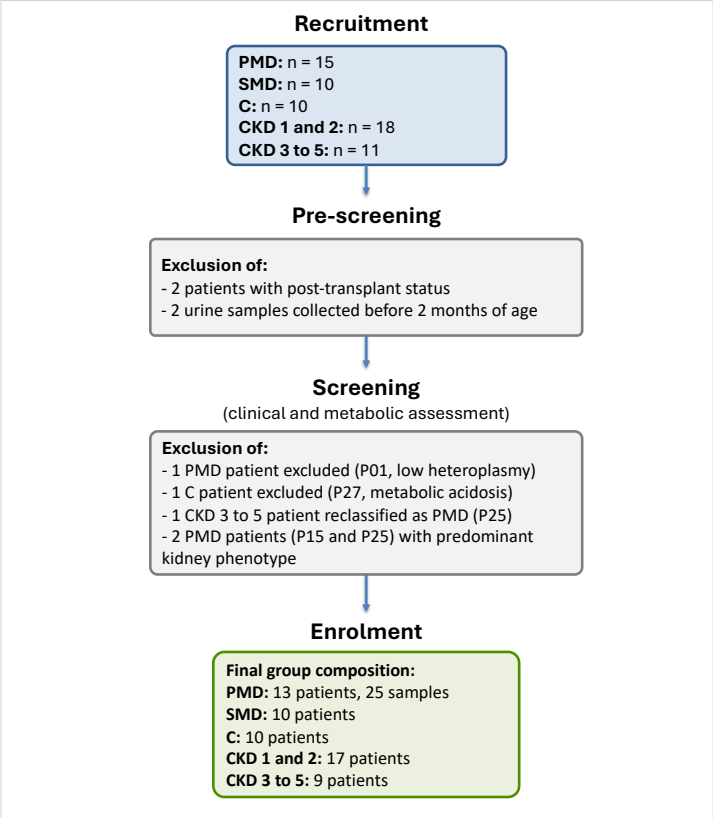


***Figure 15. Recruitment flowchart for urine $^{1}$H NMR metabolomics study.*** *Flowchart of participant selection and exclusions during the recruitment process. Final groups used in the statistical analysis are shown in the bottom box, along with the corresponding number of patients and urine samples per group.*

Baseline laboratory data confirmed the presence of kidney dysfunction in the CKD group and were within reference ranges in the PMD, SMD, and control groups as show in Table 23. Urinary biochemical parameters, including creatinine, total proteinuria were consistent with clinical group assignment. Detailed patient data are provided in Table S4.

***Table 23. Diagnosis, demographic and kidney stage by study group.***

| *Group* | *Primary mitochondrial disease (PMD) (n=13)* | *Suspected mitochondrial disease (SMD) (n=10)* | *Control (n=10)* | *CKD stages 1–2 (n=17)* | *CKD stages 3–5 (n=9)* |
|---|---|---|---|---|---|
| **Median Age** | 9.5 [1.1-18.8] | 4.1 [0.2-18.7] | 11.2 [2.7-18.2] | 12.1 [2.8–17.9] | 12.9 [4.7–15.8] |
| **Sex (M/F)** | 6/7 | 3/7 | 7/10 | 12/5 | 6/3 |
| **eGFR mean** | 153.4 ± 63.2 | 111.0 ± 18.7 | 110.4 ±9.2 | 97.8 ± 15.3 | 20.3 ± 7.0 |
| **Stage A2[1] (%)** | 0% | 0% | 0% | 59% | 33% |
| **Stage A3[1] (%)** | 0% | 0% | 0% | 41% | 44% |
| | mtDNA point mutations (n=4), SLDMD (n=1), Nuclear gene defects (n=8) | Undiagnosed (n=10) | *Minor* CAKUT (n=5), Mild vesicoureteral reflux (n=2), Other (n=3) | Glomerular (n=4), CAKUT (n=3), Tubulopathies (n=2), Other (n=8) | Glomerular (n=3), CAKUT (n=2), Nephronophthisis (n=2), Other (n=2) |

*[1]Stage based on Albumin-to-creatine ratio (ACR), classified as 30–300 mg/g (A2) and >300 mg/g (A3). Diagnoses were grouped into clinically relevant categories. Abbreviations: ACR: albumin-to-creatinine ratio; eGFR: estimated glomerular filtration rate; CKD: chronic kidney disease; SLDM: single large mtDNA deletion; SD: standard deviation; MA: microalbuminuria; mtDNA: mitochondrial DNA.*

Overall, 57 metabolites were identified (4 of them still corresponding to putative assignments, requiring additional investigation to validate their identification). The identified metabolites are consistent with the literature for NMR urine metabolomics studies[332].

Unsupervised multivariate analysis performed by PCA using all samples (Figure 16A) highlighted distinct metabolic fingerprint in CKD patients compared to other groups. PLS-DA scores plot (Figure 16B) illustrated a diverging tendency between groups, with the observation of three clusters: PMD and suspected mitochondrial disorders (SMD), CKD1-2 and Control, and CKD3-5.

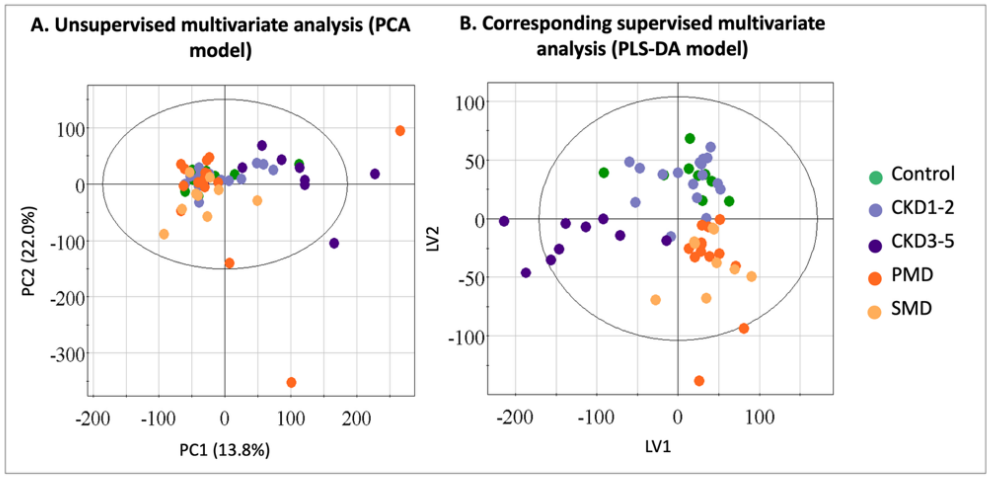


***Figure 16. Multivariate analysis of urinary 'H NMR metabolomic profiles across groups.*** *Primary Mitochondrial Disease (PMD, orange), Suspected Mitochondrial Disease (SMD, yellow), Control individuals (green), Chronic Kidney Disease (CKD) stages 1-2 (lilac) and stages 3-5 (purple). The ellipses represent 95% confidence intervals. A. Principal Component Analysis (PCA) highlights distinct metabolic fingerprint in CKD patients compared to other groups. B. Corresponding supervised model using partial least squares discriminant analysis (PLS-DA) shows metabolic divergence between groups, particularly between PMD, CKD3-5, and controls. Abbreviations: PMD: primary mitochondrial disease; SMD: suspected mitochondrial dysfunction; CKD: chronic kidney disease.*

Pairwise comparisons between groups confirmed the overall observed tendencies, with robust discrimination ($Q^2$ = 0.52) between PMD and controls (Figure 17A). Despite the clear metabolic separation between PMD patients and healthy controls (Figure 17B), multivariate analysis failed to discriminate PMD patients from individuals with SMD ($Q^2$ = 0.32). Regarding the other comparisons for PMD against the CDK group, *vs*. CKD1-2 (Figure 17C) and *vs*. CKD3-5 (Figure 17D), the PLS-DA models revealed a high predictive power ($Q^2$ = 0.62 and $Q^2$= 0.78, respectively), reflecting strong metabolic differences between these groups.

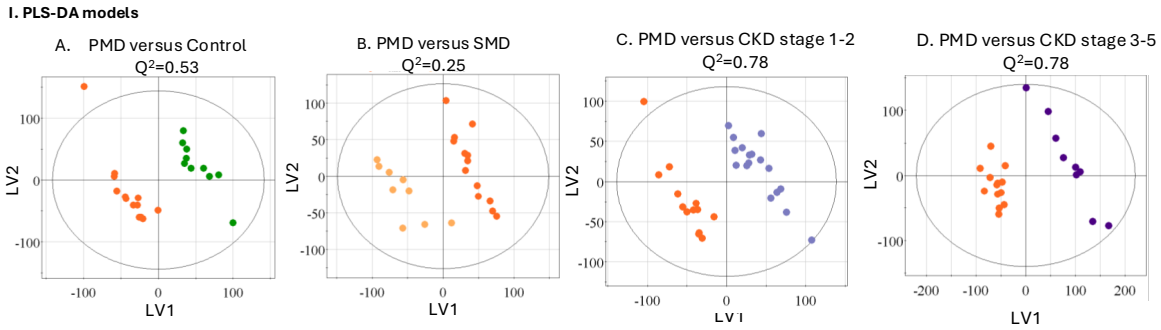


***Figure 17. Supervised multivariate models of primary mitochondrial disorders.*** *Partial Least Squares Discriminant Analysis (PLS-DA) models were generated to compare patients with PMD to: A. controls, B. SMD, C.CKD stages 1–2, and D. CKD stages 3–5. The corresponding $Q^2$ values, indicate the predictive performance of each model. Clear discrimination was observed between PMD and controls ($Q^2$ = 0.53), and both CKD subgroups ($Q^2$ = 0.62 and 0.78). In contrast, the model comparing PMD to SMD showed lower predictive performance ($Q^2$ = 0.25), reflecting significant metabolic overlap.*

Table 24 summarizes the urinary metabolites showing significant differences between PMD, CKD stage 3–5, SMD and control groups. Fold changes (FC) and Hedge's effect size (g) were calculated to assess group differences.

***Table 24. Most relevant findings of metabolite univariate analysis.***

| Compound name | ppm | FC | Hedge's (g) | *p*-value | Sign.[1] |
|---|---|---|---|---|---|
| **PMD vs Control** | | | | | |
| ***cis*-Aconitate** | 3.11 | 1.48 | 0.63 | 0.020 | * |
| **Creatine** | 3.93 | 1.84 | 0.95 | 0.020 | * |
| **Fumarate** | 6.52 | 2.22 | 0.88 | 0.006 | ** |
| **Histidine** | 7.10 | 0.36 | -0.62 | 0.038 | * |
| **Homovanillate (HVA)** | 6.94 | 1.70 | 0.99 | 0.020 | * |
| **Hypoxanthine** | 8.19 | 1.56 | 1.13 | 0.020 | * |
| **Succinate** | 2.41 | 1.52 | 0.93 | 0.010 | * |
| **Tryptophan** | 7.75 | 2.02 | 1.34 | 0.002 | ** |
| **PMD vs CKD3-5** | | | | | |
| **1-Methylnicotinamide (MNA)** | 9.29 | 2.96 | -0.95 | 0.001 | *** |
| **3-Aminoisobutyrate** | 2.20 | 1.42 | -0.94 | 0.019 | * |
| **2-Hydroxyisobutyrate** | 1.36 | 1.30 | -0.76 | 0.033 | * |
| **3-Hydroxybutyrate** | 1.23 | 1.59 | -1.01 | 0.011 | * |
| **3-Hydroxyisovalerate** | 1.27 | 2.08 | -1.98 | 0.002 | ** |
| **4-Hydroxyphenylacetate** | 7.17 | 1.34 | -0.39 | 0.045 | * |
| ***cis*-Aconitate** | 3.11 | 1.68 | -0.81 | 0.019 | * |
| **Creatine** | 3.93 | 1.80 | -1.01 | 0.033 | * |
| **Formate** | 8.46 | 2.87 | -0.62 | 0.006 | ** |
| **Glucuronate** | 5.27 | 0.09 | 1.23 | 0.003 | ** |
| **Glycolate** | 3.96 | 1.37 | -0.83 | 0.038 | * |
| **Homovanillate (HVA)** | 6.94 | 2.53 | -1.79 | 0.001 | *** |
| **Hypoxanthine** | 8.19 | 1.46 | -0.53 | 0.005 | ** |
| **Propylene glycol** | 1.14 | 2.31 | -1.25 | 0.006 | ** |
| **Succinate** | 2.41 | 1.39 | -0.86 | 0.045 | * |
| **Tryptophan** | 7.75 | 1.73 | -1.23 | 0.009 | ** |
| **Tyrosine** | 6.90 | 2.10 | -1.26 | 0.001 | *** |
| **CKD3-5 vs Control** | | | | | |
| **1-Methylnicotinamide (MNA)** | 9.29 | 0.35 | -1.25 | 0.010 | * |
| **3-Hydroxyisovalerate** | 1.27 | 0.42 | -1.01 | 0.037 | * |
| **Acetate** | 1.93 | 1.83 | 1.12 | 0.030 | * |
| **Citrate** | 2.66 | 0.59 | -0.71 | 0.004 | ** |
| **Fumarate** | 6.53 | 2.42 | 1.44 | 0.030 | * |
| **Glucuronate** | 5.27 | 24.19 | 9.21 | 0.002 | ** |
| **Lactate** | 1.34 | 2.36 | 1.53 | 0.020 | * |

*Statistical comparisons were performed using Mann-Whitney U test, normalized to PQN. Features with $p < 0.05$ but minimal variation (FC between 0.8 and 1.2) and negligible effect size (Hedge's g between –0.2 and 0.2) were excluded to avoid overinterpretation of biologically irrelevant findings. Shown chemical shifts correspond to the metabolite peaks used for integration. The table includes representative metabolites from amino acid metabolism, organic acids, and energy-related pathways, particularly those showing statistically significant differences between PMD and other groups, including controls and CKD stages 3–5. Statistical significance (Sign.): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: PMD: primary mitochondrial disease; SMD: suspected mitochondrial disorder; CKD: chronic kidney disease.*

### *Urinary Metabolomic of Primary Mitochondrial Disease differs from controls with minor differences to Suspected Mitochondrial Disease*

In PMD versus controls, several discriminatory metabolites were identified. Krebs cycle intermediates (cis-aconitate, succinate, fumarate) were consistently elevated (FC 1.48–2.22; $p < 0.02$; g 0.63–0.93), together with tryptophan (FC 2.02; $p = 0.002$; $g = 1.34$), hypoxanthine (FC 1.56; $p = 0.020$; $g = 1.13$), creatine (FC 1.84; $p = 0.020$; $g = 0.95$), and HVA (FC 1.70; $p = 0.020$; $g = 0.99$). Histidine was the only metabolite significantly reduced (FC 0.36; $p = 0.038$; $g = -0.62$). These findings highlight increased purine turnover, amino acid dysregulation, and enhanced excretion of TCA intermediates in PMD.

Comparison between PMD and Suspected MD revealed largely overlapping profiles, with no statistically robust separation. 3-hydroxybutyrate and propylene glycol were nominally higher in PMD, but values overlapped with controls, limiting discriminatory power. Histidine remained the only metabolite consistently differentiating PMD from both suspected PMD and controls.

### *Mitochondrial Impairment in Renal Disease Differs from Primary Mitochondrial Dysfunction*

The urinary metabolome of advanced CKD (stages 3–5) showed significant alterations compared with both controls and early CKD (stages 1–2). Increases in lactate, fumarate, and acetate, together with reductions in citrate and N-methylnicotinamide (MNA), were consistent with progressive renal impairment. Glucuronate was markedly elevated, distinguishing CKD3–5 from all other groups.

In contrast, PMD patients exhibited a distinct metabolic profile that clearly separated from CKD across stages ($Q^2 > 0.62$), particularly from CKD3–5. Eighteen metabolites contributed to this discrimination, including Krebs cycle intermediates (*cis*-aconitate, succinate), amino acid derivatives (tryptophan, tyrosine, HVA), and organic acids (3-hydroxyisovalerate, 2-hydroxyisobutyrate, formate, propylene glycol). Divergent patterns were evident: metabolites such as HVA, hypoxanthine, succinate, and tryptophan were consistently elevated in PMD relative to both CKD3–5 and controls, while histidine was selectively reduced in PMD. Conversely, MNA and 3-hydroxyisovaleric acid were suppressed, and glucuronate strongly elevated, in CKD3–5 but not in PMD, indicating renal-specific alterations independent of mitochondrial dysfunction.

### *Candidate biomarkers for PMD*

ROC analysis supported the discriminative potential of several urinary metabolites for PMD. Histidine (AUC = 0.78, log2FC = –1.15) and tryptophan (AUC = 0.74, log2FC = +0.56) showed the highest specificity for PMD versus other groups, aligned with univariate findings. Propylene glycol, creatine, and fumarate also showed good performance (AUC ≥ 0.71), reflecting mitochondrial dysfunction, although with less specificity.

A targeted five-metabolite panel consisting of *cis*-aconitate, fumarate, HVA, tryptophan and histidine achieved significant separation across all five diagnostic groups (PMD, SMD, CKD1–2, CKD3–5, controls) in one-way ANOVA and displayed strong classification performance in Random Forest analysis. These metabolites contributed consistently to group discrimination, supporting their potential as a biosignature for diagnostic stratification.

To further assess the potential clinical applicability, of the above metabolite signature, we constructed classification models using the most discriminative compounds. The same, five-metabolite panel achieved excellent accuracy in distinguishing PMD from controls, with AUCs of 0.949 (linear SVM), 0.938 (PLS-DA), 0.88 (random forest), and 0.836 (logistic regression). When applied to a broader comparison of PMD versus all others, it maintained strong discriminative power (logistic regression AUC = 0.783), highlighting its robustness in non-binary diagnostic classification.

Our findings reveal that the urinary metabolome of paediatric PMD patients is significantly different from healthy controls, even under basal clinical conditions. Differences were consistently observed in multivariate and univariate analyses, supporting the existence of a mitochondrial disease-specific metabolic footprint.

Key metabolic alterations in PMD included increased urinary levels of HVA, *cis*-aconitate and fumarate and decreased histidine with moderate-to-large effect sizes compared to controls and CKD stage 3–5 patients, revealing how differently PMD and CKD impact the urinary metabolome. Pathway enrichment analysis identified significant involvement of energy metabolism and redox balance, including glutathione metabolism, as well as pathways related to amino acid turnover—specifically glutamate, arginine, proline, and tryptophan metabolism—and the oxidation of branched-chain fatty acids. These findings support the biological consistency of the urinary metabolomic profile in PMD and underscore the contribution of mitochondrial energy production, amino acid catabolism, and oxidative stress response to its pathophysiology.

Substantial overlap was observed between PMD and SMD and effect sizes were generally small. These findings reflect the heterogeneity of the group and probably reflect selection bias for patients with biochemical evidence of mitochondrial dysfunction to be considered in this group. It most likely includes both true mitochondrial disorders and “mitochondrial mimics”.

Despite the shared involvement of mitochondrial pathways in both PMD and CKD, our data support the existence of distinct urinary metabolomic signatures. In PMD patients compared to controls, elevated levels of fumarate, *cis*-aconitate tryptophan and HVA were consistently observed, even in the absence of renal impairment. In contrast, these metabolites were either unchanged or

variably modulated in CKD, with *cis*-aconitate showing an inverse trend with CKD progression. CKD3–5 patients exhibited increased urinary excretion of MNA, 3-hydroxybutyrate, and other branched-chain organic acids, alongside reductions in HVA and histidine.

## 2. Urinary Metabolomic Signatures in PMD with Renal Phenotype

In a complementary metabolomic analysis, we explored the impact of kidney involvement on urinary metabolite profiles in primary mitochondrial disorders. This subset included patients with genetically confirmed PMD and clinically evident renal disease (PMD+CKD), aiming to determine whether mitochondrial dysfunction remains a dominant metabolic driver even in the presence of advanced renal impairment.

Within the cohort, two patients presented with PMD in which CKD represented a major and early manifestation, indicating predominant renal involvement. To ensure consistency with identifiers applied in the metabolomics cohort (see previous section), the correspondence with the clinical cohort (Section I) is as follows: patient P15 in the metabolomic study matches patient P60 in the clinical cohort, and patient P25 matches patient P54.

Patient P15 (female) had neonatal onset of proximal tubulopathy consistent with Fanconi syndrome, associated with CKD and glomerular proteinuria. Metabolic investigations revealed elevated lactate and organic acid abnormalities, including ketonuria, Krebs cycle intermediates, dicarboxylic acids, and increased 4-hydroxyphenyl-lactic and -pyruvic acids. Brain MRI was normal. Skeletal muscle histology was normal, whereas respiratory chain enzymology showed markedly reduced Complex I activity (12%).

The clinical diagnosis was GRACILE syndrome, with a molecular diagnosis of *BCS1L* compound heterozygosity (c.-147A>G p.? and c.166C>T, p.(Arg56Ter)). During the study period, she with stable eGFR values adequate for age (mean eGFR 77.9 mL/min/1.73 $m^2$) and marked albuminuria (mean urinary albumin-to-creatinine ratio [ACR] 278.9–604.6 mg/g). Despite variations in individual creatinine- and cystatin C-based eGFR estimates, the overall kidney function remained within CKD stage G2. Tubular proteinuria was consistently absent, while tubular dysfunction was evident. Additional clinical features included ataxia, myopathy, recurrent vomiting, FTT, short stature, hypogonadism, insulin-like growth factor 1 (IGF1) deficiency, and anaemia.

Patient P25 (male) presented at age 5 A with rapidly progressive sensorineural hearing loss. The following year, he presented with steroid-resistant nephrotic syndrome, and renal biopsy was consistent with focal segmental glomerulosclerosis with C3 and C4 deposits.

Due to exercise intolerance and a myopathic posture and given the suspicion of a primary mitochondrial disorder, coenzyme Q10 supplementation was initiated, resulting in a slight

improvement in both renal and muscle function. In the subsequent years, the neuromuscular condition worsened, with fatigue, facial paresis, bilateral ptosis with ophthalmoparesis, Gowers' sign, muscle hypotrophy, and mild scoliosis. During a few years he maintained a mean eGFR 59.5 mL/min/1.73 $m^2$ (G2) with persistent severe albuminuria (ACR 9175 mg/g), without evidence of tubulopathy. Additional findings included anaemia, hypogammaglobulinemia, and hyperCKemia. Metabolic studies revealed elevated lactate.

Extensive genetic testing, including nuclear and mitochondrial genes as well as whole-exome sequencing, with a focus on defects in CoQ10 biosynthesis, were inconclusive.

Reanalysis of a trio-exome identified him a compound heterozygosity with two VUS (c.146A>G, p.(Tyr49Cys) and c.550+3A>G p.?) in the *RRM2B* gene. Muscle biopsy showed fibber atrophy, increased lipid droplets, multiple COX-negative and SDH-positive fibbers and numerous RRF. Enzymatic respiratory chain analysis was normal. The identification of multiple large-scale deletions in mtDNA extracted from muscle tissue supported this diagnosis.

Following the age of 11 years, the patient experienced rapid progression to end-stage chronic kidney disease. At the time of the most recent assessment, the patient was undergoing dialysis.

Both patients with PMD and predominant renal involvement presented with chronic kidney disease stage G2 and albuminuria category A3, according to KDIGO classification at the time of sample collections.

### 2.1. Metabolomic profile

P15 had five urine samples collected over a 2.5-year period, enabling longitudinal metabolomic profiling. $^1$H NMR spectra (Figure 20) consistently showed a pronounced dominance of glucose resonances within the 3.2–4.0 ppm region, consistent with severe glycosuria secondary to proximal tubulopathy (Fanconi syndrome). Although glycosuria was not quantified biochemically, its persistence across multiple time points likely exerted a major influence on the multivariate positioning, driving four of the five P15 samples away from the PMD cluster towards the CKD space in total area–normalized PCA plots.

Secondary spectral features included elevated lactate, tricarboxylic acid (TCA) cycle intermediates (e.g., citrate, succinate), and aromatic metabolites (e.g., 4-hydroxyphenyllactate), consistent with underlying mitochondrial dysfunction. Notably, the P15c sample, collected at age 14.3 years, exhibited a relative reduction in glucose peak intensity compared with earlier collections, corresponding to its closer proximity to the PMD cluster in multivariate analyses.

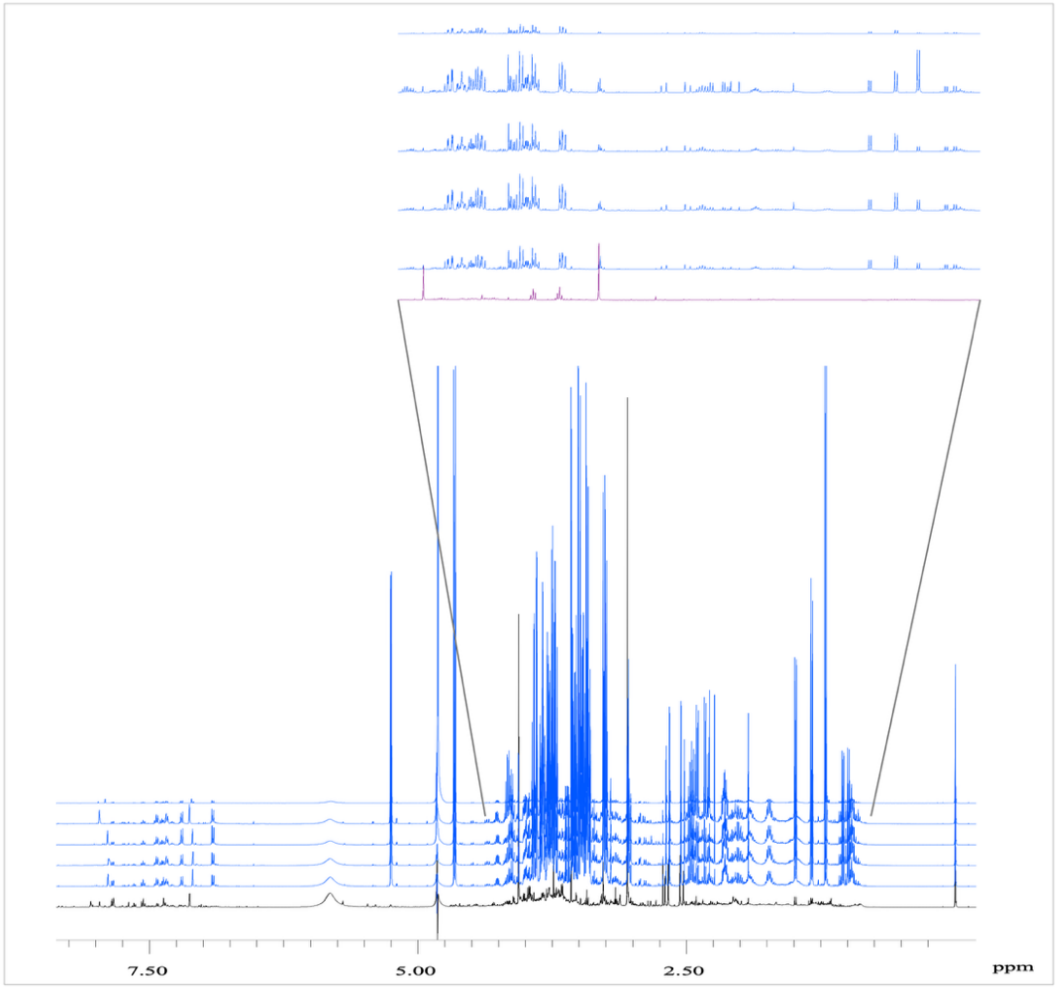


***Figure 18. Urinary $^{1}$H NMR spectra from patient P15 across five collection time points.*** *Spectra are displayed in chronological order from earliest (P15a) to latest (P15e) collection (blue) compared with a representative control spectrum (purple). The most prominent feature is the glucose multiplet spanning 3.2–4.0 ppm. Additional peaks corresponding to lactate, TCA cycle intermediates, and aromatic metabolites are also visible.*

Multivariate analyses were performed using PCA models to explore the urinary metabolic profiles of patients with primary mitochondrial disease and chronic kidney disease (PMD+CKD) in the context of the broader cohort (Figure 19). Analyses were conducted separately after normalization to total spectral area and after probabilistic quotient normalization (PQN), thereby accounting for dilutional effects and evaluating the influence of dominant metabolites on clustering behaviour. When normalized to the total spectral area, four of the five longitudinal samples from P15 were positioned far from the main PMD cluster, aligning closer to the CKD3–5 group in both PCA and PLS-DA score plots. This separation was driven predominantly by markedly elevated urinary glucose resonances (3.2–4.0 ppm), consistent with severe glycosuria due to proximal tubulopathy (Fanconi syndrome). The only sample clustering with the PMD group (P15c) corresponded to a time point with visibly reduced glucose signal intensity. P25 occupied an intermediate position between PMD and CKD3–5, suggesting a mixed metabolic signature.

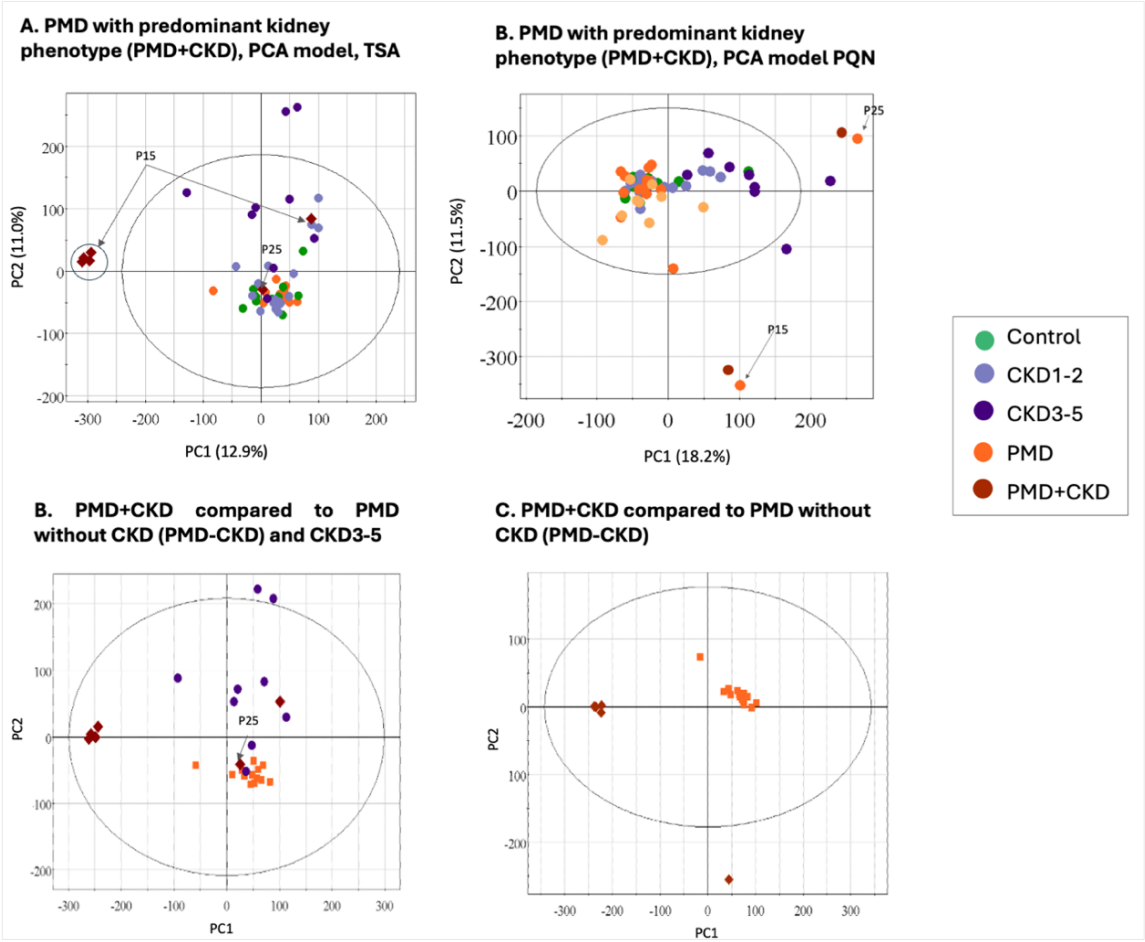


***Figure 19. Multivariate analysis of urinary $^{1}$H NMR spectra normalized by total area.*** *Models based on Principal component analysis (PCA). A. All study groups and PMD patients with a predominant kidney phenotype (PMD+CKD). B. Under Probabilist Quotient Normalization (PQN), the dispersion of P15 samples is markedly reduced compared to total area normalization, reflecting attenuation of the dominant glycosuria effect. P25 remains positioned close to the main PMD cluster. C. PMD+CKD compared with PMD without CKD (PMD–CKD) and CKD stages 3–5. D. PMD+CKD compared with PMD–CKD. Dataset is normalized to total spectral area. The strong separation of P15 samples from the PMD cluster reflects the dominant influence of glycosuria under total area normalization, while P25 positions closer to the CKD3–5 space.*

Following PQN normalization, which minimizes the disproportionate influence of high-abundance metabolites, the clustering pattern for P15 remained largely unchanged, separating from CKD in the first component (PC1) and from PMD in the second (PC2), as shown in Figure 19. In contrast, the position of P25 samples shifted: the strong overlap with PMD seen under total area normalization was attenuated, moving in the same axis (PC1) of the CKD3–5 group. These shifts highlight the substantial impact of normalization on multivariate positioning. While PQN attenuates the disproportionate effect of glycosuria, it does not fully explain the repositioning observed, since P25 also shifts relative to PMD and CKD groups.

Univariate comparisons between PMD patients with renal involvement (P15 [P60] and P25 [P54]) and reference groups confirmed several of the multivariate trends and highlighted individual metabolite changes of potential pathophysiological relevance (Figure 20).

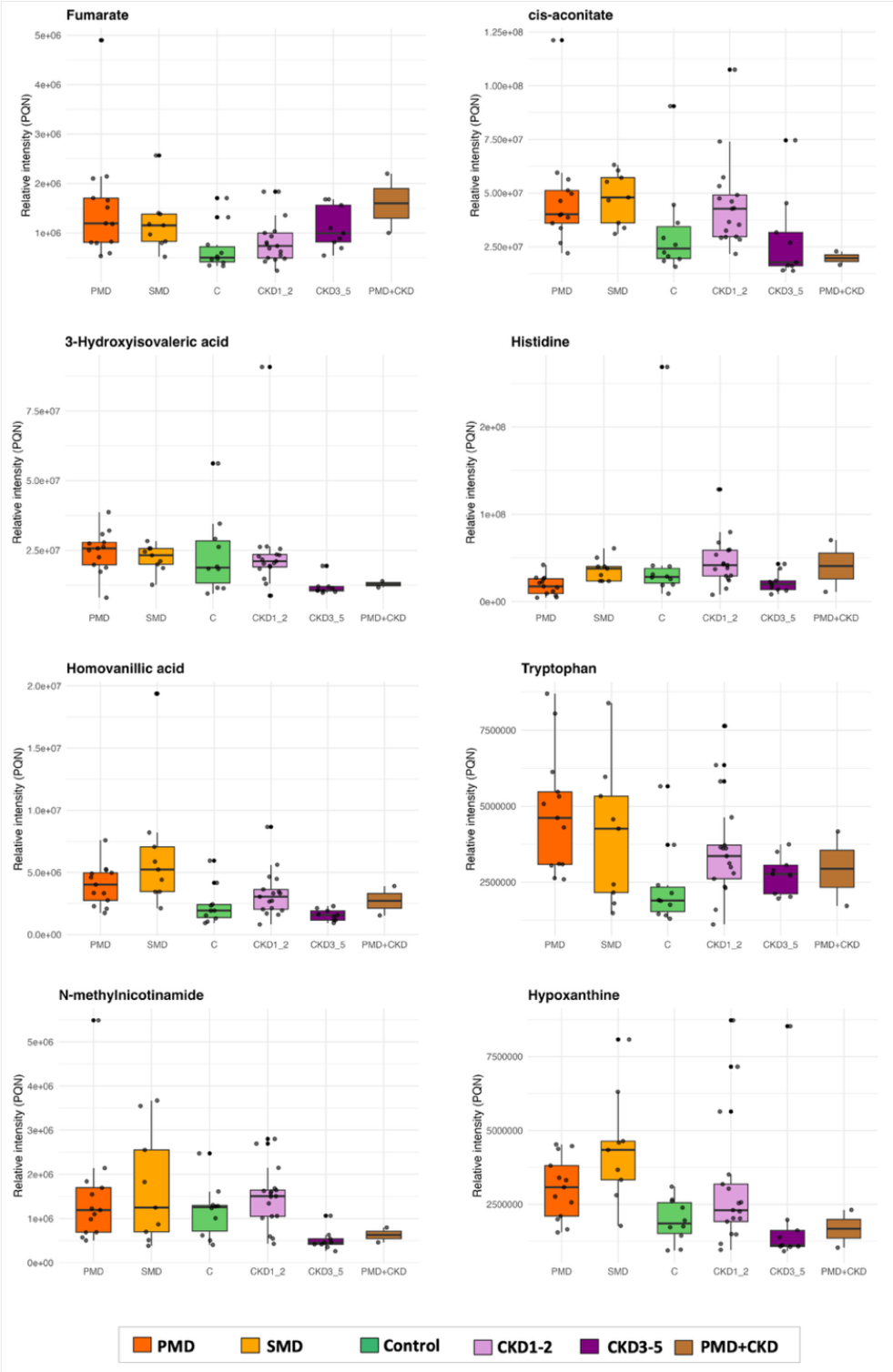


***Figure 20. Univariate analysis of selected urinary metabolites across study groups.*** *Boxplots show PQN-normalized concentrations in controls, CKD1–2, CKD3–5, PMD, and SMD, with individual values for P15 and P25 overlaid. Both patients with PMD+CKD aligned with CKD3–5 profiles for 3-hydroxyisovaleric acid, N-methylnicotinamide, and hypoxanthine, while cis-aconitate, tryptophan, and HVA remained closer to PMD distributions.*

In the subgroup of PMD+CKD, distinct metabolite patterns emerged when compared with PMD without CKD and CKD reference groups, revealing two clear patterns. Levels of 3-hydroxyisovaleric acid, N-methylnicotinamide, and hypoxanthine in both patients overlapped with the CKD3–5

distribution and were markedly reduced compared with PMD, consistent with predominant renal influence. In contrast, *cis*-aconitate, tryptophan, and HVA in the PMD+CKD subgroup remained closer to PMD values, retaining a mitochondrial-like signature despite renal impairment. Other metabolites, such as valine, histidine, and phenylacetylglycine, showed intermediate positioning, not clearly aligning with either PMD or CKD. This dual behaviour suggests that in PMD with renal involvement, some mitochondrial signatures remain preserved while others are masked by the dominant influence of reduced glomerular filtration and tubular handling.

Overall, no metabolite emerged as a specific indicator of renal involvement in PMD; instead, the subgroup was characterized by a hybrid metabolic signature combining CKD-like suppression with persisting PMD-like alterations.

When looking at individual profiles, P15 consistently showed extremely elevated urinary valine and histidine, exceeding the distribution of all comparator groups, suggesting an additional renal tubular contribution to the metabolic phenotype. In contrast, markers typically reduced in advanced CKD such as hypoxanthine, NMA, and HVA were markedly decreased in P15, positioning this profile closer to CKD3–5. Together, these findings highlight a mixed signature, with tubular metabolite losses and accumulation of specific amino acids coexisting with systemic mitochondrial dysfunction.

Patient P25 displayed a hybrid pattern distinct from P15. While levels of cis-aconitate and HVA aligned more closely with the PMD cluster, other features resembled advanced CKD, including reduced NMA, tryptophan, and particularly low phenylacetylglycine. Unlike P15, P25 did not show marked elevations in valine or histidine. Overall, P25 exhibited an intermediate metabolic signature, reflecting both mitochondrial and renal disease influences.

These observations suggest that although renal dysfunction exerts a strong impact on urinary metabolic profiles, individual trajectories within PMD+CKD may diverge, with P15 showing predominant tubular loss signatures and P25 a mixed profile with overlap to both PMD and CKD.

These results highlight that while severe CKD may obscure disease-specific signatures, characteristic profiles can still be detected in selected individuals. In P15, the dominance of glycosuria and consistent loss of TCA intermediates underscored the impact of Fanconi-type proximal tubulopathy, while the reduced excretion of tryptophan and HVA suggested a selective impairment in tubular handling of aromatic amino acids and catecholamine metabolites. Even in advanced CKD with predominantly glomerular involvement, disease-specific mitochondrial signatures can persist, aiding differential diagnosis from non-mitochondrial CKD.

Suppression of N-methylnicotinamide and hypoxanthine was a consistent finding in patients with mitochondrial and non-mitochondrial CKD. This pattern indicates that these metabolites function as markers of kidney involvement, independent of mitochondrial dysfunction, and thus represent non-specific organ markers without particular influence of mitochondrial metabolism. For PMD patients, they might provide valuable indicators of target organ damage, regardless of global metabolic status.

Together, these findings reinforce the phenotypic and biochemical distinctiveness of mitochondrial patients with renal involvement, while also illustrating how renal dysfunction can both obscure and reshape disease-specific metabolomic signatures.

# *Chapter V.* ***Discussion***

This thesis provides a multidimensional perspective on paediatric primary mitochondrial disorders, addressing persistent challenges in diagnosis, disease definition, and individualized care. By integrating longitudinal cohort analysis, detailed genotype–phenotype correlations in ultra-rare disorders, and exploratory urinary $^{1}$H NMR metabolomics, it refines our understanding of both the heterogeneity and the shared mechanisms underlying these conditions. The use of structured frameworks demonstrates how standardization can improve diagnostic consistency, reduce misclassification, and facilitate systematic re-evaluation of undiagnosed cases.

The complementary value of these methodological approaches is a central theme. Analysis of a large cohort exposed the high frequency of mitochondrial mimics and underscored the importance of rigorous diagnostic trajectories, as well as the well-recognized issue of diagnostic yields being lower than initially expected with next-generation sequencing. While cohort studies provide breadth and generalizable patterns, detailed case reports allow focused exploration of ultra-rare disorders and support the expansion of the phenotypic spectrum of individual genes, as illustrated here for *LYRM4* and *EARS2*. In parallel, the metabolomics arm added a functional dimension, showing how urinary $^{1}$H NMR profiling can capture both convergent and phenotype-specific biosignatures, even in the presence of confounders such as renal dysfunction.

Together, these elements support a shift toward integrative diagnostic strategies that combine molecular genetics, deep phenotyping, and omics approaches. Such a framework not only strengthens diagnostic consistency but also opens opportunities for incorporating biomarker-based strategies into both clinical practice and research.

## 1. Insights from the cohort

The primary aim of this work was to systematically evaluate a cohort of children with suspected primary mitochondrial disease, a population that remains particularly challenging to diagnose due to clinical heterogeneity and overlapping phenotypes. By applying uniform protocols for longitudinal follow-up and multidisciplinary assessment, we were able to generate consistent data across a large and diverse group of patients. This structured approach not only provided robust insights into the diagnostic trajectory of mitochondrial disorders but also highlighted the dual contribution of expanding the recognized phenotype spectrum and delineating the role of genetic mimics within this setting.

The heterogeneity of PMD remains the central obstacle to timely diagnosis. This complexity was also evident in our cohort, where age of onset, organ involvement, and progression varied widely, and where a considerable proportion of patients remained without a definitive molecular diagnosis.

## 1.1. The paradigm shift on diagnosis and testing strategies

Mitochondrial medicine has undergone a major paradigm shift over the last two decades. Historically, diagnosis relied on a sequential approach: clinical suspicion was first supported by biochemical markers, neuroimaging, and histological or respiratory chain activity studies, often requiring invasive tissue biopsies. Genetic testing was typically reserved for later stages and was not considered essential for a confirmed diagnosis.

This sequential pathway was not only time-consuming but frequently led to diagnoses that were later revised in light of genetic findings. The correlation between clinical or biochemical findings and true mitochondrial diseases is inconsistent. For example, some patients with genetically confirmed defect of OXPHOS subunits can have normal respiratory chain assays and multiple patients with isolated or multiple respiratory chain deficiencies do not have a PMD[35,69,333].

The advent of NGS radically altered this trajectory, enabling the parallel interrogation of hundreds of nuclear and mitochondrial genes and allowing earlier recognition of both classical PMD and their genetic mimics. These wide genetic approaches have not only accelerated the diagnostic process but also uncovered unexpected genetic aetiologies, thereby reshaping the differential diagnosis and refining the boundaries between primary mitochondrial disorders and their mitochondrial mimics or phenocopies[227,334,69]. These include congenital disorders of glycosylation, peroxisomal disorders, neurotransmitter defects, and various monogenic syndromes affecting central nervous system development, metabolism, or ion channel function[69,12,210,35].

Recent large-scale studies suggest that up to 30% of children referred for suspected MD ultimately receive alternative diagnoses, many of which mimic classical PMD in their clinical, neuroimaging, or metabolic profiles[210]. This high frequency of mimics highlights the importance of early comprehensive genetic testing and multidisciplinary evaluation. Moreover, the increasing recognition of phenocopies has catalysed a shift in diagnostic paradigms, with a growing emphasis on genome-wide sequencing approaches, integrated data interpretation, and careful re-evaluation of clinical trajectories[33,42].

This led to the re-evaluation of multiple patients previously diagnosed with mitochondrial disorders, some of whom ultimately received alternative diagnoses. As a result, a definite diagnosis is currently only recognized after genetic confirmation. Genetic testing as first tier approaches are progressively replacing traditional hierarchical algorithms, reshaping diagnostic strategies in mitochondrial medicine. Deep tissue biopsy has shifted to a secondary, complementary role in the diagnostic evaluation of PMDs, primarily contributing to differential diagnosis, ancillary histochemical or biochemical analyses, and the functional interpretation of genetic variants.

This study was conducted over this period marked by substantial shifts in diagnostic methodology, transitioning from traditional stepwise testing to NGS-based approaches. As a result, different molecular strategies were applied across the cohort, reflecting both the evolving availability of technologies and the real-life challenges of clinical practice. Despite this heterogeneity, our findings underscore the value of integrating NGS into the diagnostic pathway for suspected MD, particularly when guided by clinical phenotype and supported by multidisciplinary evaluation.

## 1.2. Diagnostic landscape

Among 178 children evaluated for suspected mitochondrial disease, 38 (21.3%) received a genetically confirmed PMD, while another 20% were diagnosed with non-mitochondrial genetic disorders.

The confirmed PMD cases in our cohort reflected the broad molecular heterogeneity of mitochondrial disease, including both mtDNA variants and nuclear-encoded defects in translation, OXPHOS assembly, mtDNA maintenance, and cofactor metabolism. This distribution mirrors contemporary reports and illustrates the value of pathway-based approaches in classifying and understanding PMD.

Notably, the incidence of mitochondrial mimics approached that of confirmed PMD, underscoring the extensive range of differential diagnoses in this context. These mimics encompassed chromosomal syndromes, neurotransmitter disorders, channelopathies, and defects of vesicular transport, reflecting the wide clinical overlap with primary mitochondrial presentations Similar proportions of phenocopies have been reported in large-scale diagnostic studies for suspected mitochondrial disease[205,335].

## 1.3. Diagnostic odyssey

The diagnostic trajectories in our cohort reflected the evolving landscape of mitochondrial medicine over the past 15 years. Early in the study period, investigations were largely driven by sequential testing of mtDNA and targeted nuclear panels, often in combination with muscle biopsy. While this approach successfully identified several mtDNA mutations and nuclear defects, it frequently prolonged the diagnostic process and left many patients undiagnosed.

The progressive introduction of NGS reshaped this paradigm. Although targeted NGS panels contributed significantly (≈20% yield), whole-exome sequencing proved particularly impactful, accounting for nearly 40% of diagnoses and uncovering both canonical PMD and a wide spectrum of genetic mimics. This underscores the limitations of restricted panels and supports genome-wide sequencing as a first-line strategy in suspected mitochondrial disease, a conclusion echoed by recent paediatric cohorts.

Muscle biopsy retained diagnostic value in a minority of cases, either by revealing characteristic histological or biochemical features, by enabling detection of mtDNA deletions, or by guiding genetic confirmation. Nonetheless, its overall yield was low, reinforcing its current role as a complementary rather than primary diagnostic tool.

The choice of genetic strategy strongly influenced diagnostic yield. While targeted panels accounted for 19.2% of diagnoses, whole-exome sequencing provided 38% and was particularly instrumental in identifying mimics that would not have been detected by panel-based testing. These findings reinforce the role of untargeted sequencing as a first-line strategy for suspected mitochondrial disease, a shift also supported by other recent studies[208,204,336,205].

Non-WES targeted panels (n=22) were most often performed as second-line investigations after clinical re-evaluation, when the initial suspicion of a PMD was not confirmed and alternative causes were prioritised. Their relatively high yield (36.4%) therefore reflects phenotype-driven test selection and pre-test enrichment rather than superior analytical performance. Conversely, *COQ8A*-related disease was identified in a first-line ataxia panel, prior to the consideration of a mitochondrial aetiology.

Mitochondrial DNA sequencing yielded 12 diagnoses (13.6%), with a higher detection rate for assays targeting recurrent pathogenic variants (38.9%) compared with full mtDNA sequencing (7.5%). This pattern likely reflects the molecular epidemiology of mtDNA disorders in childhood, where most identifiable cases are caused by well-known point mutations or single large-scale deletions rather than rare or novel variants. A stepwise approach remains appropriate: when initial screening for common mtDNA mutations is negative is first-tier, clinical re-evaluation is warranted before proceeding to full mtDNA sequencing, as certain presentations may instead result from simple mtDNA deletions or nuclear defects.

Exome-based approaches were implemented later in the diagnostic trajectory (n=50) and achieved the highest yield overall (38.0%), reflecting the broader genomic coverage of exome-based workflows.

Together, this resulted in an overall diagnostic yield of 32.6%, which is slightly lower than with recent paediatric cohorts reporting yields of 35–60% depending on case selection, severity, and methodology[204,206,209]. However, this diagnostic yield only considers definite diagnosis and not very probable. Additionally, it also likely reflects differences in case selection, disease severity, and temporal access to next-generation sequencing.

This outcome also underscores the importance of longitudinal re-evaluation, the combined use of complementary testing strategies, and the contribution of multidisciplinary review to achieving diagnostic resolution in complex or evolving phenotypes., the extended longitudinal follow-up with

systematic re-evaluation, the application of multiple testing strategies, reverse phenotyping, and regular multidisciplinary review. Collectively, these observations illustrate how testing strategies and access to emerging technologies strongly influenced diagnostic outcomes. They also highlight the importance of dynamic, multidisciplinary re-evaluation, including reverse phenotyping and periodic data review, in resolving complex or initially inconclusive cases. In fact, periodic reanalysis is recommended in clinical settings, as it improves diagnostic yield, particularly when performed within multidisciplinary teams[47,337].

The diagnostic journey in mitochondrial medicine is notoriously protracted, and our findings confirm this trend. Despite earlier symptom onset in PMD compared to non-PMD patients, referral to metabolic consultation was often delayed, resulting in overall diagnostic intervals exceeding four years. This highlights persistent barriers to early recognition in paediatric practice, even in patients who ultimately proved to have classical mitochondrial disease.

While PMD cases presented earlier, they did not experience shorter time to referral, probably emphasizing barriers in clinical recognition, access to metabolic genetics, and timely initiation of testing. In contrast, once evaluated within specialized settings, PMD patients reached a final diagnosis earlier than those with non-mitochondrial disorders. This may reflect the higher prevalence of metabolic markers and suggestive imaging (e.g., Leigh-like lesions) in true PMD, leading to higher diagnostic suspicion and investment to achieve a diagnosis. Conversely, non-PMD patients presented more heterogeneous or subtle phenotypes, probably contributes to protracted diagnostic trajectories.

A key turning point was the introduction of next-generation sequencing around 2017, which markedly reduced diagnostic intervals in both groups. This technological shift shortened the time from first metabolic evaluation to definitive diagnosis from several years to less than one year in many cases, illustrating the transformative impact of genome-wide approaches.

Similar delays have been described in other paediatric cohorts, with time-to-diagnosis often extending for years due to overlapping features with other inborn errors of metabolism or neuromuscular disorders[69,204]. Together, these observations underscore the dual challenge of improving early clinical recognition and ensuring timely access to comprehensive genomic testing to shorten the diagnostic odyssey.

Beyond its scientific and clinical implications, the process of reaching a diagnosis carries a profound personal and social burden. Families often endure years of uncertainty, repeated investigations, and inconclusive results before a definitive diagnosis is obtained. This uncertainty is not only exhausting but also affects access to care, educational planning, and psychological well-being.

Importantly, a confirmed diagnosis can lead to actionable impacts such as tailored management and treatment. Less importantly. parents and caregivers consistently report that a definite diagnosis leads to change in perception by healthcare professionals. provides validation, empowerment, and relief, even when specific therapies are not available[338,339]. These experiences underscore that the value of diagnosis should not be measured solely in terms of treatment options, but also in the sense of closure, legitimacy, and agency it provides to patients and families.

### 1.4. Undiagnosed in Mitochondrial Medicine: Toward Integrative Diagnostics

The introduction of NGS has unquestionably improved the diagnostic yield in suspected MD by enabling simultaneous analysis of numerous genes and, in selected platforms, parallel assessment of nuclear and mitochondrial genomes[329]. Nevertheless, several individuals still carry the label of mitochondrial disease despite lacking genetic confirmation, reflecting the persistence of historical definitions and the lag between evolving knowledge and changes in medical practice, particularly in low- and middle-income countries. Beyond the challenges this poses for clinical follow-up, one cannot ignore the psychological and social burden on patients and families, nor the sustained effort over recent years to resolve life-long medical mysteries.

Careful clinical phenotyping remains indispensable to guide genetic testing, contextualize inconclusive results, and inform prognosis and management. Large, well-characterized paediatric cohorts are critical to unravel diagnostic trajectories and to refine the boundaries between PMDs and their mimics in the era of high-throughput sequencing. Nevertheless, more than 60% of patients in this cohort remained without a definitive genetic diagnosis even after NGS, reflecting both the extreme heterogeneity of PMDs and the incomplete understanding of the genome–phenome relationship.

The undiagnosed group in our cohort represents a highly heterogeneous and clinically relevant subset. While some patients displayed milder phenotypes or showed clinical improvement over time, many presented with severe or progressive disease. Of note, 48 individuals remained under suspicion for PMD based on their phenotype, including 10 carrying variants in mitochondrial-related genes. Several reached high MDC scores (≥7), comparable to genetically confirmed PMDs, yet remained molecularly undiagnosed. These cases exemplify diagnostic uncertainty even in the presence of hallmark features and raise the possibility of elusive mechanisms such as deep intronic variants, structural rearrangements, epigenetic dysregulation, or polygenic contributions - beyond the scope of current testing.

Our results highlight the limitations of conventional diagnostic approaches in resolving the aetiology of suspected mitochondrial disease. Muscle biopsy, biochemical markers, and

neuroimaging often yielded inconclusive or nonspecific findings. While respiratory chain dysfunction remains a relevant clue, isolated abnormalities should be interpreted with caution, as they may reflect secondary mitochondrial impairment rather than a primary disorder. Similarly, histological hallmarks such as ragged-red fibbers or COX-deficient fibbers were rare and, when present, are not pathognomonic.

Notably, a subgroup of 13 undiagnosed patients carried variants in genes of potential clinical relevance. These included genes traditionally associated with mitochondrial function (e.g., *OPA1*, *ITPR1*, *ATAD3A*, *LRPPRC*), as well as others whose role in mitochondrial biology remains under investigation. While not sufficient to establish a definitive diagnosis, these findings underscore the grey zone between "solved" and "unsolved," highlighting the need for cautious interpretation and longitudinal monitoring as well as periodical reanalysis.

However, the translation of candidate variants into definitive diagnoses remains hampered by significant technical and logistical barriers. Functional validation—through patient-derived cellular models, transcriptomic and proteomic profiling, or enzymatic assays—requires expertise, infrastructure, and resources that are not universally available, and is often restricted to research rather than clinical settings. This diagnostic bottleneck perpetuates uncertainty for many families and highlights the gap between genetic discovery and its clinical implementation.

Systematic reanalysis of sequencing data, incorporation of updated gene–disease associations, and participation in data-sharing consortia represent cost-effective strategies to narrow this gap. Beyond genomics, integrative omics approaches may provide complementary insights by identifying disease biomarkers or perturbed pathways, thereby supporting variant prioritization and functional interpretation in ambiguous or undiagnosed cases.

## 2. Expanding the Phenotype Spectrum in Ultra-Rare Mitochondrial Disorders

Overall prevalence of PMD is around 20 to 23 cases per 100,000 individuals. Within this group, ultra-rare mitochondrial disorders, often described through isolated cases or small series, play a pivotal role in delineating phenotypic variability, refining genotype–phenotype correlations, and expanding the recognised spectrum of primary mitochondrial disease. In this thesis, detailed characterization of patients with *LYRM4* and *EARS2* defects exemplifies how deep phenotyping within longitudinal cohorts contributes to expanding the clinical boundaries of these conditions.

### 2.1. *LYRM4* (ISD11 deficiency)

The identification of pathogenic variants in *LYRM4* (ISD11 deficiency) represented the second family affected worldwide, which allowed to categorize this gene as disease-causing. Acute

presentation appears to be the common characteristic between the three known patients[313,340]. To our knowledge, no further cases were described meanwhile. Iron-sulfur clusters are well-conserved genes throughout evolution, and this extremely low prevalence may translate that alterations in this co-factor are mostly incompatible with life or may represent acute deaths that might be attributed to other factors such as *partum* complications or intercurrent disease. Intriguingly, after surviving the acute events, both P2 from Lim et al 2013 and our patient have a rather uneventful follow-up considering such severe presentations or at least no considerable neurological sequelae It is known that some mitochondrial proteins have age-dependent significance and might show improvement over time. For example, in TRMU deficiency, liver dysfunction is often transient in infancy and tends to normalize with supportive care including cysteine supplementation[341]. Some proteins are either thermal or pH-sensitive, which could also lead to a shift towards acute presentations. Also, a second-hit model can also be responsible for this particular phenotype-genotype, either extrinsic (infectious or hypoxia) or intrinsic (inflammation). Phenotypical observations, such as these can lead to pathophysiology hypothesis.

In the first report of this patient, special attention was given to 3-MGA-uria however we disregarded the significance of 3-OH-IVA, the first reported patient with such metabolic profile not diagnosed with primary 3-MGA-uria. This patient had considerable high levels of 3-MGA-uria plus 3-OH-IVA limited to metabolic crisis. She had no other indication of leucine pathway disruption and preserved ketogenesis, with moderate ketosis during crisis. More recently, a patient with a defect in same pathway (*NFS1*) was found to have a very similar clinical and biochemical profile – life-threatening events early in life during which he had 3-MGA plus 3-OH-IVA aciduria[342]. A rationale for this finding is that Krebs cycle is majorly affected by defects in Fe-S cluster biogenesis, since aconitase is also an Fe-S enzyme[19]. However, this does not account for 3-OH-IVA accumulation. In mice models, drug inhibition of HMG-CoA reductase by statins (pravastatin) induces significative inhibition of aconitase, unveiling a possible unreported pathway and a logic for such uncommon organic acids profile. Interestingly, in this research, concomitant coenzyme Q10 or creatine treatment annulled the pravastatin effect, normalizing aconitase activity levels[343].

Being a disorder related to iron metabolism, with the possibility to induce iron overload on tissues and storage depletion, evaluation of ferritin, transferrin, soluble transferrin receptor or of hepcidin, may help understand the impact on the dynamics of iron metabolism, despite no evidence of impaired iron homeostasis in our patient so far.

Since *L*-cysteine acts the major sulfur donor, supplementation with this non-essential amino acid can be beneficial, especially during periods of higher energy requirement. This rationale for treatment with *L*-cysteine or *N*-acetyl-cysteine is supported by studies on patients with mitochondrial disorders with some benefit in those with translation-related defects[313,344].

### 2.1.1. EARS2-deficiency

The present study contributes to the redefinition of EARS2 deficiency from a narrowly delineated LTBL phenotype toward a broader clinical spectrum. Our three patients, together with a systematic review of published cases, illustrate striking heterogeneity ranging from catastrophic neonatal courses to milder, static encephalopathy with partial recovery. This reinforces that EARS2-related disease exists along a phenotypic *continuum* rather than as a discrete entity, a concept increasingly recognized for mitochondrial aminoacyl-tRNA synthetase deficiencies as a group.

In the case of *EARS2*-related disease, we noticed a particularly high number of cases within our cohort (3 out of 24 nDNA). In addition, literature review showed the disproportionate representation of Portuguese cases in the literature, several of whom carried the recurrent p.Met1? or p.Ile62Phe variants. An ascertainment bias is unlikely since we are a country with limited resources, lagging behind many other European countries that perform more and broaden or untargeted genetic tests, for far more time. Geographical predominance of disorders is a well-known phenomenon, frequently due a founder effect leading to private mutations, with French-Canadian Leigh syndrome (*LRRPCC*) as the most common example[294]. However, some regions have high prevalence of a particular PMD, not entirely due to a founder effect, such as observed in Spain for *TK2*[96,272]. The recurrent variants identified in Portuguese ancestry suggest the possibility of private mutations contributing to geographic clustering, but larger population-based analyses would be required to confirm this hypothesis.

No consistent genotype–phenotype correlations were observed, with identical variants producing divergent clinical outcomes. This unpredictability underscores the limited prognostic utility of molecular data alone and highlights the importance of phenotype-based stratification at presentation. Our proposed severity classification based on early clinical features offers a pragmatic approach for anticipating disease trajectory and counselling families, particularly in settings where genetic results are inconclusive or of uncertain significance.

Finally, the clustering of presentation features with prognosis underscores the importance of detailed longitudinal follow-up and international data aggregation for ultra-rare conditions. Specialized centres play a pivotal role in recognising atypical presentations, ensuring that milder or non-classical forms are not overlooked, and refining disease definitions over time. Ultimately, these insights not only inform clinical management and prognosis but also contribute to more accurate patient stratification for future therapeutic trials.

## 3. Challenges and opportunities in Mitochondrial Medicine

The diagnosis of primary mitochondrial disorders is shaped by a unique tension between rapid advances in molecular medicine and the enduring uncertainties of clinical practice. As knowledge expands, long-standing definitions are being revised, new diagnostic tools are incorporated, and previously overlooked mechanisms emerge as relevant. These shifts create both obstacles and opportunities, challenging clinicians and researchers to constantly adapt their frameworks. They also illustrate the dynamic nature of mitochondrial medicine and underscore the need for integrative, critically informed approaches.

### 3.1. Definition and Classification of Mitochondrial Disease

A first challenge in defining PMD concerns the reclassification of genes that were historically regarded as mitochondrial. For example, *TANGO2* was initially described in association with mitochondrial dysfunction and is still included as a PMD gene in several published cohorts. However, more recent functional studies have demonstrated that its primary role lies in vesicular trafficking, leading to its reclassification outside the spectrum of canonical mitochondrial disorders. Such redefinitions are not merely semantic, but directly influence diagnostic attribution, cohort comparability, and clinical trial eligibility.

A second source of complexity arises from the lack of consensus across published case series in terms of which genes are considered mitochondrial. Many cohorts apply broad or heterogeneous definitions, which can inflate prevalence estimates and complicate comparisons between studies.

In this thesis, the definition of primary mitochondrial disease was based on the curated *PanelApp Mitochondrial panel*, which integrates multi-expert consensus and clinical evidence of gene–disease associations. This approach contrasts with studies that rely on broader functional inventories such as *MitoCarta3.0*, a comprehensive catalogue of the mitochondrial proteome comprising more than 1,100 genes with evidence of mitochondrial localization and function[86]. While *MitoCarta*-based definitions, as recently applied in predictive modelling studies[345], provide an inclusive framework that captures genes with potential mitochondrial relevance, they also encompass genes not yet validated as causative of human disease. In contrast, *PanelApp* restricts inclusion to genes with documented pathogenic variants and clinical correlation, thereby reducing the risk of overestimation but potentially excluding newly emerging disease genes. In this context, the use of *PanelApp* prioritizes clinical specificity and diagnostic applicability, while acknowledging that broader functional resources such as MitoCarta may be more sensitive for exploratory or hypothesis-generating purposes. By drawing on multi-expert consensus and frequent updates, provides a transparent and reproducible framework for gene inclusion, thereby improving diagnostic consistency and harmonization across studies[209,96,35].

The overlap between ICIMD and PanelApp classifications illustrates how mitochondrial dysfunction extends beyond canonical oxidative phosphorylation defects. While the ICIMD framework correctly categorises *SLC19A3*-related disease under "disorders of vitamin and cofactor metabolism" and *TAZ*-related disease under "disorders of organelle biogenesis, dynamics and interactions," both genes are included in the Genomics England PanelApp *Mitochondrial disorders* panel. This reflects their well-documented impact on mitochondrial function: *SLC19A3* deficiency causes secondary mitochondrial impairment due to defective thiamine transport and consequent impairment of thiamine-dependent dehydrogenase complexes, whereas *TAZ* mutations disrupt cardiolipin remodelling, compromising mitochondrial membrane integrity and respiratory chain activity. These examples emphasise that the PanelApp curation encompasses genes whose pathogenic mechanisms converge functionally on mitochondrial dysfunction, even when their primary biochemical defect lies outside the traditional boundaries of mitochondrial energy metabolism.

The blurred boundaries between mitochondrial and non-mitochondrial functions are further illustrated by *ABAT*, which is classified by the ICIMD both under "neurotransmitter metabolism" and within "disorders of mitochondrial function." This dual placement acknowledges that mitochondrial dysfunction can arise as an intrinsic component of seemingly non-mitochondrial pathways. *ABAT* encodes γ-aminobutyrate aminotransferase, the enzyme that converts γ-aminobutyric acid (GABA) to succinic semialdehyde, thereby linking GABA catabolism to the Krebs cycle. Loss-of-function variants cause GABA-transaminase deficiency, with impaired GABA degradation and accumulation of this inhibitory neurotransmitter. Notably, reports of mtDNA depletion in patients with GABA-T deficiency together with *in vitro* observations that pharmacological inhibition of GABA-transaminase with vigabatrin can induce mtDNA depletion, lead to the recognition that *ABAT* also exerts non-canonical roles within mitochondria, including in the nucleotide salvage pathway[346]. The phenotypic overlap observed in our patient is particularly noteworthy, as the presentation combined features of a neurodegenerative disorder with an unusually long survival, manifesting within a Leigh-like clinical spectrum.

### 3.2. Diagnostic suspicion

The observed heterogeneity in PMD regarding age of onset, organ involvement, and progression poses significant diagnostic challenges, especially in paediatric populations where phenotypes may be nonspecific or incompletely developed at presentation. Diagnostic suspicion is often based on neurological and/or muscular involvement, MRI findings or otherwise unexplained multisystem disease. Biochemical findings are well known to lack specificity, a limitation that was also evident in our cohort.

The Modified MDC score was adapted from a well-known structured clinical tool, the Nijmegen Mitochondrial Disease Criteria, to stratify the likelihood of PMD and guide the prioritization of diagnostic investigations[200,201]. Large genomic studies have further shown that even in highly selected cohorts, a substantial proportion of patients with elevated scores ultimately receive alternative genetic diagnoses[35,209,335,347]. In practice, many genetic phenocopies can reach high MDC values, reducing its specificity[35,333].

In our cohort, individuals with confirmed PMD generally exhibited higher cumulative scores across clinical, metabolic, and imaging domains, with over 60% of cases reaching the highest combined laboratory score. However, important exceptions were observed. Several genetically confirmed PMD patients were classified only as "Possible" (2–4 points), often due to subtle or early phenotypes lacking consistent biochemical or radiological abnormalities. Conversely, some patients with non-mitochondrial genetic disorders accumulated sufficient points to be classified as "Definite" (≥8 points), underscoring the limited specificity of MDC criteria in the presence of overlapping phenotypes. Metabolic and neuroimaging domains contributed most to discriminatory power yet remained insufficient to achieve reliable separation between groups. Retrospective application, incorporating longitudinal data, may have further inflated scores in both PMD and non-PMD patients, underscoring the methodological caveat of post hoc scoring.

Comparison with literature remains challenging, as several published cohorts continue to apply the original MDC criteria rather than the updated version[201]. This heterogeneity in diagnostic scoring systems complicates direct comparisons across studies, since the revised criteria incorporate adjustments for molecular testing and de-emphasize invasive procedures such as muscle biopsy.

Thus, the MDC score is best regarded as a tool for clinical triage and data harmonization across cohorts, rather than a determinant of diagnostic certainty. Its contemporary utility lies in enriching patient selection for genomic testing and providing a uniform language for research cohorts, but it cannot shorten the diagnostic odyssey or substitute for genomic-first approaches. In this thesis, although MDC scoring was applied systematically, its contribution to diagnostic yield was modest, reinforcing the need to prioritize genomics while using clinical scores only as supportive context.

### 3.3. Interpretation of mtDNA depletion and multiple deletions

In fact, mtDNA depletion syndromes (MDS) remain defined by diminished mtDNA copy number compared to age-matched controls. However, our cohort illustrates the pitfalls of relying on this criterion as sufficient for diagnosis, as originally proposed in our diagnostic algorithm. In practice, mtDNA quantification alone lacked both specificity and diagnostic yield: one patient with Aymé-Gripp syndrome presented with marked muscle mtDNA depletion in childhood, while another case

with severe depletion but only oligosymptomatic disease remained genetically undiagnosed. These findings highlight that mtDNA depletion should be interpreted as a biochemical signature of impaired nucleotide metabolism rather than as a disease-defining hallmark, requiring correlation with genetic and clinical data.

In disorders of mtDNA maintenance, a reduction in mtDNA copy number can be typically seen in early-onset, severe phenotypes, while the accumulation of multiple mtDNA deletions often characterizes late-onset or milder presentations. In our patient with RRM2B-related disease, for example, the detection of multiple deletions strongly supported impairment of the maintenance pathway. These observations highlight that mtDNA depletion and multiple deletions are valuable biochemical signatures of impaired nucleotide or replication pathways, but neither should be considered disease-defining in isolation. Their diagnostic value emerges only when integrated with genetic and clinical data, Together, these two signatures represent complementary biochemical readouts of mitochondrial genome instability.

### 3.4. Reframing Diagnostic Approaches: Lessons from Our Cohort

To improve diagnostic coherence and facilitate structured evaluation, we used a tiered diagnostic framework adapted to our paediatric practice. This approach, incorporating clinical, biochemical, imaging, and molecular data, is aligned with international efforts to standardize PMD diagnosis[200,201], but introduces practical adaptations for resource-limited settings and real-world clinical variability.

However, applying this framework to our cohort revealed important limitations. First, it did not adequately address critically ill or post-mortem patients, where a time-sensitive approach is required. In such cases, simultaneous biochemical characterization and untargeted genomic testing (e.g., trio-based WES/WGS) should be prioritized to avoid diagnostic delays.

Second, it failed to provide less experienced physicians with sufficient guidance on which clinical features should prompt suspicion of a mitochondrial disorder. This absence of explicitly defined red flags or emphasis on multisystemic presentations (notably the combination of neurological features and myopathy) limited its practical utility for non-mitochondrial experts these features could instead guide clinicians toward a semi-targeted genetic approach, and in rare scenarios of a well-defined classical syndrome, even elicit a targeted approach of genetic studies and/or tissue selection.

Third, although an initial stratification into possible, probable, and definite mitochondrial disease had been proposed in earlier work, its operational value proved limited in our cohort. The categories were not sufficiently defined to guide real-time decision-making.

Fourth, the reliance on biochemical and histological studies obtained from deep-tissue biopsy as part of the criteria for 'probable' disease risked both under-diagnosis (in patients with normal respiratory chain assays) and over-diagnosis (in non-PMD phenocopies with secondary changes).

Fifth and last, although in theory a repeat muscle biopsy might have provided additional information, in practice this option was never pursued in our cohort. The clinical team consistently avoided exposing patients to additional invasive procedures without clear diagnostic benefit. This real-world decision reflects a broader paradigm shift: rather than repeating biopsies, diagnostic efforts moved toward genetics-first approaches and complementary non-invasive functional assays, better aligning with both ethical considerations and patient-centred care.

Based on these findings, a revised approach is warranted, in which genetic confirmation remains the sole basis for a definite diagnosis, while the categories of possible and probable should be anchored in standardized clinical, biochemical, and imaging features, to help guide testing strategies. Clinical MDC scoring can be used to standardize phenotypic documentation and enrich case selection, but testing should be prioritized independently of MDC score category.

The diagnostic pathway should incorporate: i) clearer definitions of suspicion levels; ii) prioritization of untargeted genetic testing (e.g., trio-based WES) in critical conditions or multisystemic presentations; iii) parallel testing for biochemical and genetic testing, providing support for biochemical phenotype, reduce diagnostic delay and prioritize treatable conditions.

The overarching goal of revising our diagnostic algorithm is to align clinical practice and local contingencies with current recommendations from high-income settings, thereby reducing the diagnostic odyssey and the cumulative time burden placed on both patients and clinicians.

Access to "wider" targeted approach that the currently available ones, is priority (targeted genes panels with twice the number of genes currently covered and, preferably, WES-based strategies). While a untargeted-first approach should represent the default strategy, selective exceptions must be acknowledged. When the clinical picture is highly characteristic—such as low citrulline pointing to m.8993T>C/G, or classical features of LHON—a targeted first-line analysis remains justified.

Within this framework, muscle biopsy and respiratory chain enzymology should no longer be considered standard steps, but rather highly targeted procedures reserved for situations where genetics remains inconclusive. In the revised algorithm, muscle biopsy should no longer be regarded as a standard step but reserved for specific situations. These include (i) suspected single large-scale mtDNA deletions when blood analysis is negative, (ii) investigation of multiple deletions or mtDNA copy number defects in nuclear-encoded maintenance disorders, and (iii) functional verification of genetic variants of uncertain significance. Outside these contexts, biopsy yields limited additional benefit and exposes children to unnecessary risks. Nevertheless,

histopathology retains value in redirecting diagnoses toward alternative neuromuscular conditions when genomic analysis remains inconclusive.

This pragmatic refinement reduces unnecessary investigations, shortens diagnostic delays, and optimizes resource allocation. Rather than abandoning traditional tools, it repositions muscle biopsy and functional assays as confirmatory tests in selected contexts, where they directly inform variant interpretation or mechanistic clarification.

Taken together, this dynamic, gene-centred model reflects both the lessons learned from our cohort and the broader evolution of mitochondrial medicine. It provides a realistic and ethically grounded template for implementation in paediatric metabolic practice.

## 3.5. Insights from Mitochondrial Mimics into Mitochondrial Metabolism

To better understand the functional diversity of the identified genes and associated disorders, a structured classification by pathophysiological mechanism and molecular function was compiled (Table 25). This approach highlights shared pathways and distinct molecular disruptions, facilitating mechanistic interpretation beyond clinical labels.

This classification facilitates the functional interpretation of genetically heterogeneous disorders and supports the discussion of shared mechanisms, diagnostic overlaps, and potential targeted therapeutic strategies.

***Table 25. Functional classification of non-mitochondrial genes identified in this cohort.*** *Disease categories were primarily grouped according to molecular mechanisms and cellular pathways involved in pathogenesis.*

| Pathophysiological Mechanism | Gene basis | Molecular Function | Biological Process |
|---|---|---|---|
| **Disorders of lipid metabolism** | | | |
| **Glycerophospholipid metabolism** | *CHKB* | Choline kinase activity | Phospholipid biosynthetic process |
| **Disorders of complex molecule degradation** | | | |
| **Complex molecule degradation** | *LAMP2* | Lysosomal membrane protein | Autophagy |
| **Autophagy** | *SNX14* | Phosphatidylinositol binding | Neuronal excitability and synaptic transmission |
| **Disorders of organelle biogenesis, dynamics and interactions** | | | |
| **Vesicular trafficking** | *TANGO2* | Protein transport activity | Intracellular protein transport |
| **Disorders of extracellular matrix** | | | |
| **Collagen biosynthesis and processing** | *COL5A1* | Structural constituent of extracellular matrix | Collagen fibril organization |
| **Cytoskeletal defects** | | | |
| **Microfilaments (actin) disorders** | *FLNA* | Actin-binding protein | Regulation of cytoskeleton organization |
| **Neurotransmitter disorders** | | | |
| **Synaptic vesicle cycle** | *STXBP1* | SNARE binding | Synaptic vesicle exocytosis |
| **Glutamate neurotransmitter** | *GRIN2B* | Glutamate receptor activity | Glutamate receptor signaling pathway |
| **Channelopathies** | | | |

| | | | |
|---|---|---|---|
| **Calcium channels** | *CACNA1A* | P/Q-type voltage-gated calcium channel dysfunction | Regulation of neurotransmitter secretion |
| | *CACNA1G* | T-type calcium channel activity | Regulation of membrane potential |
| **HCN channelopathy** | *HCN1* | Cyclic nucleotide-gated channel activity | Regulation of membrane potential |
| **Neuromuscular Junction Disorders** | | | |
| **Neuromuscular junction** | *PRELP* | Extracellular matrix structural constituent | Neuromuscular junction development |
| **Muscular dystrophy** | | | |
| **Dystrophin-associated complex** | *DMD* | Structural constituent of muscle | Muscle fibber development |
| **Nuclear envelope (laminins)** | *LMNA* | Structural molecule activity | Nuclear envelope organization |
| **Disorders of nucleobase, nucleotide and nucleic acid metabolism** | | | |
| **Ectonucleotide and nucleic acid metabolism** | *ADAR* | RNA editing activity | RNA editing |
| **DNA repair disorders** | | | |
| **DNA repair** | *PNKP* | Polynucleotide kinase activity | DNA repair |
| | *PMS2* | Mismatch repair endonuclease activity | Mismatch repair |
| **Repair of DNA double-strand breaks** | *NSD2* | Histone methyltransferase activity | Histone methylation |
| **Gene expression disorders** | | | |
| **Histone modification/ chromatin remodeling** | *ANKRD11* | Transcription coregulator activity | Regulation of transcription |
| **Transcription factor** | *CRELD1* | Cell adhesion molecule binding | Heart development (calcineurin/NFAT pathway) |
| **Transcription Factor** | *MAF* | Transcription factor activity | Cell differentiation (MAF) |
| **Epigenetic disorders** | | | |
| **Imprinting** | *UBE3A* (15q11.2–q13) | Ubiquitin-protein ligase activity | Protein ubiquitination |
| | ICR1 (11p15.5) | Regulation of genomic imprinting | |
| **Chromosomal structural abnormalities** | | | |
| **Chromosomal Rearrangement** | dup22q11.2 | - | |
| | del3q29 | - | |
| **Unspecified** | | | |
| **Abnormal neuronal signal transduction** | *CDKL5* | Protein serine/threonine kinase activity | Regulation of dendrite morphogenesis |
| **Neuronal differentiation defect** | *ZC4H2* | Zinc ion binding | Regulation of neuron differentiation |

*For inherited metabolic disorders, classification follows the International Classification of Inherited Metabolic Disorders (ICIMD). The Molecular Function and Biological Process columns were annotated using terminology aligned with the Gene Ontology (GO) framework and UniProt, reflecting the biochemical activity of the gene product and the biological processes in which it is involved.*

Beyond their diagnostic implications, mitochondrial mimics provide valuable insights into mitochondrial biology. Many of the disorders identified in this cohort converge on pathways that interact with, regulate, or secondarily impact mitochondrial function.

In particular, the study of channelopathies highlights the critical role of mitochondria in calcium homeostasis. Voltage-gated calcium channel defects (CACNA1A, CACNA1G) are not primarily

mitochondrial in origin, yet they profoundly affect mitochondrial calcium uptake. Calcium homeostasis is a key regulator of mitochondrial bioenergetics. Uptake of $Ca^{2+}$ through the mitochondrial calcium uniporter (MCU) activates several Krebs cycle dehydrogenases, increasing the production of reducing equivalents (NADH, $FADH_2$) that fuel the respiratory chain and sustain respiratory chain activity. This mechanism allows mitochondria to adjust ATP synthesis in response to cellular energy demand, particularly in excitable tissues. Disruption of calcium signalling, including that caused by channelopathies, may therefore secondarily impact mitochondrial metabolism.

Similarly, autophagy-related disorders such as LAMP2 deficiency (Danon disease) or *SNX14*-associated disease illustrate the dependence of mitochondrial homeostasis on lysosomal and autophagic flux. Ineffective removal of dysfunctional mitochondria by mitophagy results in accumulation of defective organelles, reinforcing the continuum between primary and secondary mitochondrial dysfunction.

For instance, vesicular trafficking defects provide a clear example of how disturbances in inter-organelle communication can impact mitochondrial function. *TANGO2*, initially regarded as a mitochondrial disorder gene, has now been shown to mediate ER-to-mitochondria retrograde transport, a process essential for maintaining mitochondrial proteostasis and bioenergetics. Loss of TANGO2 impairs mitochondrial protein import, disrupts oxidative phosphorylation, and sensitizes cells to energetic stress, thereby explaining the metabolic crises observed in affected patients. These insights support its reclassification outside the spectrum of canonical primary mitochondrial disorders, while simultaneously highlighting the importance of inter-organelle pathways in sustaining mitochondrial homeostasis.

Other groups reveal the centrality of nucleotide and DNA metabolism in maintaining mitochondrial integrity. Genes involved in DNA repair (*PNKP*, *PMS2*) or RNA editing (*ADAR*) affect mitochondrial genome stability and gene expression, while chromatin regulators (*NSD2*, *ANKRD11*) influence the expression of nuclear-encoded mitochondrial proteins[348–350]. These findings support the view that mitochondrial dysfunction can arise from defects in nuclear processes not traditionally classified as mitochondrial.

Finally, defects in structural proteins such as *FLNA* (actin cytoskeleton) and *COL5A1* (collagen fibril organization) show how cytoskeletal integrity and extracellular matrix signalling shape mitochondrial distribution, dynamics, and interactions with other organelles[351,352]. Cytoskeletal and extracellular matrix defects indirectly affect mitochondrial dynamics and distribution, pointing to structural determinants of mitochondrial function that extend beyond the organelle itself.

By broadening the perspective to include such mimics, mitochondrial medicine gains valuable insights into how ion fluxes, signalling networks, and organelle crosstalk modulate bioenergetics.

Altogether, the study of mitochondrial mimics not only refines differential diagnosis but also expands our knowledge of mitochondrial metabolism. By situating mitochondria at the crossroads of calcium regulation, autophagy, nucleotide metabolism, and cytoskeletal architecture, these findings highlight that the spectrum of disorders relevant to mitochondrial medicine extends well beyond the canonical OXPHOS machinery.

Beyond mechanistic insights, the study of mitochondrial mimics has practical implications for both diagnostics and therapeutics. First, it provides a framework to interpret variants of uncertain significance in canonical mitochondrial genes, as similar pathophysiological mechanisms are observed across distinct genetic backgrounds. Second, it explains why non-mitochondrial disorders often present with overlapping phenotypes, such as epilepsy, myopathy, or metabolic decompensation, traditionally considered hallmarks of PMD. Finally, by uncovering convergent pathways such as calcium homeostasis, mitophagy, or nucleotide repair, mimics highlight potential therapeutic entry points that extend across diagnostic boundaries, raising the prospect of pathway-based interventions in both mitochondrial and non-mitochondrial conditions.

## 4. Urinary Metabolomics as a Tool for Biomarker Discovery

Urinary metabolomics offers a non-invasive and repeatable approach to functional phenotyping, which is particularly advantageous in paediatrics. In this work, $^{1}$H NMR was applied to characterize the urinary metabolic profiles of children with genetically confirmed primary mitochondrial disease (PMD), aiming to identify biochemical fingerprints of mitochondrial dysfunction beyond conventional genetic testing. The rationale was to explore metabolomics as a complementary tool to genomics, addressing the persistent need for functional biomarkers in disorders where clinical heterogeneity and diagnostic uncertainty remain major challenges.

To date, no prior studies have compared the urinary metabolome of paediatric PMD patients with that of children with non-mitochondrial CKD, making this study a novel contribution.

This study adopted an innovative design by including multiple comparison groups: healthy children, patients with CKD stages 1–2, patients with CKD stages 3–5, and patients with suspected but genetically unconfirmed mitochondrial disease. While this layered structure added complexity and required careful interpretation, it provided a more refined framework for distinguishing mitochondrial-driven metabolic changes from those related to renal physiology or secondary mitochondrial dysfunction. By enabling comparisons across both healthy and disease controls, the study design enriched the biological interpretation of findings and enhanced their potential clinical relevance.

Interpretation of urinary metabolomics requires consideration of both systemic biochemical pathways and renal physiology. On the one hand, alterations in metabolites such as TCA cycle intermediates or amino acids reflect mitochondrial dysfunction at the cellular level. On the other hand, the urinary excretion profile is also shaped by organ-level processes including glomerular filtration, tubular reabsorption, and secretion. This dual perspective is particularly relevant in PMD, where renal involvement may range from subtle tubular defects to overt glomerular disease. The present findings illustrate how both dimensions interact: mitochondria-driven signatures were consistently detectable, yet their expression was modulated by the type and severity of renal pathology.

The comparison with CKD groups highlighted the bidirectional relationship between renal and mitochondrial function. Several metabolic alterations observed in PMD patients overlapped with those seen in CKD, reflecting shared mechanisms such as impaired tubular reabsorption and altered energy metabolism. Importantly, certain changes were detected even in PMD patients without overt nephropathy, supporting the notion that subtle renal involvement may be an early feature of mitochondrial disease. Metabolites such as NMA and hypoxanthine emerged as kidney-driven rather than mitochondria-driven alterations, highlighting their potential as organ-damage markers rather than disease-specific biomarkers.

In this exploratory analysis, genetically confirmed PMD cases showed consistent disturbances in TCA-cycle intermediates, amino acids, and energy-related metabolites across all comparisons. A subset of discriminatory metabolites — including tryptophan, histidine, and HVA — showed alterations that were consistent in PMD but not in CKD, suggesting that these changes are primarily mitochondria-driven rather than kidney-driven. Multivariate modelling yielded a candidate multi-metabolite panel that discriminated PMD not only from healthy controls but also from CKD and suspected-but-unsolved cases, achieving high cross-validated performance ($Q^2 > 0.62$; AUC > 0.90), consistent with strong discriminative potential. These findings are preliminary and require validation in independent cohorts.

The analysis of PMD with CKD patients (PMD+CKD) further confirmed both diverging and converging signatures. In P15, with BCS1L-related Fanconi syndrome, metabolomics revealed a stable profile dominated by severe glycosuria, persistent losses of Krebs cycle intermediates, and marked reductions in tryptophan and HVA. In contrast, with *RRM2B*-related glomerulopathy, showed a mixed pattern with consistently elevated N-methylnicotinamide and branched-chain amino acid catabolites, while retaining mitochondrial-specific features despite advanced CKD and dialysis. These complementary case studies illustrate how metabolomics can capture stable and distinctive profiles in tubular versus glomerular phenotypes and highlight candidate biomarkers such as N-methylnicotinamide for further validation. For P25, the observation that his urinary

profile clustered with genetically confirmed PMD in initial unsupervised multivariate analysis reinforced the clinical suspicion of PMD with renal involvement rather than isolated CKD. This further supported trio-exome revaluation, leading to the identification of compound heterozygous variants in *RRM2B*. Although metabolomics does not currently allow functional reclassification of variants, in this context it supported clinical reasoning and helped prioritize further testing.

As a group, PMD cases without CKD shared convergent alterations in central metabolic pathways that enabled discrimination from multiple control groups, supporting the concept of mitochondria-driven urinary signatures across diverse genotypes. In contrast, patients with renal involvement demonstrated that metabolomic profiling can capture highly distinctive and stable biochemical fingerprints, shaped by the nature of the renal lesion (tubular versus glomerular) yet still retaining mitochondrial-specific features. This dual perspective highlights both the potential of metabolomics to reveal common disease-wide signatures and its ability to uncover phenotype-specific patterns that may inform individualized monitoring.

Several limitations must be acknowledged. The genetic heterogeneity of the cohort, with most genes represented by a single patient, precludes genotype-specific stratification. Confounders such as age, diet, hydration status, microbiome composition, and renal function introduce variability that may mask subtle metabolic signals. Moreover, the candidate biomarker panel was derived from a single-centre study and has not yet been tested across independent datasets. Taken together, the results should be interpreted as proof-of-concept, offering preliminary insights rather than definitive diagnostic tools.

Looking forward, urinary metabolomics has the potential to complement genomics and clinical evaluation by providing a functional readout of mitochondrial dysfunction. Its utility may be particularly relevant for patients with ambiguous clinical presentations, for longitudinal monitoring of disease stability or therapeutic response, and for recognizing specific renal phenotypes within PMD. Integration with other omics layers and the application of Artificial Intelligence (AI) and Machine Learning (ML) will be essential to refine patient stratification, uncover convergent disease pathways, and develop biomarker-based outcome measures for clinical trials. The present findings therefore represent an important step towards embedding metabolomics into diagnostic and prognostic frameworks in mitochondrial medicine.

In the longer term, initiatives such as Recon4IMD and DeepMet are pioneering the integration of metabolomics into rare disease diagnostics through AI-driven modelling and network-based analysis. These platforms aim to refine diagnostic accuracy, predict disease progression, and guide therapeutic interventions based on multi-omics stratification.

In summary, urinary metabolomics offers a non-invasive, scalable approach to biochemical phenotyping in PMDs. As data harmonization and analytical pipelines improve, this methodology

may become a core component of diagnostic and prognostic frameworks for mitochondrial disease.

## 5. Personalized Medicine in Mitochondrial Disorders

A core aspect of this thesis is the progressive shift from descriptive, syndrome-based diagnosis toward a precision medicine model in mitochondrial disorders. Given the genetic, phenotypic, and biochemical heterogeneity of PMDs, a one-size-fits-all approach is rarely effective. Instead, individualized diagnostic and therapeutic strategies are increasingly needed to guide care, monitor disease evolution, and inform clinical decision-making.

Genomic diagnosis provides the foundation for this paradigm. The systematic application of NGS, WES, and iterative reanalysis reduces diagnostic uncertainty, refines prognosis, and enables patient-specific surveillance strategies, such as anticipating cardiac, hepatic, or renal complications. In selected conditions, genetic findings may even unlock genotype-directed therapies.

Genomics alone does not fully capture disease complexity. This study demonstrates the complementary value of untargeted urinary $^{1}$H-NMR metabolomics, which provides individualized biochemical readouts of mitochondrial dysfunction. Even in genetically heterogeneous cases, metabolomics identified biosignatures that supported differential diagnosis, revealed early organ involvement, and suggested opportunities for biomarker-informed monitoring.

Organ-specific phenotyping emerged as an informative layer within this work, particularly in the exploratory analysis of renal involvement in PMD. Cases such as P15 and P25 illustrate how kidney manifestations may represent both a diagnostic clue and a potential dimension of disease heterogeneity that deserves further study. While this study does not establish organ-specific interventions, it highlights the importance of systematically capturing such phenotypes, which may, in the future, contribute to more tailored surveillance strategies and individualized management.

Importantly, personalized medicine requires dynamic monitoring rather than a static diagnostic snapshot. Longitudinal metabolomic profiling holds promise as a tool to track metabolic stability or change in parallel with clinical evolution, allowing early detection of subclinical organ involvement, identification of therapeutic windows, and adjustment of supportive care strategies over time. This is particularly relevant in paediatrics, where phenotypes evolve with growth and development, and early interventions may alter long-term outcomes.

Despite these advances, a major challenge remains in translating group-level findings into strategies that are meaningful at the individual level. Clinical trials have often struggled with

heterogeneous patient cohorts, where individuals with fundamentally different mechanisms of disease were treated under the same protocol. The metabolomic findings presented here underscore both the promise and the limitation of this approach: while shared biosignatures highlight convergent pathways of dysfunction, phenotype-specific profiles point to distinct subgroups that may require tailored monitoring and therapy. Biomarker-informed stratification, combining genotype with functional readouts, may therefore be essential to refine trial design and bring interventions closer to true precision medicine.

Looking forward, personalized medicine in mitochondrial disease should not be reduced to gene-specific therapies alone. Rather, it should be understood as a holistic framework integrating longitudinal clinical data, genomic findings, and multi-omics functional readouts into individualized therapeutic strategies. Advances in AI and ML will be crucial to integrate these complex datasets, predict disease trajectories, and anticipate treatment responses. Embedding such approaches into clinical practice ultimately offers the prospect of more precise, stratified, and effective care for patients with mitochondrial disease.

## 6. Strengths and Limitations

### 6.1. Strengths

This study presents several methodological strengths that enhance both its scientific rigor and translational relevance. A key advantage lies in its interdisciplinary design, integrating clinical, genetic, biochemical, and metabolomic data to address the diagnostic complexity of primary mitochondrial disorders (PMDs). This comprehensive approach provides a refined view of disease heterogeneity and supports the development of personalized medicine strategies.

The cohort represents one of the largest single-centre genetic series of paediatric patients with suspected PMD to date (n = 178), enabling detailed analysis of diagnostic trajectories, phenotype reclassification, and identification of mitochondrial mimics. The systematic application of a tiered diagnostic framework ensured internal consistency, facilitated case stratification, and guided prioritization for genetic testing and follow-up.

Another strength is the metabolomic arm, which constitutes the first application of untargeted $^{1}$H NMR urinary metabolomics in genetically confirmed PMD patients. This exploratory component yielded novel insights into functional biochemical alterations, particularly those related to renal involvement, and generated candidate biomarkers for further validation.

Finally, the work directly addresses a longstanding diagnostic challenge: the misclassification of patients with overlapping phenotypes as PMD. The systematic identification and categorization of

mitochondrial mimics underscore the value of broad molecular testing and structured reassessment in achieving diagnostic precision.

## 6.2. Limitations

Several limitations should be acknowledged. As a single-centre study, the genetic and phenotypic findings may reflect local referral patterns and limit external generalizability.

The retrospective characterization of phenotypes constrains causal inference and limits longitudinal monitoring. Likewise, incomplete clinical, biochemical, or genetic data in patients assessed earlier in the study period may have introduced bias and hindered uniform interpretation across all cases.

Natural history analyses and survival estimates were not included in this study. Due to the considerable genetic and phenotypic diversity of primary mitochondrial disorders, combining patients with fundamentally different molecular aetiologies in longitudinal analyses would risk biological inaccuracies and confounding results. Furthermore, most genotypes were represented by only a single individual, precluding genotype-specific analyses and limiting the ability to draw conclusions about gene–phenotype correlations or disease trajectories.

These constraints also extended to the metabolomics component, where sample size and phenotypic variability limited the depth of subgroup analyses. Although patients with CKD were included as a disease control group to account for renal confounding, the number of individuals with clearly defined tubular versus glomerular lesions was insufficient to support subtype-specific analyses. As such, the study could not assess whether distinct renal lesion types influence the urinary metabolic profile in a systematic way. Despite rigorous prospective design and standardization, the metabolomic findings are exploratory. They require validation in larger, independent datasets to ensure that the observed biochemical signatures and potential biomarkers are robust and broadly applicable within the context of primary mitochondrial disorders.

## 6.3. Mitigation Strategies

To address the limitations described above, several deliberate mitigation strategies were implemented.

In the diagnostic component, a standardized and consistently applied diagnostic pipeline was used throughout the study. This included a tiered classification system integrating clinical, biochemical, and molecular data, which promoted reproducibility and minimized inter-clinician variability. Variant interpretation was conducted in a multidisciplinary clinical setting and followed ACMG-AMP guidelines, ensuring alignment with current best practices in genetics. A stringent and

up-to-date definition of PMD-associated genes was adopted, grounded in expert-curated resources and reflecting current consensus on gene–disease associations. This approach aimed to ensure clinical validity and consistency in gene inclusion criteria throughout the analysis of the cohort and comparison with the literature.

To mitigate the impact of genetic heterogeneity, this study deliberately avoided aggregate longitudinal analyses across diverse genotypes. Instead, it focused on diagnostic trajectories and cross-sectional metabolomic patterns and addressed specific subgroups separately. Grouping biologically distinct disorders under a single umbrella, can obscure clinically meaningful patterns. Studies should ideally be stratified by genotype or by well-defined phenotypic subgroups to ensure biological coherence.

This rationale was the basis to address genotype-specific cohorts (*EARS2*) and to explore phenotype-specific dimensions (such as renal involvement) in the metabolomics component of this study.

In the metabolomic study, patients with PMD were stratified based on the presence or absence of CKD, allowing clearer interpretation of mitochondria-related urinary signatures and avoiding confounding from CKD-driven metabolic alterations. To further disentangle mitochondrial-specific signatures from those attributable to renal dysfunction, the study included disease control groups composed of patients with CKD of non-mitochondrial origin. This strategy enabled the identification of biochemical features more likely to reflect mitochondrial dysfunction rather than the general metabolic consequences of renal impairment.

Together, these strategies strengthened the internal validity of the findings and provide a robust foundation for future multicenter validation, biomarker discovery, and the clinical translation of personalized approaches to mitochondrial medicine.

## 7. Future Perspectives in Mitochondrial Medicine

Despite major advances in genetic diagnostics and supportive care, curative treatments PMDs remain elusive. The path forward lies in translating mechanistic insights into actionable therapies, improving clinical trial infrastructure, and harnessing emerging technologies for earlier diagnosis and patient stratification.

### 7.1. Expanding Pathways and pathophysiology through Multi-Omics Data Analysis

Multi-omics, particularly metabolomics, transcriptomics, and phenomics, offers new opportunities to resolve these cases and improve understanding of pathophysiology.

Metabolomics provides a functional readout of mitochondrial dysfunction, capturing biochemical signatures of disease that can aid in diagnosis, monitor therapeutic response, and differentiate PMDs from mimics. Platforms such as $^{1}$H NMR and LC-MS have revealed disease-specific alterations in MELAS, Leigh syndrome, and mitochondrial myopathies. Artificial intelligence (AI) and machine learning (ML) are now essential tools for integrating multi-omics data. These models can stratify patients, predict disease trajectories, and support biomarker discovery by identifying latent patterns inaccessible to traditional analysis.

Deep learning frameworks such as OmiEmbed combine feature embeddings from multiple omics layers to improve disease classification and subtype prediction[353] . Other approaches, including MOLI (Multi-Omics Late Integration) and iOmicsPASS, leverage complementary data modalities to uncover converging biological mechanisms and clinically relevant patient subgroups[354,355].

Together, these AI-enhanced frameworks are reshaping diagnostic strategies in PMD, offering scalable tools to analyse the complexity of rare mitochondrial diseases and accelerate precision medicine efforts. These tools may serve as decision-support systems for clinicians, enabling earlier diagnosis and reducing reliance on invasive procedures.

## 7.2. Optimizing diagnostic yield

Despite significant advances in NGS, the diagnostic yield for PMD remains suboptimal, with many patients remaining undiagnosed after exome or genome sequencing. This limitation stems from multiple factors, including the phenotypic heterogeneity of mitochondrial disease, the high prevalence of VUS, incomplete gene annotation, and the complexity of mitochondrial genetics.

Recent developments in mitochondrial genome analysis have further expanded the diagnostic toolbox for PMDs. Long-read sequencing technologies offer improved resolution for detecting complex mtDNA rearrangements and phasing heteroplasmic variants. Macken et al. (2023) highlight the diagnostic advantages of PCR-free WGS protocols, combined with enhanced bioinformatic pipelines capable of distinguishing NUMTs, quantifying mtDNA copy number, and interpreting large-scale deletions[101]. These approaches are particularly valuable in patients with muscle involvement, where mtDNA defects may be tissue-specific. As costs decline and analysis becomes more standardized, such technologies may soon complement or replace traditional targeted mtDNA assays in clinical diagnostics.

Recent innovations have sought to overcome these challenges by integrating multi-dimensional data sources and leveraging advanced computational strategies to enhance variant interpretation. Integrative approaches that combine advanced sequencing technologies, structured phenomics, functional assays, and computational pipelines are increasingly shaping the diagnostic landscape in PMD.

### 7.2.1. Integration of Phenomics for Genotype-Phenotype Correlation

Phenomics - the systematic analysis of phenotypic traits - has proven useful in interpreting complex genomic data in PMDs. Tools such as the Leigh Map, which links over 90 causative genes to 275 standardized clinical features, demonstrate that structured phenotype ontologies can improve variant interpretation and clinical correlation. In validation studies, this approach achieved over 80% predictive accuracy for gene–phenotype associations in Leigh syndrome[356].

By formalizing clinical data, phenomics integration supports molecular diagnosis, reclassification of VUS, and prioritization of therapeutic targets in rare mitochondrial disorders. One fundamental strategy involves the incorporation of structured phenotype data through the Human Phenotype Ontology (HPO). Tools such as Exomiser, PhenIX, and LIRICAL utilize semantic similarity algorithms to rank genes based on compatibility between patient phenotypes and known gene-disease associations[357–361]. These methods improve prioritization, particularly when the phenotype is well-characterized. However, their reliance on curated databases limits discovery in genetically undiagnosed or atypical cases.

AI-driven platforms such as MOON (also known as GEM), which integrate genome-wide sequencing data with structured phenotypic descriptors, have demonstrated diagnostic yields above 95% in rare diseases[362]. Although not specifically developed for PMDs, such approaches exemplify how machine learning can shorten the diagnostic odyssey in genetically heterogeneous conditions by prioritizing candidate genes through combined genotype–phenotype interpretation.

Machine learning models trained on structured clinical features have been shown to predict the likelihood of mitochondrial disease with high accuracy, complementing genomic approaches and underscoring the utility of phenomics integration in guiding variant interpretation and diagnostic prioritization[345].

### 7.2.2. Improving Functional Assays and Variant Reclassification

The interpretation of VUS remains a major bottleneck in the diagnostic workflow of mitochondrial disorders. Functional assays provide a crucial bridge between genomic data and pathogenicity inference, particularly when results involve genes not yet linked to mitochondrial phenotypes. However, traditional validation strategies, such as functional assays are labour-intensive, tissue-specific, and poorly scalable.

Recent advances in high-throughput phenotyping platforms have broadened the scope of functional validation. A promising development is the use of high-throughput imaging flow cytometry (IFC) to screen for that interrogates six cellular compartments in patient-derived fibroblasts: mitochondria, lysosomes, Golgi apparatus, endoplasmic reticulum, autophagy machinery, and NF-κB signalling in fibroblasts. This multiplexed approach identified reproducible

phenotypic signatures in individuals carrying VUS, even in genes not previously associated with mitochondrial disease, and the resulting cellular profiles correlated with disease severity. Importantly, these signatures discriminated affected individuals from healthy controls, demonstrating potential for rapid phenotypic stratification [363].

Such multiplexed, cell-based assays represent a promising avenue for semi-automated functional classification of VUS and may soon become integral to diagnostic pipelines and variant curation efforts. In the context of precision diagnostics, they could facilitate stratification for targeted therapeutic trials and support reclassification of variants within the ACMG framework. Looking forward, integration of these functional readouts with transcriptomic, proteomic, and metabolomic layers will be essential to establish robust cellular endophenotypes and clinically relevant biomarkers.

Collectively, these strategies point to a paradigm shift toward patient-specific, multi-omics, and computationally enhanced diagnostics. For mitochondrial medicine, where multisystem involvement and incomplete penetrance are the rule, such integrative approaches are essential to maximize the diagnostic potential of NGS.

### 7.2.3. Integrating Multi-Layered Data and Computational Tools

Tools such as Exomiser and MOON integrate variant annotations, population frequency, and phenotype matching, and have significantly improved diagnostic yield in rare diseases[357,359,361,362,364,365]. Exomiser applies rule-based prioritization combining variant effect prediction with HPO-driven phenotype matching, whereas MOON/GEM employs a Bayesian framework that dynamically weighs variant, inheritance, and phenotype data, enabling probabilistic diagnosis in real-world clinical genomics. However, they rely on predefined pathogenicity models and curated gene-disease associations, which may limit performance in genetically heterogeneous or atypical MD presentations.

Literature-aware methods, such as Automatic Mendelian Literature Evaluation (AMELIE), apply natural language processing to biomedical publications to dynamically associate genes with patient phenotypes[364]. This machine learning strategy enables rapid incorporation of emerging knowledge, a key advantage in rare disease diagnostics where gene-disease links are continually evolving.

A promising alternative is VIOLA (Variant prIOritization using Latent spAce), a novel pipeline that uses unsupervised learning to identify outlier variants within each patient's genome based on functional scores, phenotype similarity, and transcriptomic co-expression[366]. For example, the VIOLA model combines whole-exome sequencing with patient-specific RNA-seq from fibroblasts, identifying variants as functional outliers in a latent space derived from variant attributes. In a

proof-of-concept study involving 20 patients with suspected MD, VIOLA consistently ranked known pathogenic variants among the top five candidates and outperformed Exomiser in all validated cases. Notably, it identified disease-associated variants in genes not previously classified as mitochondrial, underscoring its exploratory potential. By integrating transcriptomic co-expression networks (MitoBook), inheritance-aware filtering, and HPO similarity, VIOLA demonstrated superior performance to Exomiser in identifying causal variants, even in genes not previously classified as mitochondrial[366].

Other tools such as GeneNetwork Assisted Diagnostic Optimization (GADO) leverage large-scale public RNA-seq datasets to infer gene co-expression patterns, allowing prioritization of genes based on tissue-relevant expression and phenotype similarity[362,367]. Similarly, network-based models integrate protein-protein interactions and disease ontologies to identify functionally coherent gene modules[368].

Finally, Bayesian frameworks like GEM synthesize variant, inheritance, frequency, and phenotype data to compute posterior probabilities of pathogenicity, providing clinically interpretable outputs suitable for diagnostic settings. GEM has been adopted in real-world clinical genomics programs and illustrates the value of integrated, automated pipelines.

In parallel, orthogonal technologies are being explored to resolve genetic diagnoses in cases where sequencing approaches remain inconclusive. In addition to prioritization at the time of initial analysis, systematic reanalysis of unsolved cases has emerged as a complementary strategy to improve diagnostic yield. Exomiser can identify new diagnoses in previously unsolved cases by integrating updated gene-disease associations and automated ACMG classification. In the 100,000 Genomes Project cohort, Exomiser reanalysis uncovered 463 new diagnoses, representing 2% of the previously undiagnosed cases. Notably, 72% of these involved variants already present in the initial data but overlooked due to panel limitations at the time[361]. These findings support the implementation of periodic, phenotype-driven reinterpretation workflows, particularly as curated disease databases continue to expand. Optical Genome Mapping (OGM), for instance, has emerged as a powerful tool to detect structural variants (SVs), including translocations, inversions, and complex rearrangements often missed by short-read sequencing. In a recent case study, OGM enabled the identification of a cryptic X;18 translocation in two siblings with syndromic features, previously undetected by exome and microarray[369]. Although challenges in variant interpretation persist, especially for SVs of uncertain significance, the integration of OGM with WGS and standardized interpretation frameworks is expected to improve diagnostic resolution in rare diseases.

## 7.3. Advances in treatment

Despite improved recognition and molecular diagnosis of PMD, curative treatments remain limited. The future of mitochondrial therapeutics lies in two converging directions: (1) adapting clinical trial designs to account for rarity, heterogeneity, and evolving biomarkers; and (2) advancing targeted therapeutic discovery through integrative, mechanism-based strategies.

### 7.3.1. Advancing Clinical Trial Design and Infrastructure

One of the major challenges in PMD research is the implementation of clinical trials within small, phenotypically heterogeneous populations. Key obstacles include limited patient numbers, variability in disease progression, lack of validated biomarkers and endpoints, and the need for long-term follow-up. International initiatives have emphasized the importance of harmonized outcome measures, dynamic trial designs, and standardized registries to overcome these limitations[232,233,278].

Regulatory bodies have increasingly accepted flexible methodologies such as adaptive protocols, basket and umbrella trials, and the integration of real-world data. International initiatives, including TREAT-Mito, E-Rare, and MITOREG, are essential to enable cross-border recruitment, natural history study integration, and longitudinal biomarker development. These collaborative frameworks are helping to standardize data collection, facilitate patient stratification, and accelerate therapeutic trial readiness. [370,370]

Progress in therapeutic innovation must be accompanied by more rigorous clinical trial design, harmonized outcome measures, and a clearer understanding of natural history[232,233,278]. In parallel, recent consensus, research priorities were defined to include biomarker discovery, disease modelling, and the development of validated, patient-centred outcome tools, particularly for paediatric populations[300,278,210].

### 7.3.2. Innovative Therapeutic Strategies

Progress in therapeutic discovery is increasingly driven by integrative approaches that combine genomic, transcriptomic, and metabolomic data with *in silico* screening and high-throughput functional validation. AI and ML are accelerating this paradigm shift by enabling data-driven compound selection, pathway prioritization, and patient stratification[286]. Several ongoing interventional trials focus on diagnostic innovation, physiological monitoring, and non-drug interventions, reflecting a broader strategy to improve disease characterization and patient management. These efforts are supported by evolving model systems, including patient-derived fibroblasts, iPSC-derived organoids, and cross-species validation platforms[371–374]

Advances also extend to the domain of digital health and remote monitoring. For example, novel biophysical and device-based methods are under investigation. Oxygen nanosensor technologies are being validated as innovative diagnostic tools to assess muscle oxygenation dynamics in mitochondrial disease (NCT04086329, NCT06819683). A diagnostic trial is addressing the current impasse in molecular diagnosis of mitochondrial diseases by integrating minimally invasive procedures such as skin biopsies and blood sampling into novel pipelines (NCT06621732).

In parallel, the application of passive exercise devices are being studied in mitochondrial myopathies, combined with cardiopulmonary exercise testing and contrast imaging (NCT05569122). One study is evaluating a home-based exercise program and smart watch technology, aiming to capture real-time functional data and enhance disease monitoring in daily life (NCT06535646).

AI-guided drug repurposing efforts have identified agents such as metformin and bezafibrate as potential modulators of mitochondrial function, while generative models and knowledge graphs are being used to prioritize novel compounds[268,375]. In parallel, experimental therapies are being developed that directly target mitochondrial pathophysiology. These include small molecules that enhance mitophagy or oxidative phosphorylation, CRISPR-based gene editing for nuclear-encoded mitochondrial genes, and mitochondrial transplantation strategies[376–378]. Notably, mitochondria-targeted zinc finger nucleases (mtZFNs) have shown promise in selectively eliminating mutated mtDNA, thereby restoring normal function in preclinical models[379].

Triheptanoin (C7 oil), a medium-chain triglyceride with odd-carbon fatty acids, replenishes tricarboxylic acid (TCA) cycle intermediates through anaplerosis and has shown improved exercise tolerance in mitochondrial myopathy, though broader efficacy remains under study[380]. Pantethine, a precursor of CoA biosynthesis, has demonstrated redox-modulating activity and potential support of mitochondrial bioenergetics in vitro[350]. Meldonium, a carnitine analogue that promotes glucose utilization over fatty acid oxidation, has been explored in ischemic and cardiovascular settings, but its role in PMDs is still experimental[381].

Early-stage and preclinical strategies further expand the therapeutic landscape. Small-molecule correctors of POLG mutations, identified through cryo-EM–guided high-throughput screening, exemplify allele-specific drug development[375]. NV354, a cell-permeable succinate prodrug, is advancing toward first-in-human trials as a targeted therapy for complex I deficiency. Gene therapy, allotopic expression, and mitochondrial augmentation therapies also remain under active investigation[382]. While these approaches are still largely preclinical, they illustrate the diversification of strategies aimed at directly correcting or bypassing mitochondrial dysfunction.

Experimental studies of hypoxia have also revealed potential protective effects. In mouse models of Leigh syndrome, intermittent hypoxia prolonged survival and improved neurological outcomes, possibly via HIF-1α–mediated metabolic reprogramming[383,384].

Omaveloxolone, an Nrf2 pathway activator recently approved for Friedreich ataxia, demonstrates the translational potential of redox-sensitive signalling in related disorders (NCT02255435).

Beyond single interventions, multi-omics integration is emerging as a central strategy to unravel convergent mechanisms and stratify patients. Large collaborative networks, including GENOMIT and RDCRN, are incorporating metabolomic biomarkers into longitudinal studies and clinical trials to standardize outcome measures and refine trial readiness.

As therapies become increasingly personalized, ethical considerations must be revisited. Germline interventions such as mitochondrial replacement therapy raise concerns regarding long-term consequences, informed consent, and intergenerational impact[385]. Responsible implementation of these technologies will require continuous dialogue among clinicians, scientists, ethicists, and patient communities.

Ultimately, the path forward in mitochondrial medicine will depend on a coordinated effort to align clinical innovation with molecular insights. Advances in trial infrastructure, biomarker discovery, therapeutic targeting, and ethical governance will be essential to translate discovery into durable benefit for individuals living with mitochondrial disease.

# *Chapter VI.* **Conclusions**

The results underscore the clinical and genetic heterogeneity characteristic of mitochondrial disease, highlight the limitations of single-modality diagnostic approaches, and demonstrate the potential of integrating genomic and metabolomic methodologies to enhance understanding and facilitate individualized patient care.

Structured diagnostic frameworks are essential to manage clinical heterogeneity and to guide the rational use of genomic tools, particularly in paediatric cohorts, while also promoting equity in access to care across physicians. A follow-up plan that combines a shared structure with individualization according to the patient's phenotype–genotype ensures comorbidities are appropriately identified and managed enhance supportive care and contribute to the understanding of natural history. Applying similarly comprehensive monitoring strategies to undiagnosed patients enables deep phenotyping and may provide critical clues for future diagnostic clarification. In this context, the implementation of a tiered, standardized diagnostic framework improved diagnostic consistency, enabled systematic re-evaluation, and supported prioritization for functional and genomic testing. The integration of clinical expertise, functional data, and longitudinal reassessment therefore remains vital.

Through the structured evaluation of a large paediatric cohort, this work highlights the diagnostic complexity of PMDs, characterized by marked phenotypic heterogeneity and frequent overlap with other genetic and metabolic syndromes. It reinforces current evidence that clinical, biochemical, and histological features, although supportive, lack specificity and cannot establish a diagnosis in isolation. Despite the progress of the past decade, many patients still carry legacy diagnoses without molecular validation, illustrating the limitations of historical diagnostic practices.

Secondary mitochondrial dysfunction and overlapping phenotypes represent a recurrent challenge in mitochondrial medicine, as they often share biochemical signatures with primary disease. Mitochondrial mimics are frequent and encompass a broad spectrum of neuromuscular, neurodevelopmental, and metabolic disorders. Phenotypic mimicry extends beyond clinical manifestations to biochemical findings, underscoring the importance of comprehensive molecular testing and diagnostic caution. Differentiating true PMDs from mimics remains a central priority for improving diagnostic accuracy and ensuring appropriate inclusion in clinical trials. Yet, even with genetic confirmation as the cornerstone of diagnosis, a substantial proportion of cases remain undiagnosed. Future studies incorporating transcriptomic approaches (e.g., RNA sequencing), long-read genome analysis, and integrative multi-omics profiling may help resolve these cases and advance diagnostic precision.

Beyond the cohort-level analysis, this study contributes original insights into rare nuclear-encoded mitochondrial disorders. The report of a second sibling pair with ISD11/*LYRM4* deficiency, the only

additional family described to date after the initial description, expands knowledge of this extremely rare condition. Its atypical presentation—restricted to a metabolic crisis with cardiorespiratory arrest during infection, discriminative 3-methylglutaconic aciduria without hyperammonaemia, absence of neurological involvement, and unexpectedly favourable outcome, illustrates the diversity of disease trajectories within mitochondrial medicine.

The characterization of *EARS2* deficiency, the most frequent nuclear-encoded cause in this cohort, was strengthened by a systematic literature review and meta-analysis that provided an overview on phenotypic spectrum and potential prognostic factors. Together, these studies underscore the complementary value of rare case reports and aggregated evidence in defining genotype–phenotype correlations and improving diagnostic precision in mitochondrial disorders.

This study demonstrates that urinary $^{1}$H NMR metabolomics can capture mitochondria-driven metabolic signatures in paediatric patients with primary mitochondrial disease, even against the confounding background of renal dysfunction and clinical heterogeneity. The inclusion of multiple comparison groups, including healthy children, CKD, and suspected but genetically unconfirmed cases, provided a nuanced framework that enriched biological interpretation. Both group-level analyses and individual case studies highlighted how metabolomics can reveal convergent disease-wide alterations while also identifying phenotype-specific fingerprints, such as those associated with tubular or glomerular pathology.

Although exploratory, these findings underscore the promise of urinary metabolomics as a non-invasive, scalable adjunct to genomic medicine. It supports the integration of metabolomics into the diagnostic and research framework of mitochondrial medicine and highlight its promise as a tool for biomarker discovery with translational potential. By supporting differential diagnosis, detecting early organ involvement, and enabling longitudinal monitoring, metabolomic profiling may contribute to future biomarker-driven stratification strategies. Validation in independent cohorts, integration with other omics layers, and the application of AI-based approaches will be critical next steps toward embedding metabolomics into diagnostic and prognostic frameworks for mitochondrial disease.

Future research should prioritize multi-omics integration, prospective organ-specific screening - especially of renal involvement - and the design of biomarker-informed clinical trials, with the goal of delivering more precise, stratified, and effective care to individuals living with mitochondrial disease.

Taken together, these contributions illustrate how systematic clinical investigation, rare disease genomics, and functional metabolomics can be combined to refine diagnosis and advance a personalized medicine approach for PMD in childhood in this new era of genome sequencing.

# References

1. Wills EJ. The Powerhouse of the Cell. *Ultrastruct Pathol*. 1992;16(3):iii-vi. doi:10.3109/01913129209061353

2. Dasgupta S. Mitochondrion: I am more than a fuel server. *Ann Transl Med*. 2019;7(20):594-594. doi:10.21037/atm.2019.08.22

3. Monzel AS, Enríquez JA, Picard M. Multifaceted mitochondria: moving mitochondrial science beyond function and dysfunction. *Nat Metab*. 2023;5(4):546-562. doi:10.1038/s42255-023-00783-1

4. Lee-Glover LP, Picard M, Shutt TE. Mitochondria – the CEO of the cell. *J Cell Sci*. 2025;138(9):jcs263403. doi:10.1242/jcs.263403

5. Picard M. Mitochondria are more than powerhouses—they're the motherboard of the cell. *Sci Am*. May 20, 2025. https://www.scientificamerican.com/article/why-mitochondria-are-more-like-a-motherboard-than-the-powerhouse-of-the-cell/

6. San-Millán I. The Key Role of Mitochondrial Function in Health and Disease. *Antioxidants*. 2023;12(4):782. doi:10.3390/antiox12040782

7. DiMauro S, Schon EA. Mitochondrial Respiratory-Chain Diseases. *N Engl J Med*. 2003;348(26):2656-2668. doi:10.1056/NEJMra022567

8. Watt IN, Montgomery MG, Runswick MJ, Leslie AGW, Walker JE. Bioenergetic cost of making an adenosine triphosphate molecule in animal mitochondria. *Proc Natl Acad Sci*. 2010;107(39):16823-16827. doi:10.1073/pnas.1011099107

9. Owen OE, Kalhan SC, Hanson RW. The Key Role of Anaplerosis and Cataplerosis for Citric Acid Cycle Function. *J Biol Chem*. 2002;277(34):30409-30412. doi:10.1074/jbc.R200006200

10. Cheng YW, Liu J, Finkel T. Mitohormesis. *Cell Metab*. 2023;35(11):1872-1886. doi:10.1016/j.cmet.2023.10.011

11. Chen S, Li Q, Shi H, Li F, Duan Y, Guo Q. New insights into the role of mitochondrial dynamics in oxidative stress-induced diseases. *Biomed Pharmacother*. 2024;178:117084. doi:10.1016/j.biopha.2024.117084

12. Stepien KM, Heaton R, Rankin S, et al. Evidence of Oxidative Stress and Secondary Mitochondrial Dysfunction in Metabolic and Non-Metabolic Disorders. *J Clin Med*. 2017;6(7):71. doi:10.3390/jcm6070071

13. Arnold PK, Finley LWS. Regulation and function of the mammalian tricarboxylic acid cycle. *J Biol Chem*. 2023;299(2):102838. doi:10.1016/j.jbc.2022.102838

14. Sano S, Inoue S, Tanabe Y, Sumiya C, Koike S. Significance of Mitochondria for Porphyrin and Heme Biosynthesis. *Science*. 1959;129(3344):275-276. doi:10.1126/science.129.3344.275

15. Houtkooper RH, Vaz FM. Cardiolipin, the heart of mitochondrial metabolism. *Cell Mol Life Sci*. 2008;65(16):2493-2506. doi:10.1007/s00018-008-8030-5

16. Miller WL. Steroid hormone synthesis in mitochondria. *Mol Cell Endocrinol*. 2013;379(1-2):62-73. doi:10.1016/j.mce.2013.04.014

17. Akram M. Citric Acid Cycle and Role of its Intermediates in Metabolism. *Cell Biochem Biophys*. 2014;68(3):475-478. doi:10.1007/s12013-013-9750-1

18. Lane AN, Fan TWM. Regulation of mammalian nucleotide metabolism and biosynthesis. *Nucleic Acids Res*. 2015;43(4):2466-2485. doi:10.1093/nar/gkv047

19. Rouault TA, Maio N. Biogenesis and functions of mammalian iron-sulfur proteins in the regulation of iron homeostasis and pivotal metabolic pathways. *J Biol Chem*. 2017;292(31):12744-12753. doi:10.1074/jbc.R117.789537

20. Joshi A, Richard TH, Gohil VM. Mitochondrial phospholipid metabolism in health and disease. *J Cell Sci*. 2023;136(17):jcs260857. doi:10.1242/jcs.260857

21. Alevriadou BR, Patel A, Noble M, et al. Molecular nature and physiological role of the mitochondrial calcium uniporter channel. *Am J Physiol-Cell Physiol*. 2021;320(4):C465-C482. doi:10.1152/ajpcell.00502.2020

22. Casalena G, Krick S, Daehn I, et al. Mpv17 in mitochondria protects podocytes against mitochondrial dysfunction and apoptosis in vivo and in vitro. *Am J Physiol-Ren Physiol*. 2014;306(11):F1372-F1380. doi:10.1152/ajprenal.00608.2013

23. Chen W, Zhao H, Li Y. Mitochondrial dynamics in health and disease: mechanisms and potential targets. *Signal Transduct Target Ther*. 2023;8(1):333. doi:10.1038/s41392-023-01547-9

24. Wang S, Long H, Hou L, et al. The mitophagy pathway and its implications in human diseases. *Signal Transduct Target Ther*. 2023;8(1):304. doi:10.1038/s41392-023-01503-7

25. Scarpulla RC. Transcriptional Paradigms in Mammalian Mitochondrial Biogenesis and Function. *Physiol Rev*. 2008;88(2):611-638. doi:10.1152/physrev.00025.2007

26. Hu MM, Shu HB. Mitochondrial DNA-triggered innate immune response: mechanisms and diseases. *Cell Mol Immunol*. 2023;20(12):1403-1412. doi:10.1038/s41423-023-01086-x

27. Tábara LC, Burr SP, Frison M, et al. MTFP1 controls mitochondrial fusion to regulate inner membrane quality control and maintain mtDNA levels. *Cell*. 2024;187(14):3619-3637.e27. doi:10.1016/j.cell.2024.05.017

28. Agresti CA, Halkiadakis PN, Tolias P. MERRF and MELAS: current gene therapy trends and approaches. *J Transl Genet Genomics*. 2018;2(7). doi:10.20517/jtgg.2018.05

29. Foo J, Bellot G, Pervaiz S, Alonso S. Mitochondria-mediated oxidative stress during viral infection. *Trends Microbiol*. 2022;30(7):679-692. doi:10.1016/j.tim.2021.12.011

30. Diokmetzidou A, Scorrano L. Mitochondria–membranous organelle contacts at a glance. *J Cell Sci*. 2025;138(9):jcs263895. doi:10.1242/jcs.263895

31. Vianello C, Cocetta V, Caicci F, et al. Interaction Between Mitochondrial DNA Variants and Mitochondria/Endoplasmic Reticulum Contact Sites: A Perspective Review. *DNA Cell Biol*. 2020;39(8):1431-1443. doi:10.1089/dna.2020.5614

32. Chen L, Zhou M, Li H, et al. Mitochondrial heterogeneity in diseases. *Signal Transduct Target Ther*. 2023;8(1):311. doi:10.1038/s41392-023-01546-w

33. Niyazov DM, Kahler SG, Frye RE. Primary Mitochondrial Disease and Secondary Mitochondrial Dysfunction: Importance of Distinction for Diagnosis and Treatment. *Mol Syndromol*. 2016;7(3):122-137. doi:10.1159/000446586

34. Baker MJ, Crameri JJ, Thorburn DR, Frazier AE, Stojanovski D. Mitochondrial biology and dysfunction in secondary

mitochondrial disease. *Open Biol*. 2022;12(12):220274. doi:10.1098/rsob.220274

35. Rouzier C, Pion E, Chaussenot A, et al. Primary mitochondrial disorders and mimics: Insights from a large French cohort. *Ann Clin Transl Neurol*. 2024;11(6):1478-1491. doi:10.1002/acn3.52062

36. Chocron ES, Munkácsy E, Pickering AM. Cause or casualty: The role of mitochondrial DNA in aging and age-associated disease. *Biochim Biophys Acta BBA - Mol Basis Dis*. 2019;1865(2):285-297. doi:10.1016/j.bbadis.2018.09.035

37. Baruteau J, Hargreaves I, Krywawych S, et al. Successful reversal of propionic acidaemia associated cardiomyopathy: Evidence for low myocardial coenzyme Q10 status and secondary mitochondrial dysfunction as an underlying pathophysiological mechanism. *Mitochondrion*. 2014;17:150-156. doi:10.1016/j.mito.2014.07.001

38. Brasil S, Richard E, Jorge-Finnigan A, et al. Methylmalonic aciduria *cblB* type: characterization of two novel mutations and mitochondrial dysfunction studies. *Clin Genet*. 2015;87(6):576-581. doi:10.1111/cge.12426

39. McCormick E, Place E, Falk MJ. Molecular Genetic Testing for Mitochondrial Disease: From One Generation to the Next. *Neurotherapeutics*. 2013;10(2):251-261. doi:10.1007/s13311-012-0174-1

40. Katsetos CD, Koutzaki S, Melvin JJ. Mitochondrial Dysfunction in Neuromuscular Disorders. *Semin Pediatr Neurol*. 2013;20(3):202-215. doi:10.1016/j.spen.2013.10.010

41. Morava É, Kozicz T. Mitochondria and the economy of stress (mal)adaptation. *Neurosci Biobehav Rev*. 2013;37(4):668-680. doi:10.1016/j.neubiorev.2013.02.005

42. Parikh S, Karaa A, Goldstein A, et al. Diagnosis of 'possible' mitochondrial disease: an existential crisis. *J Med Genet*. 2019;56(3):123-130. doi:10.1136/jmedgenet-2018-105800

43. Dawson G, Kilkus J, Siakotos AN, Singh I. Mitochondrial abnormalities in CLN2 and CLN3 forms of batten disease. *Mol Chem Neuropathol*. 1996;29(2-3):227-235. doi:10.1007/BF02815004

44. Ripolone M, Ronchi D, Violano R, et al. Impaired Muscle Mitochondrial Biogenesis and Myogenesis in Spinal Muscular Atrophy. *JAMA Neurol*. 2015;72(6):666. doi:10.1001/jamaneurol.2015.0178

45. Shulyakova N, Andreazza AC, Mills LR, Eubanks JH. Mitochondrial Dysfunction in the Pathogenesis of Rett Syndrome: Implications for Mitochondria-Targeted Therapies. *Front Cell Neurosci*. 2017;11. doi:10.3389/fncel.2017.00058

46. Buzkova J, Nikkanen J, Ahola S, et al. Metabolomes of mitochondrial diseases and inclusion body myositis patients: treatment targets and biomarkers. *EMBO Mol Med*. 2018;10(12):e9091. doi:10.15252/emmm.201809091

47. Macken WL, Falabella M, McKittrick C, et al. Specialist multidisciplinary input maximises rare disease diagnoses from whole genome sequencing. *Nat Commun*. 2022;13(1):6324. doi:10.1038/s41467-022-32908-7

48. Arena IG, Pugliese A, Volta S, Toscano A, Musumeci O. Molecular Genetics Overview of Primary Mitochondrial Myopathies. *J Clin Med*. 2022;11(3):632. doi:10.3390/jcm11030632

49. Lieber DS, Vafai SB, Horton LC, et al. Atypical case of Wolfram syndrome revealed through targeted exome sequencing in a patient with suspected mitochondrial disease. *BMC Med Genet*. 2012;13(1):3. doi:10.1186/1471-2350-13-3

50. Rossignol DA, Frye RE. A review of research trends in physiological abnormalities in autism spectrum disorders: immune dysregulation, inflammation, oxidative stress, mitochondrial dysfunction and environmental toxicant exposures. *Mol Psychiatry*. 2012;17(4):389-401. doi:10.1038/mp.2011.165

51. Kohda M, Tokuzawa Y, Kishita Y, et al. A Comprehensive Genomic Analysis Reveals the Genetic Landscape of Mitochondrial Respiratory Chain Complex Deficiencies. Barsh GS, ed. *PLOS Genet*. 2016;12(1):e1005679. doi:10.1371/journal.pgen.1005679

52. Izzo A, Mollo N, Nitti M, et al. Mitochondrial dysfunction in down syndrome: molecular mechanisms and therapeutic targets. *Mol Med*. 2018;24(1):2. doi:10.1186/s10020-018-0004-y

53. Caracausi M, Ghini V, Locatelli C, et al. Plasma and urinary metabolomic profiles of Down syndrome correlate with alteration of mitochondrial metabolism. *Sci Rep*. 2018;8(1):2977. doi:10.1038/s41598-018-20834-y

54. Zapolnik P, Sykut-Cegielska J, Pyrkosz A. Coincidence of 3-methylglutaconic aciduria and duplication 5q – a case report and literature review. *Acta Biochim Pol*. Published online June 8, 2020. doi:10.18388/abp.2020_5355

55. Sivitz WI, Yorek MA. Mitochondrial Dysfunction in Diabetes: From Molecular Mechanisms to Functional Significance and Therapeutic Opportunities. *Antioxid Redox Signal*. 2010;12(4):537-577. doi:10.1089/ars.2009.2531

56. Cunarro J, Casado S, Lugilde J, Tovar S. Hypothalamic Mitochondrial Dysfunction as a Target in Obesity and Metabolic Disease. *Front Endocrinol*. 2018;9:283. doi:10.3389/fendo.2018.00283

57. Kobayashi H, Matsubara S, Yoshimoto C, Shigetomi H, Imanaka S. A Comprehensive Review of the Contribution of Mitochondrial DNA Mutations and Dysfunction in Polycystic Ovary Syndrome, Supported by Secondary Database Analysis. *Int J Mol Sci*. 2025;26(3):1172. doi:10.3390/ijms26031172

58. Frye RE, Lionnard L, Singh I, et al. Mitochondrial morphology is associated with respiratory chain uncoupling in autism spectrum disorder. *Transl Psychiatry*. 2021;11(1):527. doi:10.1038/s41398-021-01647-6

59. Greenamyre JT. What's wrong with mitochondria in Parkinson's disease? *Mov Disord*. 2018;33(10):1515-1517. doi:10.1002/mds.98

60. Tanaka M, Szabó Á, Spekker E, Polyák H, Tóth F, Vécsei L. Mitochondrial Impairment: A Common Motif in Neuropsychiatric Presentation? The Link to the Tryptophan–Kynurenine Metabolic System. *Cells*. 2022;11(16):2607. doi:10.3390/cells11162607

61. Caielli S, Athale S, Domic B, et al. Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. *J Exp Med*. 2016;213(5):697-713. doi:10.1084/jem.20151876

62. Rath E, Moschetta A, Haller D. Mitochondrial function — gatekeeper of intestinal epithelial cell homeostasis. *Nat Rev Gastroenterol Hepatol*. 2018;15(8):497-516. doi:10.1038/s41575-018-0021-x

63. Ma C, Wang J, Hong F, Yang S. Mitochondrial Dysfunction in Rheumatoid Arthritis. *Biomolecules*. 2022;12(9):1216. doi:10.3390/biom12091216

64. Wallace DC. Mitochondria and cancer. *Nat Rev Cancer*. 2012;12(10):685-698. doi:10.1038/nrc3365

65. Khalid N, Khan MU, Rehman R, et al. Unraveling the genetic connections for mitochondrial DNA control region and breast cancer susceptibility. *Sci Rep*. 2025;15(1):4821. doi:10.1038/s41598-025-89115-9

66. Mollazadeh H, Tavana E, Fanni G, et al. Effects of statins on mitochondrial pathways. *J Cachexia Sarcopenia Muscle*. 2021;12(2):237-251. doi:10.1002/jcsm.12654

67. Kuretu A, Arineitwe C, Mothibe M, Ngubane P, Khathi A, Sibiya N. Drug-induced mitochondrial toxicity: Risks of developing glucose handling impairments. *Front Endocrinol*. 2023;14:1123928. doi:10.3389/fendo.2023.1123928

68. Videira MT. *Impairment of Mitochondrial Metabolism as Major Culprit of Misdiagnosis (Case Reports)*. Tese de Mestrado. U. Porto; 2021. https://hdl.handle.net/10216/139131

69. Pyle A, Nightingale HJ, Griffin H, et al. Respiratory chain deficiency in nonmitochondrial disease. *Neurol Genet*. 2015;1(1):e6. doi:10.1212/NXG.0000000000000006

70. Andreu AL, DiMauro S. Current classification of mitochondrial disorders. *J Neurol*. 2003;250(12):1403-1406. doi:10.1007/s00415-003-0281-3

71. Gupta R, Kanai M, Durham TJ, et al. Nuclear genetic control of mtDNA copy number and heteroplasmy in humans. *Nature*. 2023;620(7975):839-848. doi:10.1038/s41586-023-06426-5

72. Cannon SJ, Hall T, Hawkes G, et al. Penetrance and expressivity of mitochondrial variants in a large clinically unselected population. *Hum Mol Genet*. 2024;33(5):465-474. doi:10.1093/hmg/ddad194

73. Wengert S, Giannoulis X, Kreitmaier P, et al. Tissue-specific apparent mtDNA heteroplasmy and its relationship with ageing and mtDNA gene expression. *Genomics*. Preprint posted online December 16, 2024. doi:10.1101/2024.12.11.627989

74. Hathazi D, Lyons C, Lagos D, et al. Mitochondrial DNA heteroplasmy drives cortical neuronal disturbances in human organoids harbouring the common m.3243A>G mutation. *Neuroscience*. Preprint posted online March 22, 2025. doi:10.1101/2025.03.21.644499

75. Vilarinho L, Leão E, Barbot C, Santos M, Rocha H, Santorelli FM. Clinical and molecular studies in three portuguese mtdna t8993g families. *Pediatr Neurol*. 2000;22(1):29-32. doi:10.1016/S0887-8994(99)00113-7

76. Björkman K, Vissing J, Østergaard E, et al. Phenotypic spectrum and clinical course of single large-scale mitochondrial DNA deletion disease in the paediatric population: a multicentre study. *J Med Genet*. 2023;60(1):65-73. doi:10.1136/jmedgenet-2021-108006

77. Broomfield A, Sweeney MG, Woodward CE, et al. Paediatric single mitochondrial DNA deletion disorders: an overlapping spectrum of disease. *J Inherit Metab Dis*. 2015;38(3):445-457. doi:10.1007/s10545-014-9778-4

78. Rubio-Gozalbo ME, Dijkman KP, Van Den Heuvel LP, Sengers RCA, Wendel U, Smeitink JAM. Clinical differences in patients with mitochondriocytopathies due to nuclear versus mitochondrial DNA mutations. *Hum Mutat*. 2000;15(6):522-532. doi:10.1002/1098-1004(200006)15:6<522::AID-HUMU4>3.0.CO;2-Y

79. Zhang H, Burr SP, Chinnery PF. The mitochondrial DNA genetic bottleneck: inheritance and beyond. Garone C, Minczuk M, eds. *Essays Biochem*. 2018;62(3):225-234. doi:10.1042/EBC20170096

80. Chandrasegaram R, Hynes-Allen AM, Gao B, et al. Single-molecule mitochondrial DNA imaging reveals heteroplasmy dynamics shaped by developmental bottlenecks and selection in different organs *in vivo*. *Developmental Biology*. Preprint posted online January 25, 2025. doi:10.1101/2025.01.24.634671

81. Paiva Coelho M, Martins E, Vilarinho L. Diagnosis, management, and follow-up of mitochondrial disorders in childhood: a personalized medicine in the new era of genome sequence. *Eur J Pediatr*. 2019;178(1):21-32. doi:10.1007/s00431-018-3292-x

82. Grady JP, Pickett SJ, Ng YS, et al. mt DNA heteroplasmy level and copy number indicate disease burden in m.3243A>G mitochondrial disease. *EMBO Mol Med*. 2018;10(6):e8262. doi:10.15252/emmm.201708262

83. Lai M, Kim K, Zheng Y, et al. Epigenome-wide Association Analysis of Mitochondrial Heteroplasmy Provides Insight into Molecular Mechanisms of Disease. *Epidemiology*. Preprint posted online December 8, 2024. doi:10.1101/2024.12.05.24318557

84. Gupta N, Yadav DK, Gautam S, Kumar A, Kumar D, Prasad N. Nuclear Magnetic Resonance-Based Metabolomics Approach Revealed the Intervention Effect of Using Complementary and Alternative Medicine (CAM) by CKD Patients. *ACS Omega*. 2023;8(8):7722-7737. doi:10.1021/acsomega.2c06469

85. Morgenstern M, Peikert CD, Lübbert P, et al. Quantitative high-confidence human mitochondrial proteome and its dynamics in cellular context. *Cell Metab*. 2021;33(12):2464-2483.e18. doi:10.1016/j.cmet.2021.11.001

86. Rath S, Sharma R, Gupta R, et al. MitoCarta3.0: an updated mitochondrial proteome now with sub-organelle localization and pathway annotations. *Nucleic Acids Res*. 2021;49(D1):D1541-D1547. doi:10.1093/nar/gkaa1011

87. Boczonadi V, Horvath R. Mitochondria: Impaired mitochondrial translation in human disease. *Int J Biochem Cell Biol*. 2014;48:77-84. doi:10.1016/j.biocel.2013.12.011

88. Pearce S, Nezich CL, Spinazzola A. Mitochondrial diseases: Translation matters. *Mol Cell Neurosci*. 2013;55:1-12. doi:10.1016/j.mcn.2012.08.013

89. Antolínez-Fernández Á, Esteban-Ramos P, Fernández-Moreno MÁ, Clemente P. Molecular pathways in mitochondrial disorders due to a defective mitochondrial protein synthesis. *Front Cell Dev Biol*. 2024;12:1410245. doi:10.3389/fcell.2024.1410245

90. Messina M, Vaz FM, Rahman S. Mitochondrial membrane synthesis, remodelling and cellular trafficking. *J Inherit Metab Dis*. 2025;48(1):e12766. doi:10.1002/jimd.12766

91. El-Hattab AW, Craigen WJ, Scaglia F. Mitochondrial DNA maintenance defects. *Biochim Biophys Acta BBA - Mol*

*Basis Dis*. 2017;1863(6):1539-1555. doi:10.1016/j.bbadis.2017.02.017

92. Allkanjari K, Baldock RA. Beyond base excision repair: an evolving picture of mitochondrial DNA repair. *Biosci Rep*. 2021;41(10):BSR20211320. doi:10.1042/BSR20211320

93. Rahman S. Mitochondrial disease in children. *J Intern Med*. 2020;287(6):609-633. doi:10.1111/joim.13054

94. 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):753-759. doi:10.1002/ana.24362

95. Schaefer AM, McFarland R, Blakely EL, et al. Prevalence of mitochondrial DNA disease in adults. *Ann Neurol*. 2008;63(1):35-39. doi:10.1002/ana.21217

96. Bellusci M, Paredes-Fuentes AJ, Ruiz-Pesini E, et al. The Genetic Landscape of Mitochondrial Diseases in Spain: A Nationwide Call. *Genes*. 2021;12(10):1590. doi:10.3390/genes12101590

97. Schaefer AM, Taylor RW, Turnbull DM, Chinnery PF. The epidemiology of mitochondrial disorders—past, present and future. *Biochim Biophys Acta BBA - Bioenerg*. 2004;1659(2-3):115-120. doi:10.1016/j.bbabio.2004.09.005

98. Tauchmannová K, Pecinová A, Houštěk J, Mráček T. Variability of Clinical Phenotypes Caused by Isolated Defects of Mitochondrial ATP Synthase. *Physiol Res*. 2024;(Suppl 1):S243-S278. doi:10.33549/physiolres.935407

99. O'Keefe H, Queen RA, Meldau S, Lord P, Elson JL. Haplogroup Context is Less Important in the Penetrance of Mitochondrial DNA Complex I Mutations Compared to mt-tRNA Mutations. *J Mol Evol*. 2018;86(6):395-403. doi:10.1007/s00239-018-9855-7

100. Mavraki E, Labrum R, Sergeant K, et al. Genetic testing for mitochondrial disease: the United Kingdom best practice guidelines. *Eur J Hum Genet*. 2023;31(2):148-163. doi:10.1038/s41431-022-01249-w

101. Macken WL, Falabella M, Pizzamiglio C, et al. Enhanced mitochondrial genome analysis: bioinformatic and long-read sequencing advances and their diagnostic implications. *Expert Rev Mol Diagn*. 2023;23(9):797-814. doi:10.1080/14737159.2023.2241365

102. Citrigno L, Qualtieri A, Cerantonio A, et al. Genomics landscape of mitochondrial DNA variations in patients from South Italy affected by mitochondriopathies. *J Neurol Sci*. 2024;457:122869. doi:10.1016/j.jns.2024.122869

103. Damas J, Samuels DC, Carneiro J, Amorim A, Pereira F. Mitochondrial DNA Rearrangements in Health and Disease-A Comprehensive Study. *Hum Mutat*. 2014;35(1):1-14. doi:10.1002/humu.22452

104. Chen T, He J, Huang Y, Zhao W. The generation of mitochondrial DNA large-scale deletions in human cells. *J Hum Genet*. 2011;56(10):689-694. doi:10.1038/jhg.2011.97

105. Brockington M, Alsanjari N, Sweeney MG, Morgan-Hughes JA, Scaravilli F, Harding AE. Kearns-Sayre syndrome associated with mitochondrial DNA deletion or duplication: a molecular genetic and pathological study. *J Neurol Sci*. 1995;131(1):78-87. doi:10.1016/0022-510X(95)00091-F

106. Sabella-Jiménez V, Otero-Herrera C, Silvera-Redondo C, Garavito-Galofre P. Mitochondrial DNA deletion and duplication in Kearns–Sayre Syndrome (KSS) with initial presentation as Pearson Marrow-Pancreas Syndrome (PMPS): Two case reports in Barranquilla, Colombia. *Mol Genet Genomic Med*. 2020;8(11):e1509. doi:10.1002/mgg3.1509

107. Wang J, Peterson JT, Santos JDD, et al. Interpreting the clinical significance of multiple large-scale mitochondrial DNA deletions (MLSMD) in skeletal muscle tissue in the diagnostic evaluation of primary mitochondrial disease. *Front Pharmacol*. 2025;16:1507493. doi:10.3389/fphar.2025.1507493

108. Nesbitt V, Pitceathly RDS, Turnbull DM, et al. The UK MRC Mitochondrial Disease Patient Cohort Study: clinical phenotypes associated with the m.3243A>G mutation--implications for diagnosis and management. *J Neurol Neurosurg Psychiatry*. 2013;84(8):936-938. doi:10.1136/jnnp-2012-303528

109. Thompson K, Collier JJ, Glasgow RIC, et al. Recent advances in understanding the molecular genetic basis of mitochondrial disease. *J Inherit Metab Dis*. 2020;43(1):36-50. doi:10.1002/jimd.12104

110. Frazier AE, Thorburn DR, Compton AG. Mitochondrial energy generation disorders: genes, mechanisms, and clues to pathology. *J Biol Chem*. 2019;294(14):5386-5395. doi:10.1074/jbc.R117.809194

111. Fernandez-Vizarra E, Zeviani M. Mitochondrial disorders of the OXPHOS system. *FEBS Lett*. 2021;595(8):1062-1106. doi:10.1002/1873-3468.13995

112. Rahman S, Poulton J. Diagnosis of mitochondrial DNA depletion syndromes. *Arch Dis Child*. 2009;94(1):3-5. doi:10.1136/adc.2008.147983

113. El-Hattab AW, Scaglia F. Mitochondrial DNA Depletion Syndromes: Review and Updates of Genetic Basis, Manifestations, and Therapeutic Options. *Neurotherapeutics*. 2013;10(2):186-198. doi:10.1007/s13311-013-0177-6

114. Blok MJ, Van Den Bosch BJ, Jongen E, et al. The unfolding clinical spectrum of POLG mutations. *J Med Genet*. 2009;46(11):776-785. doi:10.1136/jmg.2009.067686

115. Bekheirnia MR, Zhang W, Eble T, et al. POLG mutation in a patient with cataracts, early-onset distal muscle weakness and atrophy, ovarian dysgenesis and 3-methylglutaconic aciduria. *Gene*. 2012;499(1):209-212. doi:10.1016/j.gene.2012.02.034

116. Hikmat, Omar. *The Phenotypic Spectrum of Polymerase Gamma (POLG) Disease from Birth to Late Adulthood*. 2020.

117. Dalla Rosa I, Cámara Y, Durigon R, et al. MPV17 Loss Causes Deoxynucleotide Insufficiency and Slow DNA Replication in Mitochondria. Larsson NG, ed. *PLOS Genet*. 2016;12(1):e1005779. doi:10.1371/journal.pgen.1005779

118. Del Greco C, Antonellis A. The Role of Nuclear-Encoded Mitochondrial tRNA Charging Enzymes in Human Inherited Disease. *Genes*. 2022;13(12):2319. doi:10.3390/genes13122319

119. McCormick EM, Zolkipli-Cunningham Z, Falk MJ. Mitochondrial disease genetics update: recent insights into the molecular diagnosis and expanding phenotype of primary mitochondrial disease. *Curr Opin Pediatr*. 2018;30(6):714-724. doi:10.1097/MOP.0000000000000686

120. Schlieben LD, Prokisch H. The Dimensions of Primary Mitochondrial Disorders. *Front Cell Dev Biol*. 2020;8:600079. doi:10.3389/fcell.2020.600079

121. Ferreira CR, Rahman S, Keller M, Zschocke J, ICIMD Advisory Group. An international classification of inherited metabolic disorders ( ICIMD ). *J Inherit Metab Dis*. 2021;44(1):164-177. doi:10.1002/jimd.12348

122. Morava E, Kozicz T, Wallace DC. The phenotype modifier: is the mitochondrial DNA background responsible for individual differences in disease severity. *J Inherit Metab Dis*. 2019;42(1):3-4. doi:10.1002/jimd.12050

123. Parikh S, Goldstein A, Koenig MK, et al. Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med*. 2015;17(9):689-701. doi:10.1038/gim.2014.177

124. Kim JH, Kim D, Hwang S, et al. Endocrine manifestations and long-term outcomes of patients with mitochondrial diseases. *Orphanet J Rare Dis*. 2025;20(1):235. doi:10.1186/s13023-025-03773-6

125. Zhang X, Zhang B, Tao Z, Liang J. Mitochondrial disease and epilepsy in children. *Front Neurol*. 2025;15:1499876. doi:10.3389/fneur.2024.1499876

126. Finsterer J. The Spectrum of Renal Abnormalities in Mitochondrial Disorders Is Broad. *Kidney Int Rep*. 2022;7(7):1722. doi:10.1016/j.ekir.2022.05.014

127. Gonçalves FG, Alves CAPF, Heuer B, et al. Primary Mitochondrial Disorders of the Pediatric Central Nervous System: Neuroimaging Findings. *RadioGraphics*. 2020;40(7):2042-2067. doi:10.1148/rg.2020200052

128. Huisman TAGM, Kralik SF, Desai NK, Serrallach BL, Orman G. Neuroimaging of primary mitochondrial disorders in children: A review. *J Neuroimaging*. 2022;32(2):191-200. doi:10.1111/jon.12976

129. Viering DHHM, Vermeltfoort L, Bindels RJM, Deinum J, De Baaij JHF. Electrolyte Disorders in Mitochondrial Cytopathies: A Systematic Review. *J Am Soc Nephrol*. 2023;34(11):1875-1888. doi:10.1681/ASN.0000000000000224

130. Debray FG, Lambert M, Chevalier I, et al. Long-term Outcome and Clinical Spectrum of 73 Pediatric Patients With Mitochondrial Diseases. *Pediatrics*. 2007;119(4):722-733. doi:10.1542/peds.2006-1866

131. Haas RH, Parikh S, Falk MJ, et al. Mitochondrial Disease: A Practical Approach for Primary Care Physicians. *Pediatrics*. 2007;120(6):1326-1333. doi:10.1542/peds.2007-0391

132. Boenzi S, Diodato D. Biomarkers for mitochondrial energy metabolism diseases. Garone C, Minczuk M, eds. *Essays Biochem*. 2018;62(3):443-454. doi:10.1042/EBC20170111

133. Ashfaq M, Moats AR, Northrup H, et al. Hypoglycemia in mitochondrial disorders. *Mitochondrion*. 2021;58:179-183. doi:10.1016/j.mito.2021.03.002

134. Ambrose A, Bahl S, Sharma S, et al. Genetic landscape of primary mitochondrial diseases in children and adults using molecular genetics and genomic investigations of mitochondrial and nuclear genome. *Orphanet J Rare Dis*. 2024;19(1):424. doi:10.1186/s13023-024-03437-x

135. Zhao Y, Zhao X, Ji K, et al. The clinical and genetic spectrum of mitochondrial diseases in China: A multicenter retrospective cross-sectional study. *Clin Genet*. 2024;106(6):733-744. doi:10.1111/cge.14605

136. 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. doi:10.3389/fgene.2018.00400

137. Mancuso M, Orsucci D, Angelini C, et al. Redefining phenotypes associated with mitochondrial DNA single deletion. *J Neurol*. 2015;262(5):1301-1309. doi:10.1007/s00415-015-7710-y

138. Emmanuele V, Ganesh J, Vladutiu G, et al. Time to harmonize mitochondrial syndrome nomenclature and classification: A consensus from the North American Mitochondrial Disease Consortium (NAMDC). *Mol Genet Metab*. 2022;136(2):125-131. doi:10.1016/j.ymgme.2022.05.001

139. McCormick EM, Keller K, Taylor JP, et al. Expert Panel Curation of 113 Primary Mitochondrial Disease Genes for the Leigh Syndrome Spectrum. *Ann Neurol*. 2023;94(4):696-712. doi:10.1002/ana.26716

140. Mancuso M, Lopriore P, Semmler L, Kornblum C. 280th ENMC International Workshop: The ERN EURO-NMD mitochondrial diseases working group Diagnostic criteria and outcome measures in primary mitochondrial myopathies. Hoofddorp, The Netherlands, 22-24 November 2024. *Neuromuscul Disord*. Published online March 2025:105340. doi:10.1016/j.nmd.2025.105340

141. Keshavan N, Rahman S. Natural history of mitochondrial disorders: a systematic review. Garone C, Minczuk M, eds. *Essays Biochem*. 2018;62(3):423-442. doi:10.1042/EBC20170108

142. Baldo MS, Azevedo L, Coelho MP, Martins E, Vilarinho L. A Comprehensive Approach to the Diagnosis of Leigh Syndrome Spectrum. *Diagnostics*. 2024;14(19):2133. doi:10.3390/diagnostics14192133

143. Nogueira C, Pereira C, Silva L, et al. The genetic landscape of mitochondrial diseases in the next-generation sequencing era: a Portuguese cohort study. *Front Cell Dev Biol*. 2024;12:1331351. doi:10.3389/fcell.2024.1331351

144. Thorburn DR, Smeitink J. Diagnosis of mitochondrial disorders: Clinical and biochemical approach. *J Inherit Metab Dis*. 2001;24(2):312-316. doi:10.1023/A:1010347808082

145. Shayota BJ. Biomarkers of mitochondrial disorders. *Neurotherapeutics*. 2024;21(1):e00325. doi:10.1016/j.neurot.2024.e00325

146. Chiaramello A. Mitochondrial Respiratory Disorders: A Perspective on their Metabolite Biomarkers and Implications for Clinical Diagnosis and Therapeutic Intervention. *Biomark J*. 2015;1(1).

147. Varhaug KN, Hikmat O, Nakkestad HL, Vedeler CA, Bindoff LA. Serum biomarkers in primary mitochondrial disorders. *Brain Commun*. 2021;3(1):fcaa222. doi:10.1093/braincomms/fcaa222

148. Morava E, Hogeveen M, De Vries M, Ruitenbeek W, De Boode WP, Smeitink J. Normal Serum Alanine Concentration Differentiates Transient Neonatal Lactic Acidemia from an Inborn Error of Energy Metabolism. *Neonatology*. 2006;90(3):207-209. doi:10.1159/000093590

149. Ferguson BS, Rogatzki MJ, Goodwin ML, Kane DA, Rightmire Z, Gladden LB. Lactate metabolism: historical

context, prior misinterpretations, and current understanding. *Eur J Appl Physiol*. 2018;118(4):691-728. doi:10.1007/s00421-017-3795-6

150. Wolf NI, Smeitink JAM. Mitochondrial disorders: A proposal for consensus diagnostic criteria in infants and children. *Neurology*. 2002;59(9):1402-1405. doi:10.1212/01.WNL.0000031795.91814.D8

151. Debray FG, Lambert M, Allard P, Mitchell GA. Low Citrulline in Leigh Disease: Still a Biomarker of Maternally Inherited Leigh Syndrome. *J Child Neurol*. 2010;25(8):1000-1002. doi:10.1177/0883073809351983

152. Mori M, Mytinger JR, Martin LC, Bartholomew D, Hickey S. m.8993T>G-Associated Leigh Syndrome with Hypocitrullinemia on Newborn Screening. Zschocke J, Gibson KM, Brown G, Morava E, Peters V, eds. *JIMD Rep*. 2014;17:47-51. doi:10.1007/8904_2014_332

153. Cynober L. Citrulline: just a biomarker or a conditionally essential amino acid and a pharmaconutrient in critically ill patients? *Crit Care*. 2013;17(2):122. doi:10.1186/cc12534

154. Atkuri KR, Cowan TM, Kwan T, et al. Inherited disorders affecting mitochondrial function are associated with glutathione deficiency and hypocitrullinemia. *Proc Natl Acad Sci*. 2009;106(10):3941-3945. doi:10.1073/pnas.0813409106

155. Dimmock DP, Zhang Q, Dionisi-Vici C, et al. Clinical and molecular features of mitochondrial DNA depletion due to mutations in deoxyguanosine kinase. *Hum Mutat*. 2008;29(2):330-331. doi:10.1002/humu.9519

156. Leanza L, Ferraro P, Reichard P, Bianchi V. Metabolic Interrelations within Guanine Deoxynucleotide Pools for Mitochondrial and Nuclear DNA Maintenance. *J Biol Chem*. 2008;283(24):16437-16445. doi:10.1074/jbc.M801572200

157. El-Hattab AW; Scaglia F. Deoxyguanosine Kinase Deficiency. In: *GeneReviews® [Internet]*. Adam MP; Feldman J; Mirzaa GM; et al. GeneReviews. University of Washington, Seattle; 2009. https://www.ncbi.nlm.nih.gov/books/NBK7040/

158. Majdalani M, Yazbeck N, El Harake L, Samaha J, Karam PE. Mitochondrial depletion syndrome type 3: the Lebanese variant. *Front Genet*. 2023;14:1215083. doi:10.3389/fgene.2023.1215083

159. Wortmann SB, Duran M, Anikster Y, et al. Inborn errors of metabolism with 3-methylglutaconic aciduria as discriminative feature: proper classification and nomenclature. *J Inherit Metab Dis*. 2013;36(6):923-928. doi:10.1007/s10545-012-9580-0

160. Suomalainen A. Fibroblast growth factor 21: a novel biomarker for human muscle-manifesting mitochondrial disorders. *Expert Opin Med Diagn*. 2013;7(4):313-317. doi:10.1517/17530059.2013.812070

161. Wesół-Kucharska D, Rokicki D, Greczan M, et al. The fibroblast growth factor 21 concentration in children with mitochondrial disease does not depend on the disease stage, but rather on the disease genotype. *Pediatr Endocrinol Diabetes Metab*. 2022;28(2):141-151. doi:10.5114/pedm.2022.116116

162. Davis RL, Wong SL, Carling PJ, Payne T, Sue CM, Bandmann O. Serum FGF-21, GDF-15, and blood mtDNA copy number are not biomarkers of Parkinson disease. *Neurol Clin Pract*. 2020;10(1):40-46. doi:10.1212/CPJ.0000000000000702

163. Van Hove JLK, Friederich MW, Strode DK, et al. Protein biomarkers GDF15 and FGF21 to differentiate mitochondrial hepatopathies from other pediatric liver diseases. *Hepatol Commun*. 2024;8(1):e0361. doi:10.1097/HC9.0000000000000361

164. Lin Y, Ji K, Ma X, et al. Accuracy of FGF-21 and GDF-15 for the diagnosis of mitochondrial disorders: A meta-analysis. *Ann Clin Transl Neurol*. 2020;7(7):1204-1213. doi:10.1002/acn3.51104

165. Sofou K, Shahim P, Tulinius M, et al. Cerebrospinal fluid neurofilament light is associated with survival in mitochondrial disease patients. *Mitochondrion*. 2019;46:228-235. doi:10.1016/j.mito.2018.07.002

166. Zheng YS, Sun C, Wang R, et al. Neurofilament light is a novel biomarker for mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes. *Sci Rep*. 2021;11(1):2001. doi:10.1038/s41598-021-81721-7

167. Thomas S, Khan M, Sari MC, et al. Neurofilament Light Chain Levels in a Large Idiopathic Peripheral Neuropathy Cohort. *J Peripher Nerv Syst*. 2025;30(3):e70050. doi:10.1111/jns.70050

168. El-Hattab AW, Scaglia F. Mitochondrial DNA Depletion Syndromes: Review and Updates of Genetic Basis, Manifestations, and Therapeutic Options. *Neurotherapeutics*. 2013;10(2):186-198. doi:10.1007/s13311-013-0177-6

169. Yubero D, Allen G, Artuch R, Montero R. The Value of Coenzyme Q10 Determination in Mitochondrial Patients. *J Clin Med*. 2017;6(4):37. doi:10.3390/jcm6040037

170. Paredes-Fuentes AJ, Montero R, Codina A, et al. Coenzyme Q10 Treatment Monitoring in Different Human Biological Samples. *Antioxidants*. 2020;9(10):979. doi:10.3390/antiox9100979

171. Wong SSN, Goraj B, Fung CW, et al. Radboud Centre for Mitochondrial Medicine Pediatric MRI score. *Mitochondrion*. 2017;32:36-41. doi:10.1016/j.mito.2016.11.008

172. Alves CAPF, Teixeira SR, Martin-Saavedra JS, et al. Pediatric Leigh Syndrome: Neuroimaging Features and Genetic Correlations. *Ann Neurol*. 2020;88(2):218-232. doi:10.1002/ana.25789

173. Paprocka J, Machnikowska-Sokołowska M, Gruszczyńska K, Emich-Widera E. Neuroimaging of Basal Ganglia in Neurometabolic Diseases in Children. *Brain Sci*. 2020;10(11):849. doi:10.3390/brainsci10110849

174. Yatsuga S, Povalko N, Nishioka J, et al. MELAS: A nationwide prospective cohort study of 96 patients in Japan. *Biochim Biophys Acta BBA - Gen Subj*. 2012;1820(5):619-624. doi:10.1016/j.bbagen.2011.03.015

175. Lai LM, Gropman AL, Whitehead MT. MR Neuroimaging in Pediatric Inborn Errors of Metabolism. *Diagnostics*. 2022;12(4):861. doi:10.3390/diagnostics12040861

176. Steenweg ME, Ghezzi D, Haack T, et al. Leukoencephalopathy with thalamus and brainstem involvement and high lactate 'LTBL' caused by EARS2 mutations. *Brain*. 2012;135(5):1387-1394. doi:10.1093/brain/aws070

177. Roux CJ, Barcia G, Schiff M, et al. Phenotypic diversity of brain MRI patterns in mitochondrial aminoacyl-tRNA synthetase mutations. *Mol Genet Metab*. 2021;133(2):222-229. doi:10.1016/j.ymgme.2021.04.004

178. Walker MA, Miranda M, Allred A, Mootha VK. On the dynamic and even reversible nature of Leigh syndrome: Lessons from human imaging and mouse models. *Curr Opin Neurobiol*. 2022;72:80-90. doi:10.1016/j.conb.2021.09.006

179. Senthilvelan S, Sekar SS, Kesavadas C, Thomas B. Neuromitochondrial Disorders: Genomic Basis and an Algorithmic Approach to Imaging Diagnostics. *Clin Neuroradiol*. 2021;31(3):559-574. doi:10.1007/s00062-021-01030-4

180. Mohammad SS, Angiti RR, Biggin A, et al. Magnetic resonance imaging pattern recognition in childhood bilateral basal ganglia disorders. *Brain Commun*. 2020;2(2):fcaa178. doi:10.1093/braincomms/fcaa178

181. GoMori G. A Rapid One-Step Trichrome Stain*. *Am J Clin Pathol*. 1950;20(7_ts):661-664. doi:10.1093/ajcp/20.7_ts.661

182. Bernier FP, Boneh A, Dennett X, Chow CW, Cleary MA, Thorburn DR. Diagnostic criteria for respiratory chain disorders in adults and children. *Neurology*. 2002;59(9):1406-1411. doi:10.1212/01.WNL.0000033795.17156.00

183. Challa S, Kanikannan MA, Jagarlapudi MMK, Bhoompally VR, Surath M. Diagnosis of mitochondrial diseases: Clinical and histological study of sixty patients with ragged red fibers. *Neurol India*. 2004;52(3):353-358.

184. Jou C, Ortigoza-Escobar JD, O'Callaghan MM, et al. Muscle Involvement in a Large Cohort of Pediatric Patients with Genetic Diagnosis of Mitochondrial Disease. *J Clin Med*. 2019;8(1):68. doi:10.3390/jcm8010068

185. Bourgeois JM, Tarnopolsky MA. Pathology of skeletal muscle in mitochondrial disorders. *Mitochondrion*. 2004;4(5-6):441-452. doi:10.1016/j.mito.2004.07.036

186. Rustin P, Chretien D, Bourgeron T, et al. Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta*. 1994;228(1):35-51. doi:10.1016/0009-8981(94)90055-8

187. Birchmachin MA, Briggs HL, Saborido AA, Bindoff LA, Turnbull DM. An Evaluation of the Measurement of the Activities of Complexes I-IV in the Respiratory Chain of Human Skeletal Muscle Mitochondria. *Biochem Med Metab Biol*. 1994;51(1):35-42. doi:10.1006/bmmb.1994.1004

188. Spinazzi M, Casarin A, Pertegato V, Salviati L, Angelini C. Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nat Protoc*. 2012;7(6):1235-1246. doi:10.1038/nprot.2012.058

189. Janssen AJ, Trijbels FJ, Sengers RC, et al. Measurement of the Energy-Generating Capacity of Human Muscle Mitochondria: Diagnostic Procedure and Application to Human Pathology. *Clin Chem*. 2006;52(5):860-871. doi:10.1373/clinchem.2005.062414

190. Wu M, Neilson A, Swift AL, et al. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am J Physiol-Cell Physiol*. 2007;292(1):C125-C136. doi:10.1152/ajpcell.00247.2006

191. Morten KJ, Ashley N, Wijburg F, et al. Liver mtDNA content increases during development: A comparison of methods and the importance of age- and tissue-specific controls for the diagnosis of mtDNA depletion. *Mitochondrion*. 2007;7(6):386-395. doi:10.1016/j.mito.2007.09.001

192. Poulton J, Sewry C, Potter CG, et al. Variation in mitochondrial DNA levels in muscle from normal controls. Is depletion of mtDNA in patients with mitochondrial myopathy a distinct clinical syndrome? *J Inherit Metab Dis*. 1995;18(1):4-20. doi:10.1007/BF00711367

193. O'Hara R, Tedone E, Ludlow A, et al. Quantitative mitochondrial DNA copy number determination using droplet digital PCR with single-cell resolution. *Genome Res*. 2019;29(11):1878-1888. doi:10.1101/gr.250480.119

194. Li M, Schröder R, Ni S, Madea B, Stoneking M. Extensive tissue-related and allele-related mtDNA heteroplasmy suggests positive selection for somatic mutations. *Proc Natl Acad Sci*. 2015;112(8):2491-2496. doi:10.1073/pnas.1419651112

195. Short KR, Bigelow ML, Kahl J, et al. Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci*. 2005;102(15):5618-5623. doi:10.1073/pnas.0501559102

196. Moraes CT, Shanske S, Bonilla FAE. mtDNA depletion with variable tissue expression: a novel genetic abnormality in mitochondrial diseases. *Am J Hum Genet*. Published online 1991.

197. Moraes CT, Atencio DP, Oca-Cossio J, Diaz F. Techniques and Pitfalls in the Detection of Pathogenic Mitochondrial DNA Mutations. *J Mol Diagn*. 2003;5(4):197-208. doi:10.1016/S1525-1578(10)60474-6

198. Conti F, Di Martino S, Drago F, et al. Red Flags in Primary Mitochondrial Diseases: What Should We Recognize? *Int J Mol Sci*. 2023;24(23):16746. doi:10.3390/ijms242316746

199. Walker UA, Collins S, Byrne E. Respiratory Chain Encephalomyopathies: A Diagnostic Classification. *Eur Neurol*. 1996;36(5):260-267. doi:10.1159/000117269

200. Morava E, Van Den Heuvel L, Hol F, et al. Mitochondrial disease criteria: Diagnostic applications in children. *Neurology*. 2006;67(10):1823-1826. doi:10.1212/01.wnl.0000244435.27645.54

201. Witters P, Saada A, Honzik T, et al. Revisiting mitochondrial diagnostic criteria in the new era of genomics. *Genet Med*. 2018;20(4):444-451. doi:10.1038/gim.2017.125

202. Ouyang X, Zhang Y, Zhang L, et al. Clinical Utility of Rapid Exome Sequencing Combined With Mitochondrial DNA Sequencing in Critically Ill Pediatric Patients With Suspected Genetic Disorders. *Front Genet*. 2021;12:725259. doi:10.3389/fgene.2021.725259

203. Gedikbasi A, Toksoy G, Karaca M, et al. Clinical and bi-genomic DNA findings of patients suspected to have mitochondrial diseases. *Front Genet*. 2023;14:1191159. doi:10.3389/fgene.2023.1191159

204. Wortmann SB, Koolen DA, Smeitink JA, Den Van Heuvel L, Rodenburg RJ. Whole exome sequencing of suspected mitochondrial patients in clinical practice. *J Inherit Metab Dis*. 2015;38(3):437-443. doi:10.1007/s10545-015-9823-y

205. Puusepp S, Reinson K, Pajusalu S, et al. Effectiveness of whole exome sequencing in unsolved patients with a clinical suspicion of a mitochondrial disorder in Estonia. *Mol Genet Metab Rep*. 2018;15:80-89. doi:10.1016/j.ymgmr.2018.03.004

206. Wagner M, Berutti R, Lorenz-Depiereux B, et al. Mitochondrial DNA mutation analysis from exome sequencing—A more holistic approach in diagnostics of suspected mitochondrial disease. *J Inherit Metab Dis*. 2019;42(5):909-917. doi:10.1002/jimd.12109

207. Mancuso M, Lopriore P, Lamperti C, et al. Current management of primary mitochondrial disorders in EU countries: the European Reference Networks survey. *J Neurol*. 2024;271(2):835-840. doi:10.1007/s00415-023-12017-1

208. Lieber DS, Calvo SE, Shanahan K, et al. Targeted exome sequencing of suspected mitochondrial disorders. *Neurology*. 2013;80(19):1762-1770. doi:10.1212/WNL.0b013e3182918c40

209. Schon KR, Horvath R, Wei W, et al. Use of whole genome sequencing to determine genetic basis of suspected mitochondrial disorders: cohort study. *BMJ*. Published online November 3, 2021:e066288. doi:10.1136/bmj-2021-066288

210. Forny P, Footitt E, Davison JE, et al. Diagnosing Mitochondrial Disorders Remains Challenging in the Omics Era. *Neurol Genet*. 2021;7(3):e597. doi:10.1212/NXG.0000000000000597

211. Salpietro V, Phadke R, Saggar A, et al. Zellweger syndrome and secondary mitochondrial myopathy. *Eur J Pediatr*. 2015;174(4):557-563. doi:10.1007/s00431-014-2431-2

212. Gardeitchik T, Wyckmans J, Morava E. Complex Phenotypes in Inborn Errors of Metabolism: Overlapping Presentations in Congenital Disorders of Glycosylation and Mitochondrial Disorders. *Pediatr Clin North Am*. 2018;65(2):375-388. doi:10.1016/j.pcl.2017.11.012

213. Martikainen MH, Chinnery PF. Mitochondrial disease: mimics and chameleons. *Pract Neurol*. 2015;15(6):424-435. doi:10.1136/practneurol-2015-001191

214. Keogh MJ, Daud D, Pyle A, et al. A novel de novo STXBP1 mutation is associated with mitochondrial complex I deficiency and late-onset juvenile-onset parkinsonism. *neurogenetics*. 2015;16(1):65-67. doi:10.1007/s10048-014-0431-z

215. Wortmann SB, Oud MM, Alders M, et al. How to proceed after “negative” exome: A review on genetic diagnostics, limitations, challenges, and emerging new multiomics techniques. *J Inherit Metab Dis*. 2022;45(4):663-681. doi:10.1002/jimd.12507

216. Schobers G, Schieving JH, Yntema HG, et al. Reanalysis of exome negative patients with rare disease: a pragmatic workflow for diagnostic applications. *Genome Med*. 2022;14(1):66. doi:10.1186/s13073-022-01069-z

217. Arbide D, Elkhateeb N, Goljan E, Gonzalez CP, Maw A, Park SM. A Novel Heterozygous De Novo MORC2 Missense Variant Causes an Early Onset and Severe Neurodevelopmental Disorder. Mittal B, ed. *Case Rep Genet*. 2024;2024:1-5. doi:10.1155/2024/5906936

218. Hikmat O, Naess K, Engvall M, et al. Simplifying the clinical classification of polymerase gamma (POLG) disease based on age of onset; studies using a cohort of 155 cases. *J Inherit Metab Dis*. 2020;43(4):726-736. doi:10.1002/jimd.12211

219. Beecher G, Gavrilova RH, Mandrekar J, Naddaf E. Mitochondrial myopathies diagnosed in adulthood: clinico-genetic spectrum and long-term outcomes. *Brain Commun*. 2024;6(2):fcae041. doi:10.1093/braincomms/fcae041

220. Ivaniuk A, Anselm IA, Bowen A, et al. Characterization of Factors Associated With Death in Deceased Patients With Mitochondrial Disorders: A Multicenter Cross-Sectional Survey. *Neurology*. 2025;104(4):e209779. doi:10.1212/WNL.0000000000209779

221. Keshavan N, Abdenur J, Anderson G, et al. The natural history of infantile mitochondrial DNA depletion syndrome due to RRM2B deficiency. *Genet Med*. 2020;22(1):199-209. doi:10.1038/s41436-019-0613-z

222. Whittaker RG, Blackwood JK, Alston CL, et al. URINE HETEROPLASMY IS THE BEST PREDICTOR OF CLINICAL OUTCOME IN THE m.3243A>G mtDNA MUTATION. *Neurology*. 2009;72(6):568-569. doi:10.1212/01.wnl.0000342121.91336.4d

223. Karaa A, Bertini E, Carelli V, et al. Genotype-specific effects of elamipretide in patients with primary mitochondrial myopathy: a post hoc analysis of the MMPOWER-3 trial. *Orphanet J Rare Dis*. 2024;19(1):431. doi:10.1186/s13023-024-03421-5

224. Sofou K, De Coo IFM, Isohanni P, et al. A multicenter study on Leigh syndrome: disease course and predictors of survival. *Orphanet J Rare Dis*. 2014;9(1):52. doi:10.1186/1750-1172-9-52

225. Hikmat O, Charalampos T, Klingenberg C, et al. The presence of anaemia negatively influences survival in patients with POLG disease. *J Inherit Metab Dis*. 2017;40(6):861-866. doi:10.1007/s10545-017-0084-9

226. Parasyri M, Brandström P, Uusimaa J, et al. Renal Phenotype in Mitochondrial Diseases: A Multicenter Study. *Kidney Dis*. 2022;8(2):148-159. doi:10.1159/000521148

227. Scaglia F, Towbin JA, Craigen WJ, et al. Clinical Spectrum, Morbidity, and Mortality in 113 Pediatric Patients With Mitochondrial Disease. *Pediatrics*. 2004;114(4):925-931. doi:10.1542/peds.2004-0718

228. Lim AZ, Ng YS, Blain A, et al. Natural History of Leigh Syndrome: A Study of Disease Burden and Progression. *Ann Neurol*. 2022;91(1):117-130. doi:10.1002/ana.26260

229. Rosella L, Hurst M, Buajitti E, Samson T, Young LT, Andreazza AC. A population-based cohort study of mitochondrial disease and mental health conditions in Ontario, Canada. *Orphanet J Rare Dis*. 2025;20(177). doi:https://doi.org/10.1186/s13023-025-03688-2

230. Haque S, Crawley K, Davis R, Schofield D, Shrestha R, Sue CM. Clinical drivers of hospitalisation in patients with mitochondrial diseases. *BMJ Neurol Open*. 2024;6(1):e000717. doi:10.1136/bmjno-2024-000717

231. Zhao X, Yu M, Zhang W, Hou Y, Yuan Y, Wang Z. Demographic characteristics, diagnostic challenges, treatment patterns, and caregiver burden of mitochondrial diseases: a retrospective cross-sectional study. *Orphanet J Rare Dis*. 2024;19(1):287. doi:10.1186/s13023-024-03289-5

232. Goldstein A, Rahman S. Seeking impact: Global perspectives on outcome measure selection for translational and clinical research for primary mitochondrial disorders. *J Inherit Metab Dis*. 2021;44(2):343-357. doi:10.1002/jimd.12320

233. Karaa A, MacMullen LE, Campbell JC, et al. Community Consensus Guidelines to Support FAIR Data Standards in Clinical Research Studies in Primary Mitochondrial Disease. *Adv Genet*. 2022;3(1):2100047. doi:10.1002/ggn2.202100047

234. Sue CM, Balasubramaniam S, Bratkovic D, et al. Patient care standards for primary mitochondrial disease in Australia: an Australian adaptation of the Mitochondrial Medicine Society recommendations. *Intern Med J*. 2022;52(1):110-120. doi:10.1111/imj.15505

235. Parikh S, Goldstein A, Karaa A, et al. Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. *Genet Med*. 2017;19(12):1380-1397. doi:10.1038/gim.2017.107

236. Poulton J, Finsterer J, Yu-Wai-Man P. Genetic Counselling for Maternally Inherited Mitochondrial Disorders. *Mol Diagn Ther*. 2017;21(4):419-429. doi:10.1007/s40291-017-0279-7

237. Paleologou E, Ismayilova N, Kinali M. Use of the Ketogenic Diet to Treat Intractable Epilepsy in Mitochondrial Disorders. *J Clin Med*. 2017;6(6):56. doi:10.3390/jcm6060056

238. Wesół-Kucharska D, Greczan M, Kaczor M, et al. Efficacy and Safety of Ketogenic Diet Treatment in Pediatric Patients with Mitochondrial Disease. *Nutrients*. 2024;16(6):812. doi:10.3390/nu16060812

239. Houghton D, Ng YS, Jackson MA, et al. Phase II Feasibility Study of the Efficacy, Tolerability, and Impact on the Gut Microbiome of a Low-Residue (Fiber) Diet in Adult Patients With Mitochondrial Disease. *Gastro Hep Adv*. 2022;1(4):666-677. doi:10.1016/j.gastha.2022.03.007

240. Haas RH, Barshop BA. Diet change in the management of metabolic encephalomyopathies. *BioFactors*. 1998;7(3):259-262. doi:10.1002/biof.5520070323

241. Panetta J, Smith LJ, Boneh A. Effect of high-dose vitamins, coenzyme Q and high-fat diet in paediatric patients with mitochondrial diseases. *J Inherit Metab Dis*. 2004;27(4):487-498. doi:10.1023/B:BOLI.0000037354.66587.38

242. Willemse L, Terburgh K, Louw R. The therapeutic potential of a polyunsaturated fatty acid-enriched high-fat diet in Leigh syndrome: Insights from a preclinical model. *Biochim Biophys Acta BBA - Mol Basis Dis*. 2025;1871(6):167873. doi:10.1016/j.bbadis.2025.167873

243. Murphy JL, Blakely EL, Schaefer AM, et al. Resistance training in patients with single, large-scale deletions of mitochondrial DNA. *Brain*. 2008;131(11):2832-2840. doi:10.1093/brain/awn252

244. Arribat Y, Broskey NT, Greggio C, et al. Distinct patterns of skeletal muscle mitochondria fusion, fission and mitophagy upon duration of exercise training. *Acta Physiol*. 2019;225(2):e13179. doi:10.1111/apha.13179

245. Delaney NF, Sharma R, Tadvalkar L, Clish CB, Haller RG, Mootha VK. Metabolic profiles of exercise in patients with McArdle disease or mitochondrial myopathy. *Proc Natl Acad Sci*. 2017;114(31):8402-8407. doi:10.1073/pnas.1703338114

246. Halter JP, Michael W, Schüpbach M, et al. Allogeneic haematopoietic stem cell transplantation for mitochondrial neurogastrointestinal encephalomyopathy. *Brain*. 2015;138(10):2847-2858. doi:10.1093/brain/awv226

247. Hirano M, Carelli V, De Giorgio R, et al. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE): Position paper on diagnosis, prognosis, and treatment by the MNGIE International Network. *J Inherit Metab Dis*. 2021;44(2):376-387. doi:10.1002/jimd.12300

248. Kripps KimberlyA, Nakayuenyongsuk W, Shayota BJ, et al. Successful liver transplantation in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). *Mol Genet Metab*. 2020;130(1):58-64. doi:10.1016/j.ymgme.2020.03.001

249. Finsterer J. Survival from liver transplantation in mitochondrial disorders not only depends on periprocedural complications but also on the genotype and on mitochondrion-toxic drugs. *Pediatr Transplant*. 2021;25(8):e14137. doi:10.1111/petr.14137

250. Wei LY, Chen XQ, Huang L, Shan QW, Tang Q. Liver transplantation for mitochondrial DNA depletion syndrome caused by MPV17 deficiency: a case report and literature review. *Front Surg*. 2024;11:1348806. doi:10.3389/fsurg.2024.1348806

251. De Laat P, Van Engelen N, Wetzels JF, Smeitink JAM, Janssen MCH. Five non-mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes phenotype adult patients with m.3243A>G mutation after kidney transplantation: follow-up and review of the literature. *Clin Kidney J*. 2019;12(6):840-846. doi:10.1093/ckj/sfz020

252. Nishida H, Nawano T, Fukuhara H, et al. Outcomes of Living Kidney Transplantation for Mitochondrial Disease Patients: A Case Series. *Transplant Proc*. 2022;54(2):267-271. doi:10.1016/j.transproceed.2021.12.033

253. Li Y, Godown J, Taylor CL, Dipchand AI, Bowen VM, Feingold B. Favorable outcomes after heart transplantation in Barth syndrome. *J Heart Lung Transplant*. 2021;40(10):1191-1198. doi:10.1016/j.healun.2021.06.017

254. Parikh S, Karaa A, Goldstein A, et al. Solid organ transplantation in primary mitochondrial disease: Proceed with caution. *Mol Genet Metab*. 2016;118(3):178-184. doi:10.1016/j.ymgme.2016.04.009

255. Wahedi A, Sudhakar S, Lam A, et al. Clinical Features, Biochemistry, Imaging, and Treatment Response in a Single-Center Cohort With Coenzyme $Q_{10}$ Biosynthesis Disorders. *Neurol Genet*. 2024;10(6):e200209. doi:10.1212/NXG.0000000000200209

256. Glover EI, Martin J, Maher A, Thornhill RE, Moran GR, Tarnopolsky MA. A randomized trial of coenzyme $Q_{10}$ in mitochondrial disorders. *Muscle Nerve*. 2010;42(5):739-748. doi:10.1002/mus.21758

257. Neugebauer J, Reinson K, Bellusci M, et al. Current global vitamin and cofactor prescribing practices for primary mitochondrial diseases: Results of a European reference network survey. *J Inherit Metab Dis*. 2025;48(1):e12805. doi:10.1002/jimd.12805

258. Kuszak AJ, Espey MG, Falk MJ, et al. Nutritional Interventions for Mitochondrial OXPHOS Deficiencies: Mechanisms and Model Systems. *Annu Rev Pathol Mech Dis*. 2018;13(1):163-191. doi:10.1146/annurev-pathol-020117-043644

259. El-Hattab AW, Hsu JW, Emrick LT, et al. Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation. *Mol Genet Metab*. 2012;105(4):607-614. doi:10.1016/j.ymgme.2012.01.016

260. Al Jasmi F, Al Zaabi N, Al-Thihli K, Al Teneiji AM, Hertecant J, El-Hattab AW. Endothelial Dysfunction and the Effect of Arginine and Citrulline Supplementation in Children and Adolescents With Mitochondrial Diseases. *J Cent Nerv Syst Dis*. 2020;12:117957352090937. doi:10.1177/1179573520909377

261. Enns GM, Kinsman SL, Perlman SL, et al. Initial experience in the treatment of inherited mitochondrial disease with EPI-743. *Mol Genet Metab*. 2012;105(1):91-102. doi:10.1016/j.ymgme.2011.10.009

262. Pastore A, Petrillo S, Tozzi G, et al. Glutathione: A redox signature in monitoring EPI-743 therapy in children with mitochondrial encephalomyopathies. *Mol Genet Metab*. 2013;109(2):208-214. doi:10.1016/j.ymgme.2013.03.011

263. Smeitink J. Phase 2b program with sonlicromanol in patients with mitochondrial disease due to m.3243A>G mutation. Published online 2025. doi:https://doi.org/10.1093/brain/awae277

264. Seo KS, Kim JH, Min KN, et al. KL1333, a Novel NAD+ Modulator, Improves Energy Metabolism and Mitochondrial Dysfunction in MELAS Fibroblasts. *Front Neurol*. 2018;9:552. doi:10.3389/fneur.2018.00552

265. Pizzamiglio C, Stefanetti RJ, McFarland R, et al. Optimizing rare disorder trials: a phase 1a/1b randomized study of KL1333 in adults with mitochondrial disease. *Brain*. 2025;148(1):39-46. doi:10.1093/brain/awae308

266. Reid Thompson W, Hornby B, Manuel R, et al. A phase 2/3 randomized clinical trial followed by an open-label extension to evaluate the effectiveness of elamipretide in Barth syndrome, a genetic disorder of mitochondrial cardiolipin metabolism. *Genet Med*. 2021;23(3):471-478. doi:10.1038/s41436-020-01006-8

267. Karaa A, Bertini E, Carelli V, et al. Efficacy and Safety of Elamipretide in Individuals With Primary Mitochondrial Myopathy: The MMPOWER-3 Randomized Clinical Trial. *Neurology*. 2023;101(3). doi:10.1212/WNL.0000000000207402

268. Da Rosa-Junior NT, Parmeggiani B, Da Rosa MS, et al. Bezafibrate In Vivo Administration Prevents 3-Methylglutaric Acid-Induced Impairment of Redox Status, Mitochondrial Biogenesis, and Neural Injury in Brain of Developing Rats. *Neurotox Res*. 2019;35(4):809-822. doi:10.1007/s12640-019-00019-9

269. Steele H, Gomez-Duran A, Pyle A, et al. Metabolic effects of bezafibrate in mitochondrial disease. *EMBO Mol Med*. 2020;12(3):e11589. doi:10.15252/emmm.201911589

270. Løkken N, Khawajazada T, Storgaard JH, et al. No effect of resveratrol in patients with mitochondrial myopathy: A cross-over randomized controlled trial. *J Inherit Metab Dis*. 2021;44(5):1186-1198. doi:10.1002/jimd.12393

271. Domínguez-González C, Madruga-Garrido M, Mavillard F, et al. Deoxynucleoside Therapy for Thymidine Kinase 2–Deficient Myopathy. *Ann Neurol*. 2019;86(2):293-303. doi:10.1002/ana.25506

272. Domínguez-González C, Madruga-Garrido M, Hirano M, et al. Collaborative model for diagnosis and treatment of very rare diseases: experience in Spain with thymidine kinase 2 deficiency. *Orphanet J Rare Dis*. 2021;16(1):407. doi:10.1186/s13023-021-02030-w

273. Pekeles H, Berrahmoune S, Dassi C, et al. Safety and efficacy of deoxycytidine/deoxythymidine combination therapy in POLG-related disorders: 6-month interim results of an open-label, single arm, phase 2 trial. *eClinicalMedicine*. 2024;74:102740. doi:10.1016/j.eclinm.2024.102740

274. Biousse V, Newman NJ, Yu-Wai-Man P, et al. Long-Term Follow-Up After Unilateral Intravitreal Gene Therapy for Leber Hereditary Optic Neuropathy: The RESTORE Study. *J Neuroophthalmol*. 2021;41(3):309-315. doi:10.1097/WNO.0000000000001367

275. Ding Y, Ye Y, Li M, Xia B, Leng J. Mitochondrial tRNAAla 5601C>T variant may affect the clinical expression of the LHON-related ND4 11778G>A mutation in a family. *Mol Med Rep*. Published online November 22, 2019. doi:10.3892/mmr.2019.10844

276. Jacoby E, Bar-Yosef O, Gruber N, et al. Mitochondrial augmentation of hematopoietic stem cells in children with single large-scale mitochondrial DNA deletion syndromes. *Sci Transl Med*. 2022;14(676):eabo3724. doi:10.1126/scitranslmed.abo3724

277. Brestoff JR, Singh KK, Aquilano K, et al. Recommendations for mitochondria transfer and transplantation nomenclature and characterization. *Nat Metab*. 2025;7(1):53-67. doi:10.1038/s42255-024-01200-x

278. Thomas RH, Hunter A, Butterworth L, et al. Research priorities for mitochondrial disorders: Current landscape and patient and professional views. *J Inherit Metab Dis*. 2022;45(4):796-803. doi:10.1002/jimd.12521

279. Pfeffer G, Horvath R, Klopstock T, et al. New treatments for mitochondrial disease—no time to drop our standards. *Nat Rev Neurol*. 2013;9(8):474-481. doi:10.1038/nrneurol.2013.129

280. Sharma K, Karl B, Mathew AV, et al. Metabolomics Reveals Signature of Mitochondrial Dysfunction in Diabetic Kidney Disease. *J Am Soc Nephrol*. 2013;24(11):1901-1912. doi:10.1681/ASN.2013020126

281. Gooding JR, Agrawal S, McRitchie S, et al. Predicting and Defining Steroid Resistance in Pediatric Nephrotic Syndrome Using Plasma Metabolomics. *Kidney Int Rep*. 2020;5(1):81-93. doi:10.1016/j.ekir.2019.09.010

282. Esterhuizen K, Van Der Westhuizen FH, Louw R. Metabolomics of mitochondrial disease. *Mitochondrion*. 2017;35:97-110. doi:10.1016/j.mito.2017.05.012

283. Liu X, Xu G. Recent advances in using mass spectrometry for mitochondrial metabolomics and lipidomics - A review. *Anal Chim Acta*. 2018;1037:3-12. doi:10.1016/j.aca.2017.11.080

284. Kimura T, Yasuda K, Yamamoto R, et al. Identification of biomarkers for development of end-stage kidney disease in chronic kidney disease by metabolomic profiling. *Sci Rep*. 2016;6(1):26138. doi:10.1038/srep26138

285. Liu Y, Wang Y, Ding W, Wang Y. Mito-TEMPO Alleviates Renal Fibrosis by Reducing Inflammation, Mitochondrial Dysfunction, and Endoplasmic Reticulum Stress. Daiber A, ed. *Oxid Med Cell Longev*. 2018;2018(1):5828120. doi:10.1155/2018/5828120

286. Jacob M, Nimer RM, Alabdaljabar MS, et al. Metabolomics Profiling of Nephrotic Syndrome towards Biomarker Discovery. *Int J Mol Sci*. 2022;23(20):12614. doi:10.3390/ijms232012614

287. Boelaert J, Lynen F, Glorieux G, Schepers E, Neirynck N, Vanholder R. Metabolic profiling of human plasma and urine in chronic kidney disease by hydrophilic interaction liquid chromatography coupled with time-of-flight mass spectrometry: a pilot study. *Anal Bioanal Chem*. 2017;409(8):2201-2211. doi:10.1007/s00216-016-0165-x

288. Lucio-Gutiérrez JRicardo, Cordero-Pérez P, Farías-Navarro IC, et al. Using nuclear magnetic resonance urine metabolomics to develop a prediction model of early stages of renal disease in subjects with type 2 diabetes. *J Pharm Biomed Anal*. 2022;219:114885. doi:10.1016/j.jpba.2022.114885

289. Hao XD, Yang YL, Tang NLS, Kong QP, Wu SF, Zhang YP. Mitochondrial DNA haplogroup Y is associated to Leigh syndrome in Chinese population. *Gene*. 2013;512(2):460-463. doi:10.1016/j.gene.2012.10.054

290. Kazubek-Zemke M, Rybka J, Marchewka Z, Rybka W, Pawlik K, Długosz A. Preliminary study on application of urine amino acids profiling for monitoring of renal tubular injury using GLC-MS. *Postępy Hig Med Dośw*. 2014;68:1299-1311. doi:10.5604/17322693.1128846

291. Smirnov D, Konstantinovskiy N, Prokisch H. Integrative omics approaches to advance rare disease diagnostics. *J Inherit Metab Dis*. 2023;46(5):824-838. doi:10.1002/jimd.12663

292. Labory J, Fierville M, Ait-El-Mkadem S, Bannwarth S, Paquis-Flucklinger V, Bottini S. Multi-Omics Approaches to Improve Mitochondrial Disease Diagnosis: Challenges, Advances, and Perspectives. *Front Mol Biosci*. 2020;7:590842. doi:10.3389/fmolb.2020.590842

293. Alston CL, Stenton SL, Hudson G, Prokisch H, Taylor RW. The genetics of mitochondrial disease: dissecting mitochondrial pathology using multi-omic pipelines. *J Pathol*. 2021;254(4):430-442. doi:10.1002/path.5641

294. Thompson Legault J, Strittmatter L, Tardif J, et al. A Metabolic Signature of Mitochondrial Dysfunction Revealed through a Monogenic Form of Leigh Syndrome. *Cell Rep*. 2015;13(5):981-989. doi:10.1016/j.celrep.2015.09.054

295. Esterhuizen K, Lindeque JZ, Mason S, et al. A urinary biosignature for mitochondrial myopathy, encephalopathy, lactic acidosis and stroke like episodes (MELAS). *Mitochondrion*. 2019;45:38-45. doi:10.1016/j.mito.2018.02.003

296. Esterhuizen K, Lindeque JZ, Mason S, et al. One mutation, three phenotypes: novel metabolic insights on MELAS, MIDD and myopathy caused by the m.3243A > G mutation. *Metabolomics*. 2021;17(1):10. doi:10.1007/s11306-020-01769-w

297. Shaham O, Slate NG, Goldberger O, et al. A plasma signature of human mitochondrial disease revealed through metabolic profiling of spent media from cultured muscle cells. *Proc Natl Acad Sci*. 2010;107(4):1571-1575. doi:10.1073/pnas.0906039107

298. Reinecke CJ, Koekemoer G, Van Der Westhuizen FH, et al. Metabolomics of urinary organic acids in respiratory chain deficiencies in children. *Metabolomics*. 2012;8(2):264-283. doi:10.1007/s11306-011-0309-0

299. Hall AM, Vilasi A, Garcia-Perez I, et al. The urinary proteome and metabonome differ from normal in adults with mitochondrial disease. *Kidney Int*. 2015;87(3):610-622. doi:10.1038/ki.2014.297

300. Rahman J, Rahman S. Mitochondrial medicine in the omics era. *The Lancet*. 2018;391(10139):2560-2574. doi:10.1016/S0140-6736(18)30727-X

301. Pereira C, Nogueira C, Barbot C, et al. Identification of a new mtDNA mutation (14724G>A) associated with mitochondrial leukoencephalopathy. *Biochem Biophys Res Commun*. 2007;354(4):937-941. doi:10.1016/j.bbrc.2007.01.068

302. Lopes T, Coelho M, Bordalo D, et al. Síndrome de Leigh: a propósito de um caso clínico com mutação no DNA mitocondrial. *Rev Paul Pediatr*. 2018;36(4):519-523. doi:10.1590/1984-0462/;2018;36;4;00003

303. Nogueira C, Silva L, Pereira C, et al. Targeted next generation sequencing identifies novel pathogenic variants and provides molecular diagnoses in a cohort of pediatric and adult patients with unexplained mitochondrial dysfunction. *Mitochondrion*. 2019;47:309-317. doi:10.1016/j.mito.2019.02.006

304. Baldo MS, Nogueira C, Pereira C, et al. Leigh Syndrome Spectrum: A Portuguese Population Cohort in an Evolutionary Genetic Era. *Genes*. 2023;14(8):1536. doi:10.3390/genes14081536

305. Fan L, Yao YG. MitoTool: A web server for the analysis and retrieval of human mitochondrial DNA sequence variations. *Mitochondrion*. 2011;11(2):351-356. doi:10.1016/j.mito.2010.09.013

306. Castellana S, Fusilli C, Mazzoccoli G, et al. High-confidence assessment of functional impact of human mitochondrial non-synonymous genome variations by APOGEE. Bromberg Y, ed. *PLOS Comput Biol*. 2017;13(6):e1005628. doi:10.1371/journal.pcbi.1005628

307. Sonney S, Leipzig J, Lott MT, et al. Predicting the pathogenicity of novel variants in mitochondrial tRNA with MitoTIP. Poisot T, ed. *PLOS Comput Biol*. 2017;13(12):e1005867. doi:10.1371/journal.pcbi.1005867

308. Preste R, Vitale O, Clima R, Gasparre G, Attimonelli M. HmtVar: a new resource for human mitochondrial variations and pathogenicity data. *Nucleic Acids Res*. 2019;47(D1):D1202-D1210. doi:10.1093/nar/gky1024

309. 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):405-424. doi:10.1038/gim.2015.30

310. Levin A, Ahmed SB, Carrero JJ, et al. Executive summary of the KDIGO 2024 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease: known knowns and known unknowns. *Kidney Int*. 2024;105(4):684-701. doi:10.1016/j.kint.2023.10.016

311. Engelke UFH, Kremer B, Kluijtmans LAJ, et al. NMR spectroscopic studies on the late onset form of 3-methylglutaconic aciduria type I and other defects in leucine metabolism. *NMR Biomed*. 2006;19(2):271-278. doi:10.1002/nbm.1018

312. Page MJ, Moher D, Bossuyt PM, et al. PRISMA 2020 explanation and elaboration: updated guidance and exemplars for reporting systematic reviews. *BMJ*. Published online March 29, 2021:n160. doi:10.1136/bmj.n160

313. Lim SC, Friemel M, Marum JE, et al. Mutations in LYRM4, encoding iron–sulfur cluster biogenesis factor ISD11, cause deficiency of multiple respiratory chain complexes. *Hum Mol Genet*. 2013;22(22):4460-4473. doi:10.1093/hmg/ddt295

314. Roper T, Harber M, Jones G, Pitceathly RDS, Salama AD. Delayed diagnoses of mitochondrial cytopathies in patients presenting with end stage kidney disease: two case reports. *BMC Nephrol*. 2020;21(1):361. doi:10.1186/s12882-020-02002-5

315. Finsterer J, Scorza F. Renal manifestations of primary mitochondrial disorders. *Biomed Rep*. 2017;6(5):487-494. doi:10.3892/br.2017.892

316. Martín-Hernández E, García-Silva MT, Vara J, et al. Renal pathology in children with mitochondrial diseases. *Pediatr Nephrol*. 2005;20(9):1299-1305. doi:10.1007/s00467-005-1948-z

317. Jotwani V, Yang SY, Thiessen-Philbrook H, et al. Mitochondrial genetic variation and risk of chronic kidney disease and acute kidney injury in UK Biobank participants. *Hum Genet*. 2024;143(2):151-157. doi:10.1007/s00439-023-02615-4

318. Duann P, Lin PH. Mitochondria Damage and Kidney Disease. Santulli G, ed. *Mitochondrial Dyn Cardiovasc Med*. 2017;982:529-551. doi:10.1007/978-3-319-55330-6_27

319. Rao M, Jaber BL, Balakrishnan VS. Chronic kidney disease and acquired mitochondrial myopathy: *Curr Opin Nephrol Hypertens*. 2018;27(2):113-120. doi:10.1097/MNH.0000000000000393

320. Galvan DL, Green NH, Danesh FR. The hallmarks of mitochondrial dysfunction in chronic kidney disease. *Kidney Int*. 2017;92(5):1051-1057. doi:10.1016/j.kint.2017.05.034

321. Kuhn C, Mohebbi N, Ritter A. Metabolic acidosis in chronic kidney disease: mere consequence or also culprit? *Pflüg Arch - Eur J Physiol*. 2024;476(4):579-592. doi:10.1007/s00424-024-02912-5

322. Hu Q, Ren J, Ren H, et al. Urinary Mitochondrial DNA Identifies Renal Dysfunction and Mitochondrial Damage in Sepsis-Induced Acute Kidney Injury. Da Silva J, ed. *Oxid Med Cell Longev*. 2018;2018(1):8074936. doi:10.1155/2018/8074936

323. Bhatia D, Capili A, Choi ME. Mitochondrial dysfunction in kidney injury, inflammation, and disease: Potential therapeutic approaches. *Kidney Res Clin Pract*. 2020;39(3):244-258. doi:10.23876/j.krcp.20.082

324. Hao X, Liu X, Wang W, et al. Distinct Metabolic Profile of Primary Focal Segmental Glomerulosclerosis Revealed by NMR-Based Metabolomics. Rastaldi MP, ed. *PLoS ONE*. 2013;8(11):e78531. doi:10.1371/journal.pone.0078531

325. Posada-Ayala M, Zubiri I, Martin-Lorenzo M, et al. Identification of a urine metabolomic signature in patients with advanced-stage chronic kidney disease. *Kidney Int*. 2014;85(1):103-111. doi:10.1038/ki.2013.328

326. Kobayashi T, Yoshida T, Fujisawa T, et al. A metabolomics-based approach for predicting stages of chronic kidney disease. *Biochem Biophys Res Commun*. 2014;445(2):412-416. doi:10.1016/j.bbrc.2014.02.021

327. Kwan B, Fuhrer T, Zhang J, et al. Metabolomic Markers of Kidney Function Decline in Patients With Diabetes: Evidence From the Chronic Renal Insufficiency Cohort (CRIC) Study. *Am J Kidney Dis*. 2020;76(4):511-520. doi:10.1053/j.ajkd.2020.01.019

328. Hong J, Shao X, Jia S, et al. The Mitochondria-Targeted Metabolic Tubular Injury in Diabetic Kidney Disease. *Cell Physiol Biochem*. 2019;52(2):156-171. doi:10.33594/000000011

329. Smuts I, Louw R, Du Toit H, Klopper B, Mienie LJ, Van Der Westhuizen FH. An overview of a cohort of South African patients with mitochondrial disorders. *J Inherit Metab Dis*. 2010;33(S3):95-104. doi:10.1007/s10545-009-9031-8

330. Venter L, Lindeque Z, Jansen Van Rensburg P, Van Der Westhuizen F, Smuts I, Louw R. Untargeted urine metabolomics reveals a biosignature for muscle respiratory chain deficiencies. *Metabolomics*. 2015;11(1):111-121. doi:10.1007/s11306-014-0675-5

331. Barshop BA. Metabolomic approaches to mitochondrial disease: correlation of urine organic acids. *Mitochondrion*. 2004;4(5-6):521-527. doi:10.1016/j.mito.2004.07.010

332. Bouatra S, Aziat F, Mandal R, et al. The Human Urine Metabolome. Dzeja P, ed. *PLoS ONE*. 2013;8(9):e73076. doi:10.1371/journal.pone.0073076

333. Grigalionienė K, Burnytė B, Ambrozaitytė L, Utkus A. Wide diagnostic and genotypic spectrum in patients with suspected mitochondrial disease. *Orphanet J Rare Dis*. 2023;18(1):307. doi:10.1186/s13023-023-02921-0

334. Haas RH, Parikh S, Falk MJ, et al. The in-depth evaluation of suspected mitochondrial disease. *Mol Genet Metab*. 2008;94(1):16-37. doi:10.1016/j.ymgme.2007.11.018

335. Riley LG, Cowley MJ, Gayevskiy V, et al. The diagnostic utility of genome sequencing in a pediatric cohort with suspected mitochondrial disease. *Genet Med*. 2020;22(7):1254-1261. doi:10.1038/s41436-020-0793-6

336. Pronicka E, Piekutowska-Abramczuk D, Ciara E, et al. New perspective in diagnostics of mitochondrial disorders: two years' experience with whole-exome sequencing at a national paediatric centre. *J Transl Med*. 2016;14(1):174. doi:10.1186/s12967-016-0930-9

337. Olimpio C, Paramonov I, Matalonga L, et al. Increased Diagnostic Yield by Reanalysis of Whole Exome Sequencing Data in Mitochondrial Disease. *J Neuromuscul Dis*. 2024;11(4):767-775. doi:10.3233/JND-240020

338. Thompson JLP, Karaa A, Pham H, et al. The evolution of the mitochondrial disease diagnostic odyssey. *Orphanet J Rare Dis*. 2023;18(1):157. doi:10.1186/s13023-023-02754-x

339. Heath O, Hammerl E, Spitzinger A, Wortmann SB. Ending an Odyssey? The Psychosocial Experiences of Parents after the Genetic Diagnosis of a Mitochondrial Disease in Children. *J Pers Med*. 2024;14(5):523. doi:10.3390/jpm14050523

340. Coelho MP, Correia J, Dias A, et al. Iron-sulfur cluster ISD11 deficiency ( *LYRM4* gene) presenting as cardiorespiratory arrest and 3-methylglutaconic aciduria. *JIMD Rep*. 2019;49(1):11-16. doi:10.1002/jmd2.12058

341. Zeharia A, Shaag A, Pappo O, et al. Acute Infantile Liver Failure Due to Mutations in the TRMU Gene. *Am J Hum Genet*. 2009;85(3):401-407. doi:10.1016/j.ajhg.2009.08.004

342. Hershkovitz T, Kurolap A, Tal G, et al. A recurring NFS1 pathogenic variant causes a mitochondrial disorder with variable intra-familial patient outcomes. *Mol Genet Metab Rep*. 2021;26:100699. doi:10.1016/j.ymgmr.2020.100699

343. Marques AC, Busanello ENB, De Oliveira DN, Catharino RR, Oliveira HCF, Vercesi AE. Coenzyme Q10 or Creatine Counteract Pravastatin-Induced Liver Redox Changes in Hypercholesterolemic Mice. *Front Pharmacol*. 2018;9:685. doi:10.3389/fphar.2018.00685

344. Bartsakoulia M, Müller JS, Gomez-Duran A, Yu-Wai-Man P, Boczonadi V, Horvath R. Cysteine Supplementation May be Beneficial in a Subgroup of Mitochondrial Translation Deficiencies. *J Neuromuscul Dis*. 2016;3(3):363-379. doi:10.3233/JND-160178

345. Kuo CW, Chen HA, Hsu RH, et al. Machine learning to predict mitochondrial diseases by phenotypes. *Mitochondrion*. 2025;84:102061. doi:10.1016/j.mito.2025.102061

346. Besse A, Wu P, Bruni F, et al. The GABA Transaminase, ABAT, Is Essential for Mitochondrial Nucleoside Metabolism. *Cell Metab*. 2015;21(3):417-427. doi:10.1016/j.cmet.2015.02.008

347. Rius R, Compton AG, Baker NL, et al. The Australian Genomics Mitochondrial Flagship: A national program delivering mitochondrial diagnoses. *Genet Med*. 2025;27(1):101271. doi:10.1016/j.gim.2024.101271

348. Izumi K. Disorders of Transcriptional Regulation: An Emerging Category of Multiple Malformation Syndromes. *Mol Syndromol*. 2016;7(5):262-273. doi:10.1159/000448747

349. Jeffries L, Mis EK, McWalter K, et al. Biallelic CRELD1 variants cause a multisystem syndrome, including neurodevelopmental phenotypes, cardiac dysrhythmias, and frequent infections. *Genet Med*. 2024;26(2):101023. doi:10.1016/j.gim.2023.101023

350. Zhang F, Dorn T, Gnutti B, et al. Pantethine ameliorates dilated cardiomyopathy features in PPCS deficiency disorder in patients and cell line models. *Commun Med*. 2025;5(1):323. doi:10.1038/s43856-025-01017-z

351. Reyes A, He J, Mao CC, et al. Actin and myosin contribute to mammalian mitochondrial DNA maintenance. *Nucleic Acids Res*. 2011;39(12):5098-5108. doi:10.1093/nar/gkr052

352. Van Damme T, Colman M, Syx D, Malfait F. The Ehlers–Danlos Syndromes against the Backdrop of Inborn Errors of Metabolism. *Genes*. 2022;13(2):265. doi:10.3390/genes13020265

353. Zhang X, Xing Y, Sun K, Guo Y. OmiEmbed: A Unified Multi-Task Deep Learning Framework for Multi-Omics Data. *Cancers*. 2021;13(12):3047. doi:10.3390/cancers13123047

354. Sharifi-Noghabi H, Zolotareva O, Collins CC, Ester M. MOLI: multi-omics late integration with deep neural networks for drug response prediction. *Bioinformatics*. 2019;35(14):i501-i509. doi:10.1093/bioinformatics/btz318

355. Koh HWL, Fermin D, Vogel C, Choi KP, Ewing RM, Choi H. iOmicsPASS: network-based integration of multiomics data for predictive subnetwork discovery. *Npj Syst Biol Appl*. 2019;5(1):22. doi:10.1038/s41540-019-0099-y

356. Rahman J, Noronha A, Thiele I, Rahman S. Leigh map: A novel computational diagnostic resource for mitochondrial disease. *Ann Neurol*. 2017;81(1):9-16. doi:10.1002/ana.24835

357. Smedley D, Jacobsen JOB, Jäger M, et al. Next-generation diagnostics and disease-gene discovery with the Exomiser. *Nat Protoc*. 2015;10(12):2004-2015. doi:10.1038/nprot.2015.124

358. Smedley D, Robinson PN. Phenotype-driven strategies for exome prioritization of human Mendelian disease genes. *Genome Med*. 2015;7(1):81. doi:10.1186/s13073-015-0199-2

359. Cipriani V, Pontikos N, Arno G, et al. An Improved Phenotype-Driven Tool for Rare Mendelian Variant Prioritization: Benchmarking Exomiser on Real Patient Whole-Exome Data. *Genes*. 2020;11(4):460. doi:10.3390/genes11040460

360. Robinson PN, Ravanmehr V, Jacobsen JOB, et al. Interpretable Clinical Genomics with a Likelihood Ratio Paradigm. *Am J Hum Genet*. 2020;107(3):403-417. doi:10.1016/j.ajhg.2020.06.021

361. Vestito L, Jacobsen JOB, Walker S, et al. Efficient reinterpretation of rare disease cases using Exomiser. *Npj Genomic Med*. 2024;9(1):65. doi:10.1038/s41525-024-00456-2

362. De La Vega FM, Chowdhury S, Moore B, et al. Artificial intelligence enables comprehensive genome interpretation and nomination of candidate diagnoses for rare genetic diseases. *Genome Med*. 2021;13(1):153. doi:10.1186/s13073-021-00965-0

363. Muffels IrenaJJ, Rodenburg R, Willemen HLD, et al. Imaging flow cytometry reveals divergent mitochondrial phenotypes in mitochondrial disease patients. *iScience*. 2025;28(1):111496. doi:10.1016/j.isci.2024.111496

364. Birgmeier J, Haeussler M, Deisseroth CA, et al. AMELIE speeds Mendelian diagnosis by matching patient phenotype and genotype to primary literature. *Sci Transl Med*. 2020;12(544):eaau9113. doi:10.1126/scitranslmed.aau9113

365. Denommé-Pichon AS, Matalonga L, De Boer E, et al. A Solve-RD ClinVar-based reanalysis of 1522 index cases from ERN-ITHACA reveals common pitfalls and misinterpretations in exome sequencing. *Genet Med*. 2023;25(4):100018. doi:10.1016/j.gim.2023.100018

366. Labory J, Boulaimen Y, Singh J, et al. Accelerate the discovery of genetic variants in mitochondrial diseases with VIOLA: Variant PrIOritization using Latent space.

367. Deelen P, Van Dam S, Herkert JC, et al. Improving the diagnostic yield of exome- sequencing by predicting gene–phenotype associations using large-scale gene expression analysis. *Nat Commun*. 2019;10(1):2837. doi:10.1038/s41467-019-10649-4

368. De La Fuente L, Del Pozo-Valero M, Perea-Romero I, et al. Prioritization of New Candidate Genes for Rare Genetic Diseases by a Disease-Aware Evaluation of Heterogeneous Molecular Networks. *Int J Mol Sci*. 2023;24(2):1661. doi:10.3390/ijms24021661

369. Kovanda A, Miljanović O, Lovrečić L, Maver A, Hodžić A, Peterlin B. Value of Optical Genome Mapping (OGM) for Diagnostics of Rare Diseases: A Family Case Report. *Balk J Med Genet*. 2024;27(2):87-93. doi:10.2478/bjmg-2024-0021

370. Pitceathly RDS, Keshavan N, Rahman J, Rahman S. Moving towards clinical trials for mitochondrial diseases. *J Inherit Metab Dis*. 2021;44(1):22-41. doi:10.1002/jimd.12281

371. Falk MJ. The pursuit of precision mitochondrial medicine: Harnessing preclinical cellular and animal models to optimize mitochondrial disease therapeutic discovery. *J Inherit Metab Dis*. 2021;44(2):312-324. doi:10.1002/jimd.12319

372. Hatakeyama H, Goto Y ichi. Concise Review: Heteroplasmic Mitochondrial DNA Mutations and Mitochondrial Diseases: Toward iPSC-Based Disease Modeling, Drug Discovery, and Regenerative Therapeutics. *Stem Cells*. 2016;34(4):801-808. doi:10.1002/stem.2292

373. Gropman A, Chandra B. Mitochondrial disorders: Emerging paradigms and the road ahead to personalized medicine. *Neurotherapeutics*. 2024;21(1):e00332. doi:10.1016/j.neurot.2024.e00332

374. Heiduschka S, Prigione A. iPSC models of mitochondrial diseases. *Neurobiol Dis*. 2025;207:106822. doi:10.1016/j.nbd.2025.106822

375. Valenzuela S, Zhu X, Macao B, et al. Small molecules restore mutant mitochondrial DNA polymerase activity. *Nature*. Published online April 9, 2025. doi:10.1038/s41586-025-08856-9

376. Hashimoto M, Bacman SR, Peralta S, et al. MitoTALEN: A General Approach to Reduce Mutant mtDNA Loads and Restore Oxidative Phosphorylation Function in Mitochondrial Diseases. *Mol Ther*. 2015;23(10):1592-1599. doi:10.1038/mt.2015.126

377. Cho SI, Lee S, Mok YG, et al. Targeted A-to-G base editing in human mitochondrial DNA with programmable deaminases. *Cell*. 2022;185(10):1764-1776.e12. doi:10.1016/j.cell.2022.03.039

378. Hosseinian S, Ali Pour P, Kheradvar A. Prospects of mitochondrial transplantation in clinical medicine: Aspirations and challenges. *Mitochondrion*. 2022;65:33-44. doi:10.1016/j.mito.2022.04.006

379. Ghifari AS, Ott M. A decisive technical leap forward for personalized medicine to treat mitochondrial diseases. *EMBO Mol Med*. Published online April 9, 2025. doi:10.1038/s44321-025-00232-4

380. Lynch M, Manoy S, Sly PD, et al. Phase 2a/b randomised placebo-controlled dose-escalation trial of triheptanoin for ataxia-telangiectasia: treating mitochondrial dysfunction with anaplerosis. *eBioMedicine*. 2025;118:105840. doi:10.1016/j.ebiom.2025.105840

381. Gureev AP, Nesterova VV, Babenkova PI, Ivanov ME, Plotnikov EY, Silachev DN. L-Carnitine and Mildronate Demonstrate Divergent Protective Effects on Mitochondrial DNA Quality Control and Inflammation Following Traumatic Brain Injury. *Int J Mol Sci*. 2025;26(7):2902. doi:10.3390/ijms26072902

382. Soldatov VO, Kubekina MV, Skorkina MYu, et al. Current advances in gene therapy of mitochondrial diseases. *J Transl Med*. 2022;20(1):562. doi:10.1186/s12967-022-03685-0

383. Jain IH, Zazzeron L, Goli R, et al. Hypoxia as a therapy for mitochondrial disease. *Science*. 2016;352(6281):54-61. doi:10.1126/science.aad9642

384. Wang H, Miranda M, Marutani E, et al. Therapeutic hypoxia for mitochondrial disease via enhancement of hemoglobin affinity and inhibition of HIF-2α. *J Clin Invest*. 2024;134(23):e185569. doi:10.1172/JCI185569

385. Ishii T. Should Long-Term Follow-up Post-Mitochondrial Replacement be Left up to Physicians, Parents, or Offspring? *New Bioeth*. 2019;25(4):318-331. doi:10.1080/20502877.2019.1654672

386. Simões M, Santos MJ, Martins S, et al. Challenges in Genetic Diagnosis of Mitochondrial Diseases: What CanFunctional Genomics' Studies Do? *Endocr Metab Immune Disord - Drug Targets*. 2023;24. doi:10.2174/0118715303273290231211062420

# *Publications*

# Publications included in this thesis

1. Paiva Coelho M, Martins E, Vilarinho L. **Diagnosis, management, and follow-up of mitochondrial disorders in childhood: a personalized medicine in the new era of genome sequence**. *Eur J Pediatr*. 2019;178(1):21-32. doi:10.1007/s00431-018-3292-x

2. Coelho MP, Correia J, Dias A, et al. **Iron-sulfur cluster ISD11 deficiency (*LYRM4* gene) presenting as cardiorespiratory arrest and 3-methylglutaconic aciduria.** *JIMD Reports*. 2019;49(1):11-16. doi:10.1002/jmd2.12058

3. Pelayo G, Paiva Coelho M, Correia J, et al. **Phenotyping mitochondrial glutamyl-tRNA synthetase deficiency (EARS2): A case series and systematic literature review**. *Neurobiology of Disease*. 2024;200:106644. doi:10.1016/j.nbd.2024.106644

4. Coelho MP, Costa T, Dias A, et al. **NMR-based Urinary Biomarkers in Pediatric Primary Mitochondrial Disorders and Chronic Kidney Disease: Shared Mitochondrial Dysfunction, Diverging Biosignatures.** *Metabolomics* 22 (1): 17. https://doi.org/10.1007/s11306-025-02363-8.

# Diagnosis, management and follow-up of mitochondrial disorders in childhood

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REVIEW

# Diagnosis, management, and follow-up of mitochondrial disorders in childhood: a personalized medicine in the new era of genome sequence

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**Abstract**

Primary mitochondrial disorders are highly variable in clinical presentation, biochemistry, and molecular etiology. Mitochondrial disorders can be caused by genetic defects in the mitochondrial, in nuclear genome, or in the interplay between the two genomes. Biochemical screening tests may be inconclusive or misleading since patients, with confirmed mitochondrial disorders specially in pediatric age, may exhibit normal routine biochemistry, muscle histology, or enzymatic analysis of the mitochondrial respiratory chain. Diagnosis is often challenging even with combination of multiple criteria (clinical, biochemical, histological, and functional), as innumerous conditions cause secondary mitochondrial dysfunction. Nowadays, a definite diagnosis is only possible by genetic confirmation since no single score system is satisfactorily accurate, being sensitive but not specific.

*Conclusion*: Awareness between physicians is of major importance considering that clinical suspicion may not be obvious regarding the heterogenicity in presentation and biochemical features of mitochondrial disorders. In this review, we provide information on diagnosis approach to patients suspected for mitochondrial disorders as well as management on chronic and acute settings. Follow-up should provide comprehensive information on patient's status, since intervention on these diseases is mostly supportive and prognosis is variable and sometimes unpredictable.

**What is Known:**
- *Mitochondrial disorders are heterogenous and may present at any age, with any symptoms and any type of inheritance.*
- *Mitochondrial disorders may be due to pathogenic variants in mitochondrial DNA (mtDNA) or nuclear genes (nDNA).*

**What is New:**
- *Since no single score system is satisfactorily accurate, a definite diagnosis is only possible with genetic studies with gene panels proving to be a cost-effective approach.*
- *Clinical and biochemical features of patients without a confirmed diagnosis must be reviewed and other diagnosis must be considered. A wider genetic approach may be applied (WES or WGS).*

**Keywords** Mitochondria · Mitochondrial disease · mtDNA · Respiratory chain deficiency · Genetic diagnosis

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**Abbreviations**

| | |
|---|---|
| ATP | Adenosine triphosphate |
| DNA | Deoxyribonucleic acid |
| LHON | Leber hereditary optic neuropathy |
| MELAS | Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes |
| MERRF | Myoclonic epilepsy with red ragged fibers |
| MRI | Magnetic resonance imaging |
| mtDNA | Mitochondrial DNA |
| NARP | Neuropathy with ataxia and retinitis pigmentosa |
| nDNA | Nuclear DNA |
| OXPHOS | Oxidative phosphorylation |
| PEO | Progressive external ophthalmoplegia |
| RRF | Red ragged fiber |
| WES | Whole exome sequence |
| WGS | Whole genome sequence |

## Introduction

Mitochondria are dynamic and complex organelles located in the cytoplasm with autonomous replication from mitosis. Mitochondria have independent genetic material (mtDNA): a double-stranded circular DNA molecule with 16,569 DNA base pairs containing 37 genes [37], representing 0.4% of total cell DNA. Mitochondrial function and replication are not autonomous from the nucleus, following multiple nuclear signals for mitochondrial protein synthesis and replication and incorporating proteins synthetized in the cytoplasm [1, 37, 38]. An efficient protein synthesis and assembly depends on nuclear (nDNA) and mitochondrial DNA—mitochondrial dual genetic control [37]. Pathways involved in the nucleus–mitochondria communication are complex and over 1500 proteins are listed in the mitochondrial proteome [22, 34].

Mitochondrial metabolism is complex and comprises energy production through oxidative phosphorylation, pyruvate oxidation through Krebs cycle, fatty acid beta-oxidation, amino acid catabolism, apoptosis, autophagy, and signal transduction [14, 23]. Hence, mitochondrial dysfunction may supervene in innumerous situations.

Oxidative phosphorylation (OXPHOS) is obtained through the respiratory chain, formed by five complexes. Its main purpose is to create an electrochemical gradient to allow ATP (adenosine triphosphate) synthesis, allowing the complete oxidation of 1 mol of glucose to have a net gain of 31 ATP molecules (previously considered a net gain of 38 molecules) [35]. A simplified vision of the mitochondrial metabolism and details on the respiratory chain are shown in Fig. 1.

Strictly speaking, mitochondrial disorders primarily affect OXPHOS and have an estimated incidence of 1:5000 to 1:8500 live births [3, 9, 23, 37]. Genetic defects (point mutations or deletions) that can result in a mitochondrial disorder can affect either mitochondrial or nuclear genome or the interplay between the two genomes leading to defects of assembly, mtDNA transcription, translation and maintenance, or mitochondrial fusion and fission [14, 25, 37].

Large-scale mtDNA deletions and rearrangements (breakpoints occurring within directly repeated sequences which allow the mtDNA molecule to maintain stability) are usually sporadic and have a broad phenotypic spectrum [21].

Mitochondrial disorders can also be due to a quantitative defect, i.e., reduced number of copies of mtDNA and are known as mitochondrial DNA (mtDNA) depletion syndromes [30].

Nuclear genome defects are responsible for the majority (80–90%) of mitochondrial disorders manifested in childhood [31, 34].

Mitochondrial disorders may show any kind of inheritance: mitochondrial, recessive, dominant, or X-linked [14]. Mitochondrial DNA transmission has higher variability since mitochondrial genome may be similar in all copies (homoplasmy) or may differ between copies (heteroplasmy). This may have an effect on clinical phenotype (threshold effect) [14] and accounts for the variability observed between offspring of an affected mother (mitochondrial genetic bottleneck), represented in Fig. 2 [37]. Heteroplasmy has unexpected effects in offspring and the heteroplasmy rate cannot always correlate with clinical phenotype.

## Objective

This systematic review aims to summarize clinical features and workup and presents a diagnostic approach of mitochondrial disorders. It also reviews management on chronic and acute settings for patients with suspected or confirmed mitochondrial disorders and provides comprehensive follow-up recommendations based on a personalized medicine approach.

## Clinical presentation

Mitochondrial disorders are highly heterogeneous with acute, static, or progressive symptoms at any age of onset [1, 3, 9, 15, 45]. As expected, tissues with higher energy demand, such as brain, heart, skeletal muscle, liver, kidney, and eye, will be more affected [34]. Nonetheless, signs and symptoms may associate in an unpredictable manner and patients may be oligosymptomatic.

Clinical suspicion of a respiratory chain disorder must be considered in the presence of the following: (1) consanguineous parents (consider a founder effect on parents from the same birthland); (2) evidence of maternal inheritance; (3) more than one affected system (especially if not embryologically related); (4) progressive disease; (5) worsening with

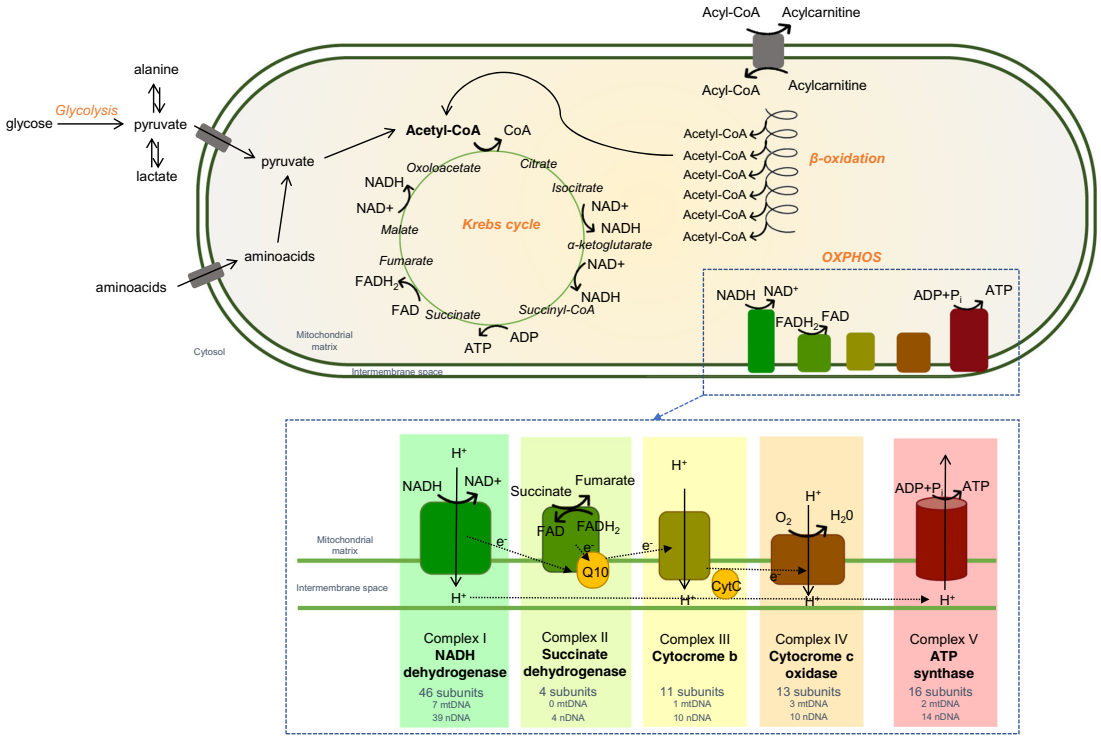


**Fig. 1** Mitochondrial metabolism, production of energy through the respiratory chain, and dual regulation of synthesis of respiratory chain complexes (OXPHOS, oxidative phosphorylation; ATP, adenosine triphosphate)

energetic imbalance (in catabolic states such as vomiting, diarrhea, dehydration, fever, prolonged fasting, surgeries); or with the use of drugs metabolized at the mitochondria [14].

Highly suggestive symptoms for mitochondrial disorder in childhood are the following: epilepsy (intractable or dependent on energetic status), encephalopathy, stroke, basal ganglia, thalamus, and/or cerebellum hyperintensities in T2 and FLAIR, neurosensorial deafness, hypertrophic cardiomyopathy, hypotonia, myopathy, ophthalmoplegia, and tubulopathy. A detailed description of possible clinical, biochemical, and histological manifestations of mitochondrial disorders is listed in Table 1, including chronic or acute signs and symptoms [1, 4, 9, 11, 15, 17, 34].

Some patients present with a cluster of clinical features [14, 34] which allowed to describe classical syndromes with a certain genotype–phenotype correlation. Few patients will fit in classical syndromes being oligosymptomatic or having a poorly defined phenotype with overlapping features. The more commonly observed in childhood are Leigh syndrome (subacute necrotizing encephalomyelopathy with typical lesions on brain MRI) and mitochondrial depletion syndromes.

Other syndromes can present in childhood or adolescence and progress to complete clinical phenotypes [3, 14] such as mitochondrial encephalomyophalopathy, lactic acidosis, and stroke-like episodes (MELAS) [40]; myoclonic epilepsy with red ragged fibers (MERRF); neuropathy with ataxia and retinitis pigmentosa (NARP); Leber hereditary optic neuropathy (LHON) with loss of central vision usually starting in adolescence; progressive external ophthalmoplegia (PEO) and Kearns-Sayre syndrome (pigmentary retinopathy and progressive external ophthalmoplegia with onset earlier than 20 years of age combined with cardiac conduction defects and ataxia or elevated cerebrospinal fluid protein) [39]; Pearson syndrome (sideroblastic anemia and exocrine pancreatic dysfunction) and Alpers syndrome (neurological presentation later complicated by liver disease). Coenzyme Q10 deficiency may present heterogeneously with neurological symptoms, renal (steroid-resistant nephrotic syndrome), and rhabdomyolysis [3, 10, 21].

## Biochemical features

There is still no single diagnostic biomarker of mitochondrial disorder despite multiple biochemical features may be evidence of mitochondrial dysfunction [34]. A probable

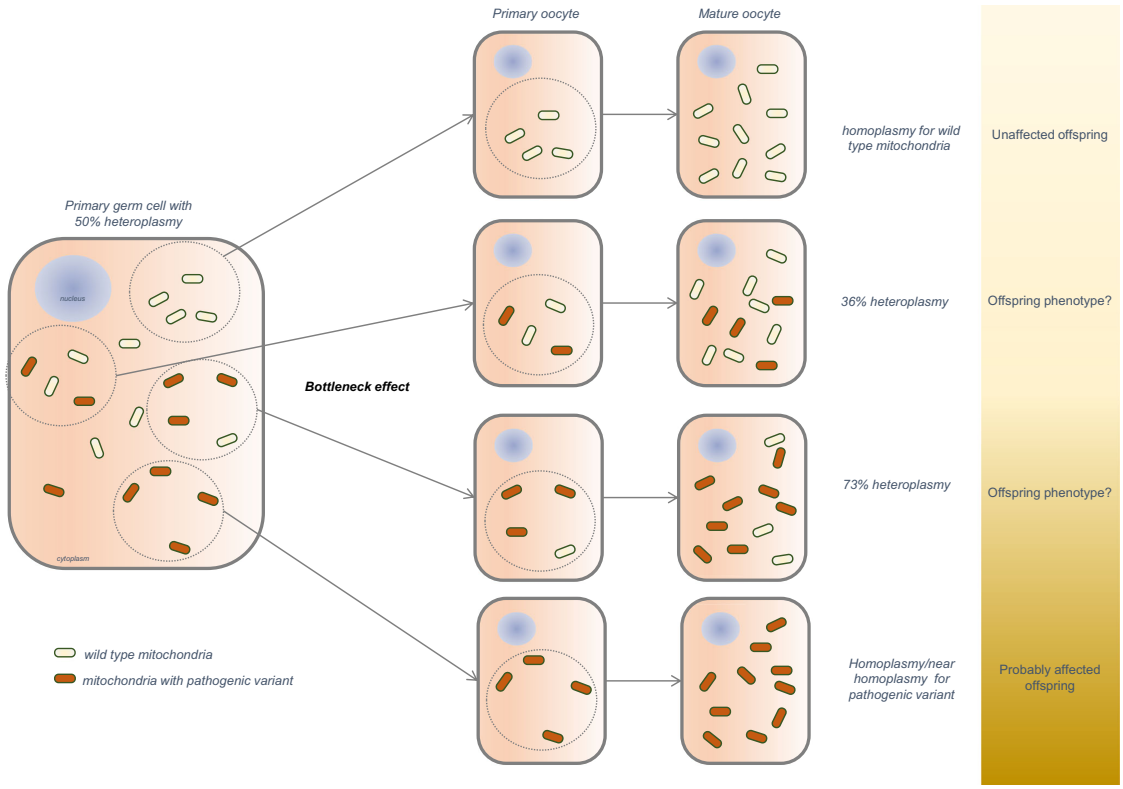


**Fig. 2** The threshold effect and the unpredictable effect on offspring

diagnosis is considered with the combination of possible symptoms with biochemical mitochondrial dysfunction.

Metabolic workup can show persistent or recurrent metabolic acidosis and/or hyperlactacidemia (above 2.0 μmol/L) with normo- or hypoglycemia. Lactate may rise in the postprandial period or after a glucose loading test (2 g/kg, maximum 50 g). Pyruvate may be elevated (> 130 μmol/L) but is not specific. Proportion of lactate/pyruvate (L/P ratio) reflects the cytoplasmic redox state and may be considered when lactate level is elevated. For calculating L/P ratio, lactate and pyruvate must be measured at the sample collected without tourniquet. A L/P above 20 (some authors support a cutoff ranging from 17 [3] to 30 [23]) is suggestive of a respiratory chain disorder while a L/P ratio lower than 10 is suggestive of pyruvate dehydrogenase deficiency.

Ketonemia is also a feature of mitochondrial disorders with a β-hydroxybutyrate (βOHBA) higher than acetoacetate (AcAc). βOHBA/AcAc ratio reflects mitochondrial redox state with a ratio above 3 or a paradoxal postprandial increase found to be very suggestive of mitochondrial disease.

Plasma amino acids may show elevation of alanine [34] (above 450 μmol/L or higher than 20% of upper normal limit). Other clues may be present, such as low citrulline in Leigh syndrome [12], mainly in those with the variants m.8993T>G or m.8993T>C. Urinary amino acids may reveal a generalized hyperaminoaciduria.

Urine organic acid cromatography may show elevation of lactic, 3-methylglutaconic, ethylmalonic and/or 2-ethylhydracrilic acids, Krebs cycle metabolites (as fumaric, succinic, and malic acids), and ketonuria (βOHBA and AcAc).

Cerebrospinal fluid (CSF) may be useful in the absence of plasma biomarkers, since elevation of lactate, pyruvate, or alanine can be evident even without plasma elevation.

Novel biomarkers for mitochondrial disorders have promising results. Fibroblast growth factor 21 (FGF-21) and growth and differentiation factor 15 (GDF-15) appear to be useful in the differentiation between primary and secondary mitochondrial dysfunctions [28].

Cerebral magnetic resonance imaging (MRI) can be useful in cases of neurological involvement showing white matter alteration, evidence of stroke (that may or not follow a vascular distribution), basal ganglia, thalamus, and/or cerebellum hyperintensities in T2 and FLAIR. Cerebral magnetic resonance with spectroscopy can demonstrate a characteristic inverted lactate peak. Cerebral MRIs should be thoroughly

**Table 1** Possible manifestations of mitochondrial disorders

| | |
|---|---|
| Central nervous system | Encephalopathy and/or coma* |
| | Epilepsy* |
| | Myoclonus |
| | Ataxia* |
| | Dystonia |
| | Migraine* |
| | Development delay or regression |
| | Behavioral disorders |
| | White matter alteration |
| | Stroke* (following vascular distribution or not) |
| | Stroke-like* |
| | Basal ganglia, thalamus, and/or cerebellum hyperintensities in T2 and FLAIR |
| | Cerebral magnetic resonance with (1.5 or 3 T) spectroscopy showing inverted lactate peak |
| Audiology | Progressive or congenital neurosensorial deafness (regardless of history of aminoglycoside treatment as precipitating factor) |
| Ophthalmologic | Ptosis |
| | Ophthalmoplegia |
| | Retinitis pigmentosa |
| | Optic atrophy |
| | Cataracts |
| Endocrine | Diabetes mellitus type 1 or 2 |
| | Hypothyroidism |
| | Hypoparathyroidism |
| | Adrenal insufficiency |
| | Short stature |
| | Growth hormone deficiency |
| | Primary or secondary hypogonadism |
| | Premature ovarian failure/premature menopause |
| Cardiac | Cardiomyopathy (mostly hypertrophic) |
| | Heart arrhythmia (specially atrioventricular block)* |
| | Cardiac failure* |
| Hepatology | Elevated liver enzymes |
| | Liver failure* |
| | Reye syndrome* |
| | Cholestasis |
| Gastrointestinal | Failure to thrive |
| | Recurrent vomiting* |
| | Pancreatic dysfunction |
| | Gastric dysmotility |
| | Pseudo-intestinal obstruction* |
| Cutaneous and subcutaneous | Hypertrichosis |
| | Lipomas |
| Kidney | Proximal tubulopathy |
| | Steroid-resistant nephrotic syndrome |
| | Tubulointerstitial nephritis |
| | Chronic kidney disease of unknown etiology |
| Hematological | Sideroblastic anemia |
| | Aplasic anemia |
| | Neutropenia |
| | Thrombocytopenia |
| Neuromuscular | Peripheral neuropathy (distal, axonal, or demyelinating; subclinical) |
| | Hypotonia |
| | Muscular weakness* (proximal and symmetrical) |
| | Myalgias* |
| | Exercise intolerance |
| | Rhabdomyolysis/myoglobinuria* |
| | Elevation of creatine kinase (may be asymptomatic) |
| | Muscle MRI spectroscopy with > Pi/Pcr |
| Metabolic | Hypoglycemia* |
| | Metabolic acidosis* |
| | Persistent or recurrent hyperlactacidemia* (at least 3 different measures with lactate above > 2.0 mmol/L)—worsened by fasting and after a glucose load test |

*Possibly features in acute presentation or decompensation

examined by an experienced radiologist or neuroradiologist. Follow-up MRIs may provide further information about relative patterns of disease progression although the risk of an eventual anesthesia in a non-compliant patient should be considered.

Histologic studies and tissue immunohistochemical assays are crucial to the diagnosis [8, 44]. Biopsy sample may also be used to functional studies of the respiratory chain. Virtually all tissues can be used in these studies but tissues highly dependent on mitochondrial metabolism are more useful. Liver, heart, kidney, and skeletal muscle are the best examples, with muscle as the most convenient and easily obtained. Histology must include a modified Gomori trichrome and may show mitochondrial proliferation and enlargement, with accumulation in the subsarcolemical space, abnormal fiber size variation, and red-ragged fibers (fibers with massive accumulation of mitochondria with disrupted structure and loss of cytoplasmic membrane integrity) [4, 44]. Red-ragged fibers higher than 3% in children are considered to be very specific for mitochondrial disorders (cutoff point for adults is 5%, considering age-related muscle modifications).

Cytochrome oxidase (COX) stain may show COX-negative fibers. Succinate dehydrogenase (SDH) stain reflects complex II activity (encoded only by nuclear genes), and strongly positive SDH fibers may be more evident around vessels in MELAS patients [4].

Even though these findings may be highly specific for mitochondrial disorders, it is important to keep in mind that disorders with secondary mitochondrial dysfunction may induce the same histological appearance and some cases of primary mitochondrial disorders may have normal muscle histology (consider muscle-sparing phenotype or age-related alterations).

Electron microscopy can provide more information about mitochondrial structure showing abnormal shape, number, hypertrophy, abnormal architecture of the cristae, or inclusions. None of these features is specific of primary mitochondrial disease.

Functional biochemical testing of complexes of the respiratory chain (global and individually) must be performed in muscle tissue, preferably in fresh tissue (if not possible, the sample can be immediately frozen at − 80 °C until analysis) [34]. Functional assays may show partial or complete deficiencies in a single complex or as a combined deficiency (more than one complex involved). A coenzyme Q10 (ubiquinone) measure in muscle lower than 50% of control reflects Q10 deficiency.

Functional analysis of the OXPHOS chain is a complex and expensive procedure that must be performed in experienced centers with good quality control. Therefore, muscle biopsy must be carefully considered in selected cases and always include histological and functional tests.

## Diagnosis

Diagnosis of mitochondrial disorders is challenging, especially in pediatric patients. It is estimated that 30–40% of affected patients, mainly children, do not have a known genetic diagnosis and there is limited knowledge of the biological factors causing their wide clinical heterogeneity [7, 43]. Some authors (such as Wolf [42] and Bernier [2]) attempted to create a diagnostic methodology for mitochondrial disorders, both including clinical, biochemical, and functional criteria [2, 42]. However, patients with confirmed mitochondrial disorders may exhibit normal routine biochemistry, muscle histology, or enzymatic analysis of the respiratory chain [1, 14].

Although sensitive, these criteria are not specific as mitochondrial dysfunction is present in several diseases and is not entirely related to primary mitochondrial respiratory chain disorders [6, 13, 26]. In the event of abnormal or equivocal biochemistry, it can be difficult to differentiate between a primary disorder of the respiratory chain from secondary mitochondrial dysfunction (for instance, in multisystemic disorders like congenital defects of glycosylation, organic acidurias, or neuronal ceroid lipofuscinosis). No single biomarker or functional study accurately distinguishes a primary defect in respiratory chain of secondary mitochondrial dysfunction [5, 7, 9, 11, 36].

A combination of classically described symptoms or biochemical signals can lead an experienced clinician to suspect certain candidate genes, eventually leading to a molecular diagnosis.

Recent development of high-throughput, next-generation sequence (NGS) technology has revolutionized the research and molecular diagnosis of human genetic disease. The ability to generate enormous amount of sequence data in a short time at an affordable cost makes this approach ideal for a wide range of applications from sequencing a group of candidate genes, all coding regions (whole exome sequencing, WES) to the entire human genome (whole genome sequencing, WGS) [7, 27, 43]. NGS may be used in gene panels which may be designed with the more common genes related to a specific disease or clinical presentation and may include a few to hundreds of genes, making it a very attractive cost-affordable option, as opposed to a gene-by-gene Sanger sequencing of individual DNA fragment strategy that is time-consuming and laborious. There are several commercial kits for the diagnosis of mitochondrial disorders. Since multiple genes can be involved, a comprehensive panel should be performed. An example is listed in Table 2, including 209 nuclear genes known to be associated with mitochondrial diseases. It is a customized panel targeting nuclear genes using Suredesign software (Agilent Technologies), initially applied in research.

**Table 2** Nuclear genes involved in mitochondrial disorders

| | |
|---|---|
| OXPHOS subunits | |
| Complex I | *NDUFS1*; *NDUFS2*; *NDUFS3*; *NDUFS4*; *NDUFS6*; *NDUFS7*; *NDUFS8*; *NDUFV1*; *NDUFV2*; *NDUFA1*; *NDUFA10*; *NDUFA11*; *NDUFA12*; *NDUFA2*; *NDUFA9*; *NDUFB3*; *NDUFB9* |
| Complex II | *SDHA*; *SDHB*; *SDHC*; *SDHD* |
| Complex III | *CYC1*; *LYRM7*; *UQCRQ*; *UQCRC2*; *UQCRB* |
| Complex IV | *COX5A*; *COX5B*; *COX4I2*; *COX6A1*; *COX6B1*; *COX7B*; *NDUFA4* |
| Complex V | *ATP5A1*; *ATP5E* |
| OXPHOS assembly factors | |
| Complex I | *ACAD9*; *FOXRED1*; *NDUFAF1*; *NDUFAF2*; *NDUFAF3*; *NDUFAF4*; *NDUFAF5*; *NDUFAF6*; *NUBPL* |
| Complex II | *SDHAF1*; *SDHAF2* |
| Complex III | *BCS1L*; *HCCS*; *TTC19* |
| Complex IV | *COA3*; *COA5*; *COX10*; *COX15*; *COX14*; *COX20*; *FASTKD2*; *SCO1*; *SCO2*; *SURF1* |
| Complex V | *ATPAF2*; *TMEM70* |
| mtDNA maintenance | *C10ORF2*; *DNA2*; *DGUOK*; *FBXL4*; *POLG*; *MGME1*; *MPV17*; *POLG2*; *SLC25A4*; *SUCLA2*; *SUCLG1*; *TYMP*; *TK2*; *RRM2B* |
| Mitochondrial translation | *AARS2*; *C12ORF65*; *CARS2*; *DARS2*; *EARS2*; *ELAC2*; *FARS2*; *GFM1*; *GTPBP3*; *HARS2*; *IARS2*; *KARS*; *LARS*; *LARS2*; *LRPPRC*; *MARS2*; *MRPL23*; *MRPL3*; *MRPL44*; *MRPL50*; *MRPL57*; *MRPS16*; *MRPS22*; *MTFMT*; *MTO1*; *MTPAP*; *NARS2*; *PARS2*; *PNPT1*; *PUS1*; *RARS2*; *RMND1*; *RNASEH1*; *SARS2*; *TACO1*; *TARS2*; *TRMU*; *TRNT1*; *TSFM*;*TUFM*; *VARS2*; *WARS2*; *YARS2* |
| Membrane function and import | *AGK*; *C19ORF12*; *CISD2*; *DNAJC19*; *DNM1L*; *GDAP1*; *GFER*; *MFF*; *MFN1*; *MFN2*; *MICU1*; *MPC1*; *OPA1*; *SERAC1*; *SLC25A22*; *SLC25A3*; *SLC25A42*; *SLC4A3*; *SLC52A1*; *SLC52A2*; *SLC52A3*; *TAZ*; *TIMM8A*; *TMEM126A*; *TMEM126B* |
| Cofactor biosynthesis (CoQ10, lipoic acid, FeS clusters, others) | *ABCB7*; *ADCK3*; *BOLA3*; *COQ2*; *COQ4*; *COQ6*; *COQ9*; *FDX1L*; *GLRX5*; *IBA57*;*ISCU*; *LIAS*; *LIPT1*; *LYRM4*; *NFU1*; *PDSS1*; *PDSS2*; *TPK1* |
| Others | *ACO2*; *AIFM1*; *ALAS2*; *ALG13*; *APTX*; *AUH*; *C9ORF116*; *CHKB*; *CLN3*; *CLN5*; *CLN6*; *CLN8*; *CLPB*; *CTSD*; *CWF19L1*; *DLAT*; *DLD*; *DNAJC5*; *DOLK*; *ECHS1*; *ETFA*; *ETFB*; *ETFDH*; *ETHE1*; *FLAD1*; *HACE1*; *HIBCH*; *KCTD7*; *KIF5A*; *MEGF10*; *MFSD8*; *MPI*; *NOTCH3*; *OPA3*; *PC*; *PCK2*; *PDHA1*; *PDHB*; *PDHX*; *PDP1*; *PLD1*; *PMM2*; *PPT1*; *SLC19A3*; *SPATA5*; *TPP1*; *WDR45*; *WFS1* |

NGS has been successfully applied to the analysis of whole mtDNA which greatly facilitates the characterization of patients with probable mitochondrial disease in a timely and cost-effective approach [11, 25].

Considering that a *definite diagnosis* is only possible with genetic confirmation (in mitochondrial or nuclear genes) [5, 7, 24, 25, 45] or in the presence of a primary mitochondrial DNA depletion syndrome (> 70% depletion), establishing the likelihood of mitochondrial disease is helpful in deciding which patients to undergo further studies [34]. If genetic studies are not conclusive, one should reconsider clinical presentation and biochemical features, and eventually consider other diagnosis.

A diagnosis is *possible* if presentation is suggestive (particularly if neurological and/or muscular) and may be accompanied by biochemical mitochondrial dysfunction (as previously mentioned).

A *probable diagnosis* is considered with the combination of clinical presentation, biochemical workup and/or imagiological findings and/or immunohistochemical findings or functional studies show decreased function of one or more complexes of the respiratory chain.

A diagnostic approach is proposed in Fig. 3.

If after genetic testing (including mitochondrial and nuclear genes through NGS) a definite diagnosis is not possible, a mitochondrial disorder must not be excluded but other

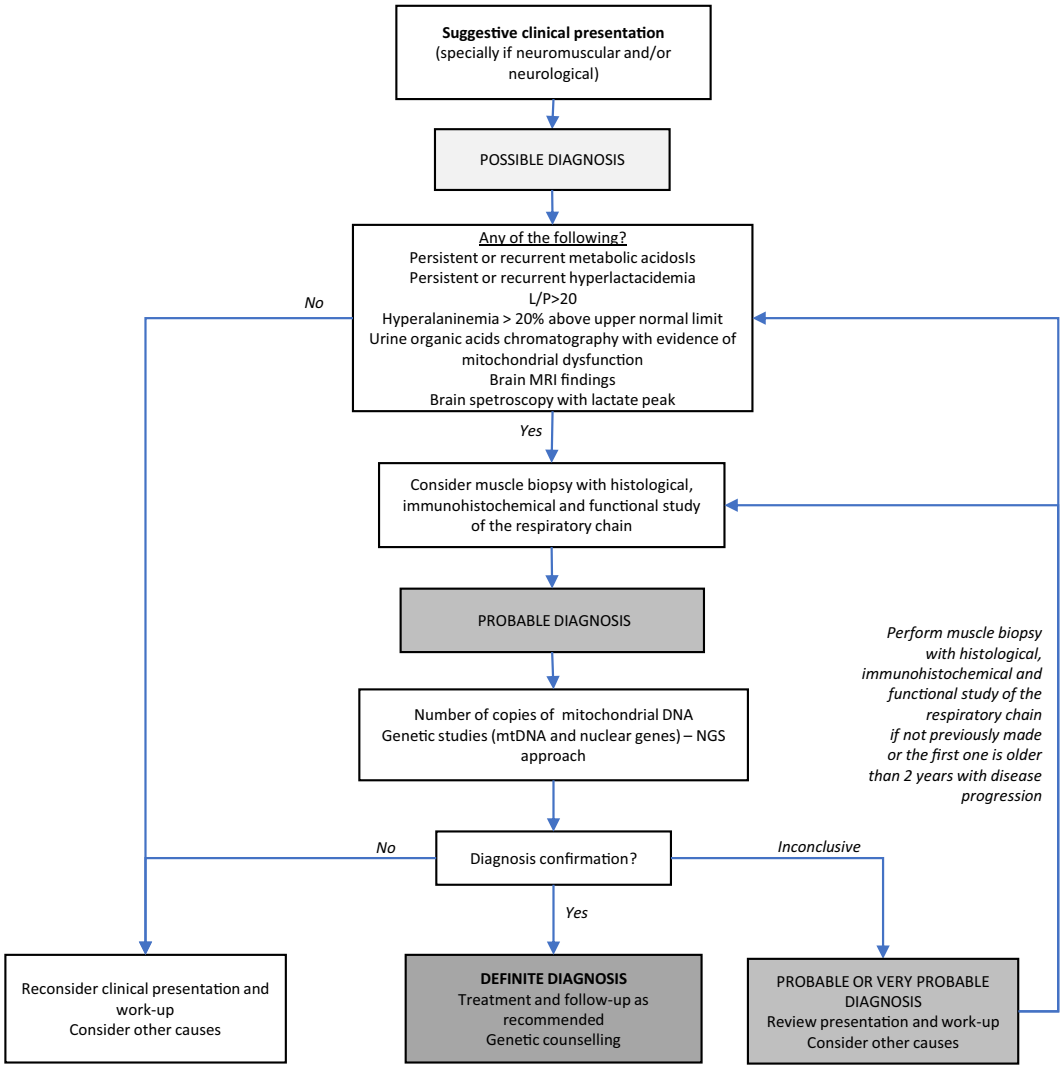


**Fig. 3** Algorithm for the diagnosis of a mitochondrial disorder

diagnoses must be considered after reviewing clinical and workup findings.

## Treatment

Specific treatment of mitochondrial disorders (disease-modifying treatment) is based on different strategies such as increasing mitochondrial biogenesis, boost of respiratory chain function, energy buffering, scavenging toxic compounds, vascular effects, or alteration of mitochondrial dynamics [18, 41].

Coenzyme Q10 is a component of the respiratory chain and can be used for augmenting its function. It can be effective in cases of Q10 deficiency administered 5 mg/kg/day (60 a 250 mg/day) orally, with an expected neuromuscular improvement but without a clear neurological benefit (poor crossing of the blood–brain barrier). For those without confirmed Q10 deficiency, it may prescribed with limited clinical evidence. Idebenone is a coenzyme Q10 analog and evidence of its value is limited [18].

L-Arginine 150–300 mg/kg/day orally is recommended in the cases of MELAS or previous history of stroke-like episodes. It acts as a vasoactive through the nitric oxide pathway [18].

Riboflavin (B2) is the flavin adenine dinucleotide precursor and doses of 10–100 mg/day have some evidence in the treatment of complex I deficiency.

L-Carnitine is important for fatty acid transport to the mitochondria and may be used to enhance fatty acid oxidation or

**Table 3** Clinical, biochemical, and imaging follow-up of mitochondrial disorders in childhood

| Interval | Workup |
|---|---|
| Annual | Blood count |
| | Plasma: glycemia, liver enzymes, urea, creatinine, creatine-kinase, sodium, potassium, chloride, total calcium, magnesium, phosphorus, vitamin D |
| | Acid–base balance and lactate |
| | Urine: microalbuminuria, alpha-1-microglobulin, beta-2-microglobulin, creatinine, proteinuria, amino acids, phosphorus, sodium, calcium, and glycose |
| | HbA1c, ACTH, cortisol, IGF1, PTH, FT4, and TSH |
| | ECG and echocardiogram |
| | Clinical neurological evaluation |
| | EEG if epilepsy |
| Every 2 years | Scoliosis, contractures, luxation, limb deformities |
| | Bone densitometry (earlier if bone fractures) |
| | Chromatography of urine organic acids and plasma amino acids |
| | Visual acuity, visual fields, eye movements, ptosis, and fundoscopy |
| | Hearing evaluation |
| Every 5 years | Brain MRI (with spectroscopy if not previously made)—earlier if acute neurological symptoms or development regression |
| | Formal developmental or cognitive evaluation |
| | Neuropsychological evaluation |

in secondary carnitine deficiency; usual dose is 50 mg/kg/day (maximum 3 g/day) [18].

Other supplements, such as thiamine and creatine, may be used per os as chronic treatment with controversial or limited clinical benefit. Thiamine (B1) is a cofactor of the pyruvate dehydrogenase complex and may have an effect on decreasing lactate while increasing acetyl-CoA; it may be used at maximum 900 mg/day. Creatine phosphate (maximum 10 g/day) may be administered in the presence of myopathy or cardiomyopathy and aims to improve ATP storage via creatine phosphatase system.

Other therapeutics include cysteine to increase the availability of antioxidant glutathione peroxidase, nuclear respiratory factors (NRF1 and 2), peroxisomal proliferator activator receptors (PPAR α, β, and γ), and their agonists to positively influence the transcription of OXPHOS genes. EPI-743 (the parabenzoquinone analog) is a novel promising therapeutic approach with some evidence of reversion of disease [16, 18]. Undergoing clinical trials also include deoxycytidine monophosphate (dCMP) and deoxythymidine monophosphate (dTMP) in thymidine kinase 2 (PK2) deficiency, a disorder leading to mtDNA instability and depletion [41].

Enzyme replacement, shift of heteroplasmy rate, and stem cell and gene therapy are still experimental.

Despite several clinical trials, there is no specific treatment for mitochondrial disorders and their management is mainly supportive [16, 18, 33]. Symptomatic treatment of mitochondrial disorders includes specific-organ management such as a pacemaker for rhythm disease, dialysis for renal insufficiency, ventilatory support, or invasive procedures such as surgical ptosis correction. Organ transplantation may be considered depending on global patient status and comorbidities.

## General recommendations

Avoidance of the following drugs are strongly recommended: (1) those metabolized at the mitochondria (sodium valproate and barbiturates as phenobarbital); (2) mitochondrial protein synthesis inhibitors (gentamycin, tetracyclines, and chloramphenicol); and (3) inhibitors of the respiratory chain (statins, metformin, phenytoin, propofol) [19, 33]. A high glucose infusion rate is not recommended (above 5% dextrose), except for immediate hypoglycemia treatment (see below).

Surgeries and anesthesia pose a particular problem within these patients, with respiratory failure, neurological lesions, cardiac depression, conduction defects, and hypoglycemia as the main problems [29]. Volatile anesthetics and propofol are more likely to depress mitochondrial function. Patients should own a written plan in the event of any surgery or need of anesthesia and start intravenous fluids with normal saline and 5% dextrose during fasting and avoid temperature variations [29].

Physical exercise is recommended, adjusted to the patient's capacity and tolerance [3, 32].

## Management in the acute setting

In the presence of acute presentation or decompensation of mitochondrial disorders, acid–base balance, lactate, ammonia,

liver enzymes, creatine-kinase, myoglobin, electrolytes, and ketonemia should be assessed [3]. Urinalysis may also be informative. If diagnosis is not definite, plasma amino acids, acylcarnitine profile, and urine organic acids chromatography should also be considered.

Intravenous fluids should be started while avoiding glycose overload. Vomiting should be treated and ondansetron (0.1 mg/kg, maximum 8 mg) is a safe option. If shock or severe dehydration, isotonic crystalloid or colloid bolus 10–20 mL/kg must be the first approach.

Hypoglycemia (lower than 45 mg/dL) can be treated with intravenous glucose 2 mL/kg and severe hyperlactacidemia (lactate > 7 mmol/L or bicarbonate < 10 mmol/L) can be treated with intravenous sodium bicarbonate at a low infusion rate (1–2 mEq/kg in 12 h) [3, 23].

In case of seizures, valproic acid and phenobarbital are contraindicated; benzodiazepines and levetiracetam are safe options [20]. In stroke or stroke-like episode, start intravenous L-arginine (150–500 mg/kg/day) straight away and reassess after 3 days [32]. Brain MRI should be performed in every de novo neurological deficits or seizures or change in the pattern of a previously known epilepsy.

If antibiotics are prescribed, aminoglycosides (especially in neonates), tetracyclines, and chloramphenicol should be avoided [32].

Rhabdomyolysis must be actively treated with 120–150% of daily needs avoiding glucose overload (preference for normal saline with 5% dextrose) while maintaining normoglycemia.

In the event of an eminent death or immediately postmortem, muscle and skin biopsies must be performed as well as blood collection for posterior genetic studies.

## Follow-up

Every patient with a very likely or definite diagnosis should be handed a medical report and acute setting and surgery guidelines [32]. Genetic counseling should be offered to the individual and their family, particularly in face of a new pregnancy (or desire to have more children) or reaching adulthood [11]. Pneumococcus and seasonal flu vaccination should be proposed.

Phenotypical variants and poor correlation of genotype–phenotype justify similar follow-up for all mitochondrial disorders. At each consultation, assessment must include auxological data (weight, height, BMI, and head circumference), blood pressure, heart frequency, complete physical exam, and development milestones. Regular evaluation must include annual workup and cardiac and neurological evaluations [33].

Ophthalmologic and hearing evaluations should be as regular as every 2 years. Some comorbidities will require specific care, namely, nutritional support (with supplements such as medium chain triglycerides), enteric nutrition through nasogastric tube or gastrostomy if feeding difficulties, respiratory support if respiratory insufficiency or sleep apnea, and immunological evaluation if recurrent or severe infections.

A recommendation on complete evaluation is presented in Table 3 and implies the collaboration in multidisciplinary teams (nephrology, endocrine, cardiology, neurology, orthopedics, ophthalmology, otorhinolaryngology, etc.). Most recommendations can be applied to adult patients.

## Conclusions

Mitochondrial disorders are highly variable in clinical presentation, biochemistry, and molecular etiology. Biochemical screening tests may be inconclusive or misleading, since patients with confirmed mitochondrial disorders may exhibit normal routine biochemistry, muscle histology, or enzymatic analysis of the respiratory chain, especially within pediatric patients.

Awareness between physicians is of major importance regarding clinical heterogenicity and biochemical features of mitochondrial disorders. Prompt diagnosis and intervention are crucial for optimizing care, considering the possibility of a rapidly progressive and devastating disease course in infancy or early childhood, where therapy options are still rather ineffective.

Molecular diagnosis of mitochondrial disorders is a great challenge with gene panels proving to be a cost-effective approach. Clinical and biochemical features of patients without a confirmed diagnosis must be reviewed and other diagnoses must be considered. A wider genetic approach may be applied (WES or WGS).

Follow-up should provide comprehensive information on patient's status, since intervention on these diseases is mostly supportive and prognosis is variable and sometimes unpredictable.

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Esmeralda Martins: critical manuscript review
Laura Vilarinho: critical manuscript review

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### Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interests** The authors declare that they have no conflict of interest.

## References


1. Alston CL, Rocha MC, Lax NZ, Turnbull DM, Taylor RW (2017) The genetics and pathology of mitochondrial disease. J Pathol 241: 236–250. https://doi.org/10.1002/path.4809
2. Bernier FP, Boneh A, Dennett X, Chow CW, Cleary MA, Thorburn DR (2002) Diagnostic criteria for respiratory chain disorders in adults and children. Neurology 59:1406–1411. https://doi.org/10.1212/01.WNL.0000033795.17156.00
3. Blau N, Duran M, Gibson KM, Dionisi Vici C (2014) Physician's guide to the diagnosis, treatment, and follow-up of inherited metabolic diseases. Springer Berlin Heidelberg, Berlin
4. Bourgeois JM, Tarnopolsky MA (2004) Pathology of skeletal muscle in mitochondrial disorders. Mitochondrion 4:441–452. https://doi.org/10.1016/j.mito.2004.07.036
5. Calvo SE, Compton AG, Hershman SG, Lim SC, Lieber S, Tucker EJ, Laskowski A, Garone C, Liu S, Jaffe DB, Christodoulou J, Fletcher JM, Bruno DL (2012) Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing. Sci Transl Med 4:1–28. https://doi.org/10.1126/scitranslmed.3003310.Molecular
6. Caracausi M, Ghini V, Locatelli C, Mericio M, Piovesan A, Antonaros F, Pelleri MC, Vitale L, Vacca RA, Bedetti F, Mimmi MC, Luchinat C, Turano P, Strippoli P, Cocchi G (2018) Plasma and urinary metabolomic profiles of Down syndrome correlate with alteration of mitochondrial metabolism. Sci Rep 8:1–16. https://doi.org/10.1038/s41598-018-20834-y
7. Carroll CJ, Brilhante V, Suomalainen A (2014) Next-generation sequencing for mitochondrial disorders. Br J Pharmacol 171: 1837–1853. https://doi.org/10.1111/bph.12469
8. Challa S, Kanikannan MA, Murthy JM, Bhoompally VR, Surath M (2004) Diagnosis of mitochondrial diseases: clinical and histological study of sixty patients with ragged red fibers. Neurol India 52: 353–358
9. Chi C-S (2015) Diagnostic approach in infants and children with mitochondrial diseases. Pediatr Neonatol 56:7–18. https://doi.org/10.1016/j.pedneo.2014.03.009
10. Chow J, Rahman J, Achermann JC, Dattani MT, Rahman S (2017) Mitochondrial disease and endocrine dysfunction. Nat Rev Endocrinol 13:92–104. https://doi.org/10.1038/nrendo.2016.151
11. Clinical Molecular Genetics Society (2008) Practice guidelines for the molecular diagnosis of mitochondrial diseases. Available online: http://www.acgs.uk.com/media/774659/mito_2008.pdf
12. Debray FG, Lambert M, Allard P, Mitchell GA (2010) Low citrulline in leigh disease: still a biomarker of maternally inherited leigh syndrome. J Child Neurol 25:1000–1002. https://doi.org/10.1177/0883073809351983
13. Demine S, Reddy N, Renard P, Raes M, Arnould T (2014) Unraveling biochemical pathways affected by mitochondrial dysfunctions using metabolomic approaches. Metabolites 4:831–878. https://doi.org/10.3390/metabo4030831
14. DiMauro S, Schon EA (2003) Mitochondrial respiratory-chain diseases. N Engl J Med 348:2656–2668. https://doi.org/10.1056/NEJMra022567
15. Emma F, Montini G, Parikh SM, Salviati L (2016) Mitochondrial dysfunction in inherited renal disease and acute kidney injury. Nat Rev Nephrol 12:267–280. https://doi.org/10.1038/nrneph.2015.214
16. Enns GM, Cohen BH (2017) Clinical trials in mitochondrial disease. J Inborn Errors Metab Screen 5:232640981773301. https://doi.org/10.1177/2326409817733013
17. Feichtinger RG, Sperl W, Bauer JW, Kofler B (2014) Mitochondrial dysfunction: a neglected component of skin diseases. Exp Dermatol 23:607–614. https://doi.org/10.1111/exd.12484
18. Finsterer J, Bindu PS (2015) Therapeutic strategies for mitochondrial disorders. Pediatr Neurol 52:302–313. https://doi.org/10.1016/j.pediatrneurol.2014.06.023
19. Finsterer J, Segall L (2010) Drugs interfering with mitochondrial disorders. Drug Chem Toxicol 33:138–151. https://doi.org/10.3109/01480540903207076
20. Finsterer J, Zarrouk Mahjoub S (2012) Mitochondrial toxicity of antiepileptic drugs and their tolerability in mitochondrial disorders. Expert Opin Drug Metab Toxicol 8:71–79. https://doi.org/10.1517/17425255.2012.644535
21. Grady JP, Campbell G, Ratnaike T, Blakely EL, Falkous G, Nesbitt V, Schaefer AM, Mcnally RJ, Gorman GS, Taylor RW, Turnbull DM, Mcfarland R (2014) Disease progression in patients with single, large-scale mitochondrial DNA deletions. Brain 137:323–334. https://doi.org/10.1093/brain/awt321
22. Kohda M, Tokuzawa Y, Kishita Y, Nyuzuki H, Moriyama Y, Mizuno Y, Hirata T, Yatsuka Y, Yamashita-Sugahara Y, Nakachi Y, Kato H, Okuda A, Tamaru S, Borna NN, Banshoya K, Aigaki T, Sato-Miyata Y, Ohnuma K, Suzuki T, Nagao A, Maehata H, Matsuda F, Higasa K, Nagasaki M, Yasuda J, Yamamoto M, Fushimi T, Shimura M, Kaiho-Ichimoto K, Harashima H, Yamazaki T, Mori M, Murayama K, Ohtake A, Okazaki Y (2016) A comprehensive genomic analysis reveals the genetic landscape of mitochondrial respiratory chain complex deficiencies. PLoS Genet 12:1–31. https://doi.org/10.1371/journal.pgen.1005679
23. Lamari F, Saudubray J-M, Mitchell GA (2016) Inborn metabolic diseases, 6th edn. Springer Berlin Heidelberg, Berlin
24. Legati A, Reyes A, Nasca A, Invernizzi F, Lamantea E, Tiranti V, Garavaglia B, Lamperti C, Ardissone A, Moroni I, Robinson A, Ghezzi D, Zeviani M (2016) New genes and pathomechanisms in mitochondrial disorders unraveled by NGS technologies. Biochim Biophys Acta Bioenerg 1857:1326–1335. https://doi.org/10.1016/j.bbabio.2016.02.022
25. Liang C, Ahmad K, Sue CM (2014) The broadening spectrum of mitochondrial disease: shifts in the diagnostic paradigm. Biochim Biophys Acta Gen Subj 1840:1360–1367. https://doi.org/10.1016/j.bbagen.2013.10.040
26. Liu JJ, Ghosh S, Kovalik JP, Ching J, Choi HW, Tavintharan S, Ong CN, Sum CF, Summers SA, Tai ES, Lim SC (2017) Profiling of plasma metabolites suggests altered mitochondrial fuel usage and remodeling of sphingolipid metabolism in individuals with type 2 diabetes and kidney disease. Kidney Int Reports 2:470–480. https://doi.org/10.1016/j.ekir.2016.12.003
27. McCormick E, Place E, Falk MJ (2013) Molecular genetic testing for mitochondrial disease: from one generation to the next. Neurotherapeutics 10:251–261. https://doi.org/10.1007/s13311-012-0174-1
28. Montero R, Yubero D, Villarroya J, Henares D, Jou C, Rodríguez MA, Ramos F, Nascimento A, Ortez CI, Campistol J, Perez-due B (2016) GDF-15 is elevated in children with mitochondrial diseases and is induced by mitochondrial dysfunction. 1–15. https://doi.org/10.1371/journal.pone.0148709
29. Niezgoda J, Morgan PG (2013) Anesthetic considerations in patients with mitochondrial defects. Paediatr Anaesth 23:785–793. https://doi.org/10.1111/pan.12158
30. Nogueira C, Almeida LS, Nesti C, Pezzini I, Videira A, Vilarinho L, Santorelli FM (2014) Syndromes associated with mitochondrial DNA depletion. Ital J Pediatr 40:1–10. https://doi.org/10.1186/1824-7288-40-34
31. Ohtake A, Murayama K, Mori M, Harashima H, Yamazaki T, Tamaru S, Yamashita Y, Kishita Y, Nakachi Y, Kohda M, Tokuzawa Y, Mizuno Y, Moriyama Y, Kato H, Okazaki Y (2014) Diagnosis and molecular basis of mitochondrial respiratory chain disorders: exome sequencing for disease gene identification. Biochim Biophys Acta Gen Subj 1840:1355–1359. https://doi.org/10.1016/j.bbagen.2014.01.025

32. Parikh S, Goldstein A, Koenig MK, Scaglia F, Enns GM, Saneto R, Anselm I, Cohen BH, Falk MJ, Greene C, Gropman AL, Haas R, Hirano M, Morgan P, Sims K, Tarnopolsky M, Van Hove JLK, Wolfe L, DiMauro S (2015) Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. Genet Med 17:689–701. https://doi.org/10.1038/gim.2014.177
33. Parikh S, Goldstein A, Karaa A, Koenig MK, Anselm I, Brunel-Guitton C, Christodoulou J, Cohen BH, Dimmock D, Enns GM, Falk MJ, Feigenbaum A, Frye RE, Ganesh J, Griesemer D, Haas R, Horvath R, Korson M, Kruer MC, Mancuso M, McCormack S, Raboisson MJ, Reimschisel T, Salvarinova R, Saneto RP, Scaglia F, Shoffner J, Stacpoole PW, Sue CM, Tarnopolsky M, Van Karnebeek C, Wolfe LA, Cunningham ZZ, Rahman S, Chinnery PF (2017) Patient care standards for primary mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. Genet Med 19. https://doi.org/10.1038/gim.2017.107
34. Rahman S, Wolf NI (2017) Diagnostic Workup of Patients with Mitochondrial Diseases. In: Hoffmann G, Zschocke J, Nyhan W (eds) Inherited Metabolic Diseases. Springer, Berlin
35. Salway JG (2017) Metabolism at a glance, 4th edn. Wiley-Blackwell Publishing, Oxford
36. Shaham O, Slate NG, Goldberger O, Xu Q, Ramanathan A, Souza AL, Clish CB, Sims KB, Mootha VK (2010) A plasma signature of human mitochondrial disease revealed through metabolic profiling of spent media from cultured muscle cells. Proc Natl Acad Sci 107:1571–1575. https://doi.org/10.1073/pnas.0906039107
37. Taylor RW, Turnbull DM (2005) Mitochondrial DNA mutations in human disease. Nat Rev Genet 6:389–402. https://doi.org/10.1038/nrg1606
38. Vilarinho L (2000) Genética Mitocondrial Humana (Doctoral dissertation), Universidade do Porto
39. Vilarinho L, Santorelli FM, Cardoso ML, Coelho T, Guimarães A, Coutinho P (1998) Mitochondrial DNA analysis in ocular myopathy. Eur Neurol 39:148–153. https://doi.org/10.1159/000007925
40. Vilarinho L, Santorelli FM, Coelho I, Rodrigues L, Maia M, Barata I, Cabral P, Dionísio A, Costa A, Guimarães A, Dimauro S (1999) The mitochondrial DNA A3243G mutation in Portugal: clinical and molecular studies in 5 families. J Neurol Sci 163:168–174. https://doi.org/10.1016/S0022-510X(99)00030-1
41. Viscomi C, Bottani E, Zeviani M (2015) Emerging concepts in the therapy of mitochondrial disease. Biochim Biophys Acta Bioenerg 1847:544–557. https://doi.org/10.1016/j.bbabio.2015.03.001
42. Wolf NI, Smeitink JAM (2002) Mitochondrial disorders: a proposal for consensus diagnostic criteria in infants and children. Neurology 59:1402–1405. https://doi.org/10.1212/01.WNL.0000031795.91814.D8
43. Wong LJ (2013) Next generation molecular diagnosis of mitochondrial disorders. Mitochondrion 13:379–387. https://doi.org/10.1016/j.mito.2013.02.001
44. Wortmann SB, Mayr JA, Nuoffer JM, Prokisch H, Sperl W (2017) A guideline for the diagnosis of pediatric mitochondrial disease: the value of muscle and skin biopsies in the genetics era. Neuropediatrics 48:309–314. https://doi.org/10.1055/s-0037-1603776
45. Zhang H, Wang F, Xiao H, Yao Y (2018) The ratio of urinary α1-microglobulin to microalbumin can be used as a diagnostic criterion for tubuloproteinuria. Intractable Rare Dis Res 7:46–50. https://doi.org/10.5582/irdr.2017.01079

# Iron-sulfur cluster ISD11 deficiency (LYRM4 gene) presenting as cardiorespiratory arrest and 3- methylglutaconic aciduria

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CASE REPORT

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# Iron-sulfur cluster ISD11 deficiency (*LYRM4* gene) presenting as cardiorespiratory arrest and 3-methylglutaconic aciduria

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**Abstract**

In the era of genomics, the number of genes linked to mitochondrial disease has been quickly growing, producing massive knowledge on mitochondrial biochemistry. *LYRM4* gene codifies for ISD11, a small protein (11 kDa) acting as an iron-sulfur cluster, that has been recently confirmed as a disease-causing gene for mitochondrial disorders. We present a 4-year-old girl patient, born from non-consanguineous healthy parents, with two episodes of cardiorespiratory arrest after respiratory viral illness with progressive decreased activity and lethargy, at the age of 2 and 3 years. She was asymptomatic between crisis with regular growth and normal development. During acute events of illness, she had hyperlactacidemia (maximum lactate 5.2 mmol/L) and urinary excretion of ketone bodies and 3-methylglutaconic acid, which are normalized after recovery. A Next Generation Sequence approach with a broad gene panel designed for mitochondrial disorders revealed a novel probably pathogenic variant in homozygosity in the *LYRM4* gene [p.Tyr31Cys (c.92A>G)] with Mendelian segregation. Functional studies in the skeletal muscle confirmed a combined deficiency of the mitochondrial respiratory chain (I, II, and IV complexes). To our knowledge, this is the third case of *LYRM4* deficiency worldwide and the first with 3-methylglutaconic aciduria, not reported in any Fe-S cluster deficiency. Remarkably, it appears to be no neurological involvement so far, only with life-threating acute crisis triggered by expectably benign autolimited illnesses. Respiratory chain cofactors and chaperones are a new field of knowledge and can play a remarkable effect in system homeostasis.

**KEYWORDS**

3-methylglutaconic aciduria, Fe-S clusters, ISD11, *LYRM4*, mitochondrial disorder

**Abbreviations:** 3-HMG, 3-hydroxymethylglutaric acid; 3-MGA, 3-methylglutaconic acid; 3-MGA-uria, 3-methylglutaconic aciduria; 3-OH-IVA, 3-hydroxyisovaleric acid; ACP, acyl carrier protein; COX, cytochrome oxidase; CS, citrate synthetase; CSF, cerebrospinal fluid; ISC, iron-sulfur cluster; ISCU, iron-sulfur cluster assembly scaffold protein; ISD11, ISC biogenesis desulfurase-interacting protein 11; *LYRM4*, LYR motif-containing protein 4; MRI, magnetic resonance imaging; NFS1, cysteine desulfurase; NGS, Next Generation Sequence; NR, normal range; OXPHOS, oxidative phosphorylation.

## 1 | INTRODUCTION

In the era of genomics, the classical interpretation that mitochondrial disorders comprised those with a deficiency in one or more subunits of the respiratory chain was broadened and nowadays includes defects of complex assembly, mitochondrial DNA transcription, translation and maintenance,

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membrane function and import, mitochondrial fusion and fission, and cofactor biosynthesis (eg, coenzyme $Q_{10}$, lipoic acid, or iron-sulfur clusters).[1,2] Iron-sulfur clusters (ISCs) are crucial cofactors for mitochondrial function and facilitate the electron transport chain. More than 50 different ISCs have been identified and localized in mitochondria, nucleus, and cytosol.[3] Mitochondrial ISCs are synthesized in the mitochondrial matrix although mitochondria also interfere in nuclear ISC assembly.[4] Extramitochondrial functions include DNA replication, damage repair, and telomere maintenance.[5]

ISC biogenesis machinery is complex and not fully understood. It first requires the synthesis of a [2Fe-2S] cluster by cysteine desulfurase complex (NFS1-ISD11-ACP), which allows cysteine to act as sulfur donor (desulfuration of cysteine to alanine) while frataxin may act as the iron donor.[3,4,6,7] This process relies on the action of the iron-sulfur cluster assembly scaffold protein (ISCU) and electron chain of NAD(P)H, ferredoxin reductase, and ferredoxin.[4,8] Along with chaperones, ISCU then releases the ISC to apoproteins in an energy-dependent reaction.[4] Later, [4Fe-4S] clusters will be formed, and by the action of several ISC targeting factors, they will be inserted into specific apoproteins.[4]

Twenty known genes have a direct role in mitochondrial ISC biogenesis and 17 ISC-related genes are implicated in human disease.[9,10] For example, ISCU interacts with frataxin through ISD11, and frataxin deficiency has a negative impact on ISCs' assembly.[11]

ISD11 is a 11 kDa protein found at both mitochondria and nucleus[3] and is codified by the well-conserved LYR motif-containing protein 4 gene (*LYRM4*) located in the chromosome 6p25.1. As an early intervenient in ISC biogenesis, ISD11 deficiency can have a negative interference in multiple systems and results in combined OXPHOS deficiency (MIM#615595—COXPD19). ISD11 also plays a key role in iron homeostasis as a controller of the intracellular iron trafficking, and its deficiency leads to mitochondrial iron overload.[3,4,11,12]

To our knowledge, there are only two published patients with *LYRM4* deficiency.[9] They were consanguineous first-degree cousins with Lebanese/Sirian origin and a missense mutation (c.203G>T, p.R68L) leading to a nonfunctional NFS1-ISD11 complex. The proband had a neonatal failure to thrive severe lactic acidosis, metabolic acidosis, respiratory distress, apnea, and elevated liver enzymes, all of which progressively improved, remaining healthy at the age of 20 years. His female cousin had respiratory distress right after birth and hyperlactacidemia; at 2 months of life she had a similar crisis, evolving to respiratory arrest and later died in intensive care unit. Organic acid analysis showed lactate excretion and ketosis, without other metabolites. Both had combined deficiency of complexes I, II, and III in skeletal muscle biopsy.

## 2 | METHODS

Chromatography of urinary organic acids was performed by gas chromatography mass spectrometry; after acidification, salting and extraction with trimethylsilyl in a sample volume are equivalent to a creatinine volume of 5 mmol/L. Two internal standards were used (3-phenylbutyric acid and 3-tricarballylic acid).

Histological studies were performed on tissue from the deltoid muscle collected by open biopsy under general anesthesia. Sample was preserved in saline-moistened gauze and immediately processed. Histological studies included modified Gomori trichrome, cytochrome oxidase (COX), and succinate dehydrogenase stains.

Spectrophotometric measurements of respiratory chain enzymes and citrate synthase were carried out in skeletal muscle homogenates as previously described,[13] in a sample of deltoid muscle, immediately frozen at −80°C until analysis.

Genetic studies were performed by a Next Generation Sequence (NGS) approach with a gene panel designed for mitochondrial disorders including 209 nuclear genes known to be associated with mitochondrial diseases (Table S1). Genes of interest were captured using the SureSelectQXT kit (Agilent Technologies), followed by sequencing on the Illumina MiSeq platform. This study was approved by the Ethics Committee of Centro Hospitalar do Porto. Variants were filtered taking into account: (a) the type of pathogenic variant (missense, frameshift, stop-gain or stop-loss, and splice-site variants), (b) in silico predictors (SIFT, PolyPhen-2, MutationTaster) and their presence in databases (dbSNP, 1000 Genomes, HGMD professional, ClinVar, ExAC, OMIM, gnomAD), and (c) the population frequency [variants with a minor allele frequency (MAF) <1% in the 1000 Genomes Project (http://www.1000genomes.org) and Exome Variant Server databases (http://evs.gs.washington.edu) were filtered out]. Sanger sequencing was used to validate the mutation and to study Mendelian segregation in parents.

## 3 | CASE REPORT

We present the case of a 4-year-old girl, first child of a non-consanguineous healthy Portuguese couple, with normal growth and neurodevelopment. She had an episode of cardiorespiratory arrest at the age of 2 years after a respiratory viral infection. Microbiological studies identified respiratory syncytial virus (RSV) in respiratory secretions and Human herpesvirus 6 (HVV6) in cerebrospinal fluid (CSF). Work-up during recovery showed a persistent increased lactate (maximum 5 mmol/L, normal range [NR] <2.0 mmol/L). Her electrocardiogram, echocardiogram, and brain magnetic resonance imaging (MRI) were normal. She was discharged with pending metabolic results and was lost in the follow-up.

At the age of 3 years, she was admitted to our hospital after cardiac arrest following acute respiratory insufficiency. Similar to

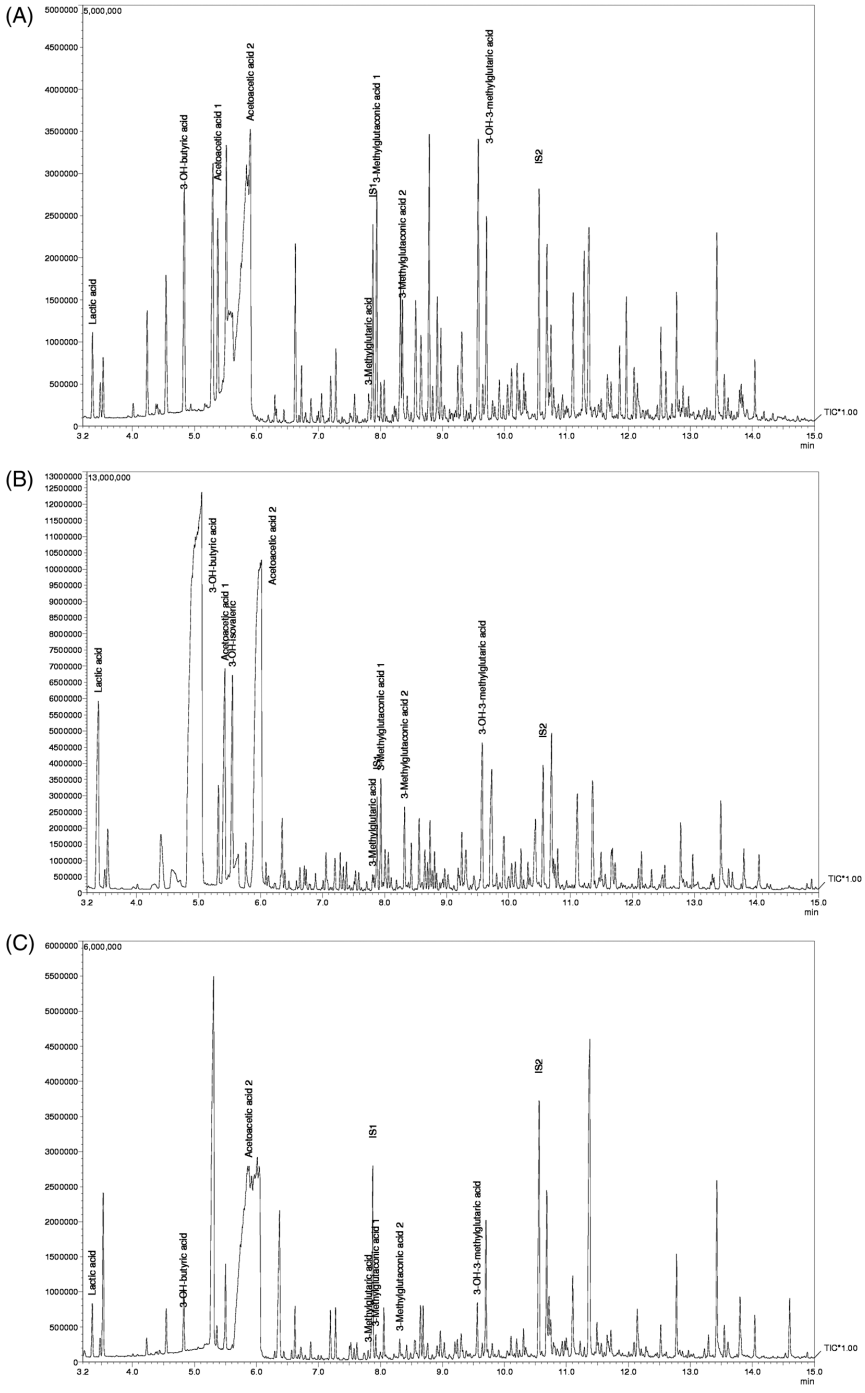


**FIGURE 1** Chromatograms of urinary organic acids showing excretion of 3-methylglutaconic acid during acute crises: A, cardiorespiratory arrest, B, during febrile mild illness without clinical deterioration, and C, during an intercrisis interval. IS1, internal standard 1 (3-phenylbutyric acid); IS2: internal standard 2 (3-tricarballylic acid)

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                                    p.Tyr31Cys
                                        ⇩
Human           MAASSRAQVLSLYRAMLRESKRFSAYNYRTYAVRRIRDAF
Zebrafish       MASCSRAQVISLYRMLMKESKKFPSYNYRTYALRRVKDGF
Chicken         MAASSRAQVLRLYRALLRESQRFSGYNYRTYAIRRIRDAF
Pig             MAASSRAQVLDLYRAMLRESKHFSAYNYRTYAIRRIRDAF
Cow             MAASSRAQVLDLYRAMLRESKRFGAYNYRTYAIRRIRDAF
Goat            MAASSRAQVLDLYRAMLRESKRFGAYNYRTYAIRRIRDAF
Horse           MAASSRAQVLDLYRAMLRESKHFSAYNYRTYAVRRIRDAF
Rat             MAASSRAQVLDLYRAMMRESKHFSAYNYRMYAVRRIRDAF
                **  *****  ***    **  *  **** ** **  * *
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**FIGURE 2** Conservation of p.Tyr31 across species. The amino acid sequence of 1-40 of human *LYRM4* is shown and compared to the corresponding sequences of eight species. Amino acid positions identical to human genome are represented as *. The p.Tyr31Cys variant is conserved among all species examined

the first episode history, parents reported nasal discharge, cough, fever, and reduced appetite for the previous 4 days, with a progressive decrease of activity. That day she had been lethargic and presented with difficulty to breath prior to the cardiorespiratory arrest.

After resuscitation, apart from pulmonary auscultation with rhonchi, her physical examination was normal. Diagnostic investigation revealed persistently high lactate (2.3-4.1 mmol/L, NR <2.0 mmol/L), normal blood and CSF counts (lactate in CSF was not performed), negative C-reactive protein, and sterile blood culture. She had no hypoglycemia and her initially elevated liver enzymes and creatine kinase (CK) (3-4 times the upper normal limit) rapidly returned to normal values. Chest X-ray showed no pneumoniae and RSV was isolated in respiratory secretions. Cardiac evaluation (electrocardiogram, echocardiogram, and continuous electrocardiographic recording) was normal and an immunodeficiency was excluded (normal immunoglobulins, flow cytometry, and adequate response to tetanus and pneumococcus vaccines).

Plasma amino acids were normal and acylcarnitines showed only low free carnitine (6.5 μM, NR 19-48 μM) with normal acetylcarnitine. Urinary organic acids on admission showed ketonuria and moderate excretion of 3-methylglutaconic acid (3-MGA) with 154 μmol/mmol creatinine (NR <19 μmol/mmol creatinine) and mild excretion of 3-hydroxyisovaleric acid (3-OH-IVA) with 85 μmol/mmol creatinine (NR 10.4-67.0 μmol/mmol creatinine) and 3-hydroxymethylglutaric acid (3-HMG) with 93 μmol/mmol creatinine (NR 6.2-49.7 μmol/mmol creatinine), as shown in Figure 1.

At this point, she enrolled in a research study for the diagnosis of mitochondrial disorders by an NGS approach. Retrieving information from the first episode showed that 2 weeks after the cardiorespiratory arrest she had mild excretion of 3-MGA (27 μmol/mmol creatinine) accompanied by multiple other metabolites, such as 4-hydroxyphenyllactic and 3-hydroxyadipic acids and traces of 3-methylglutaric, 2-OH-IVA, and sebacic acids.

After 5 days on mechanical ventilation and vasoactive support, she was weaned to spontaneous breathing and after 24 hours on noninvasive ventilation. Asymptomatic and with a normal neurologic examination, she was discharged after 2 weeks under carnitine per os. After recovery, lactate and organic acids completely normalized as well as carnitine levels, even after suspension of carnitine supplementation.

A close follow-up was guaranteed. During the subsequent episode of fever with reduced oral intake, without evidence of significant general status or neurological deterioration, CK levels and liver enzymes were normal but her urinary profiling again revealed marked ketosis and mild excretion of lactate, 3-MGA (129 μmol/mmol creatinine), 3-OH-IVA (162 μmol/mmol creatinine), and 3-HMG (102 μmol/mmol creatinine) (Figure 1). She has been admitted again to the pediatric ward for two other occasions: one for fever with reduced oral intake and lethargy and another for a wheezing episode with hypoxia, both with full recovery without any complications.

During an intercritical and asymptomatic interval, metabolic re-evaluation had no hyperlactacidemia, ketosis, elevated CK, or altered organic acid excretion (Figure 1).

At the age of 4 years, she currently attends preschool and has ballet lessons, which she completes without difficulty and both parents and patient do not report any complaints. Her physical examination remains normal.

Currently, the patient is not under any treatment. Follow-up includes regular clinical and biochemical evaluations along with general recommendations. Special caution is given during catabolic states with admittance and intravenous fluid intake (5% dextrose in normal saline). Brain MRI evaluation was delayed pondering the general anesthetic risk vs her normal neurological status.

## 4 | RESULTS

NGS for mitochondrial disorders revealed a probably pathogenic novel variant in homozygosity in the *LYRM4* gene [p.Tyr31Cys (c.92A>G)], with heterozygous parents for the same variant. The pathogenicity was predicted bioinformatically by SIFT, PolyPhen-2, and MutationTaster as probably pathogenic variants. The p.Tyr31Cys variant affects an amino acid that is highly conserved in different species through evolution (Figure 2) and was absent in 100 alleles from Portuguese subjects and is not reported in public SNP databases, including dbSNP, the Exome Variant Server, and gnomAD (without any reported homozygote).

A muscle biopsy was performed. Histology showed occasional atrophy of muscle fibers and frequent accumulation of lipid droplets, without variation on fiber size or COX negative or red-ragged fibers. Functional assay of the respiratory chain showed elevated citrate synthetase activity (354.3 nmol/min/mg, NR 85.0-180 nmol/min/mg) and confirmed the diagnosis with the evidence of a combined deficiency of complexes I, II, and IV with 40, 23, and 31% of residual activity, respectively (Table 1).

## 5 | DISCUSSION

This case broadens the phenotype knowledge on ISD11 deficiency being the third case worldwide with a novel pathogenic variant on *LYRM4*. Assumptions based on a small series must be cautious, but within these three patients, rapid clinical status deterioration with respiratory failure and arrest in the course of an infectious intercurrence appear to be a common feature. When surviving these acute crises, apparently asymptomatic periods follows. Lim et al hypothesized that hepatic cystathionase activity is prone to be lower during the neonatal period subsequent to low availability of cysteine as sulfur donor, explaining both neonatal onset and ability to survive after this period.[9] However, this justification does not account for the late onset and repeated episodes on this patient.

As in other cases with ISD11 deficiency, hyperlactacidemia and ketosis where present, although not as elevated as reported. Combined respiratory chain deficiency is similar to findings in other patients with iron-sulfur deficiencies.

A particular characteristic of this patient is the consistent and significant (>40 µmol/mmol creatinine) excretion of 3-MGA during acute events, not previously reported in patients with any Fe-S cluster deficiency. 3-MGA-uria can be found in various inborn metabolic disorders commonly along with excretion of other metabolites (fatty acid oxidation disorder, methylmalonic and propionic aciduria, glycogen storage disease, or urea cycle disorder). It can be a feature of mitochondrial disorders present in about 10% of patients,[14] but usually is not the prominent hallmark of disease and excretion is only slightly elevated and as an accompanying finding.[15] It represents not an impairment on the leucine pathway but an alternative route for acetyl-CoA degradation.[16]

Inborn errors of metabolism with 3-MGA-uria as discriminative feature (isolated, significantly or repeatedly elevated) can be classified as primary or secondary 3-MGA-uria.[15] Primary 3-MGA-uria is caused by 3-methylglutaconyl-CoA hydratase deficiency, an inborn error of the leucine pathway. Known diseases with secondary 3-MGA-uria can be due to the following[14,15,17-19]: (a) defective phospholipid remodeling: Barth syndrome (*TAZ* gene) and MEGDEL syndrome (*SERAC1* gene); (b) mitochondrial membrane-associated disorder: Costeff syndrome (*OPA3*), TMEM70 defect (*TMEM70*), and DCMA syndrome (*DNAJC19*); (c) unknown mechanism: Sengers syndrome (*AGK*), CLPB deficiency (*CLPB*), TIMM50 deficiency (*TIMM50*), HTRA2 defect (*HTRA2*), and short-chain enoyl-CoA hydratase deficiency (*ECHS1*).

This is the first case of Fe-S cluster with 3-MGA-uria, extending the differential diagnosis of 3-MGA-uria as the presenting feature.

Being a disorder related to iron metabolism, with the possibility to induce iron overload on tissues and storage depletion, evaluation of ferritin, transferrin, soluble transferrin receptor, or hepcidin may help understand the impact on the dynamics of iron metabolism, despite no evidence of impaired iron homeostasis in our patient so far.

**TABLE 1** Respiratory chain functional assay in skeletal muscle

| | Measured activity (nmol/min/mg NPC/CS) | Residual activity (%) | Reference values (nmol/min/mg NPC/CS) |
|---|---|---|---|
| Complex I | 8.0 | 40 | 8.8-30.8 |
| Complex II | 5.4 | 23 | 12.0-35.0 |
| Complex III | 21.1 | 50 | 22.2-62.2 |
| Complex II + III | 1.3 | 17 | 2.6-12.0 |
| Complex IV | 7.1 | 31 | 11.5-34.5 |

Abbreviations: CS, citrate synthetase; NPC, noncollagenous proteins.

Since cysteine acts the major sulfur donor, supplementation with this nonessential amino acid can be beneficial, especially during periods of higher energy requirement. The rationale for treatment with L-cysteine or *N*-acetylcysteine is supported by studies on patients with mitochondrial disorders with some benefit in those with translation-related defects.[20] It can be expected that a corresponding reduction in iron overload diminishes its deleterious effects.

Raised awareness and a low threshold to hospital admittance in patients with ISD11 deficiency may be crucial to improve survival. Routine neurological and neurodevelopment evaluation must be granted.

This case illustrates the importance of metabolic work-up to be performed as soon as possible, while the patient is acutely ill, as it may be a unique opportunity to diagnose a critical condition. Moreover, life-threatening events disproportional to symptoms and medical history should elicit the possibility of energy-related inherited metabolic disorder. Acute presentations of mitochondrial disorders will become more common with the improved diagnostic yield of genetic studies.

## AUTHOR CONTRIBUTIONS

M.P.C. was involved in patient diagnosis, literature review, and drafting of the manuscript. J.C. was involved in patient follow-up and investigation, and drafting of the manuscript. A.D. worked on chromatography of urinary organic acids, interpretation, and figure construction. C.N. worked on genetic studies and interpretation. A.B. and E.M. were involved in patient's work-up and did critical manuscript review. L.V. worked on functional and genetic studies interpretation and did critical manuscript review.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## COMPLIANCE WITH ETHICAL STANDARDS

The study "Genetic Defects of Mitochondrial Diseases: a Next Generation Sequencing Approach" (FCT PTDC/DTP-PIC/2020/2014) was approved by the Ethics Committee of Centro Hospitalar do Porto (REF. 2016.194(164-DEFI/153-CES). Informed consent from patient's parents was obtained.

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## REFERENCES

1. DiMauro S, Schon EA. Mitochondrial respiratory-chain diseases. *N Engl J Med*. 2003;348:2656-2668. https://doi.org/10.1056/NEJMra022567.
2. Liang C, Ahmad K, Sue CM. The broadening spectrum of mitochondrial disease: shifts in the diagnostic paradigm. *Biochim Biophys Acta*. 2014;1840:1360-1367. https://doi.org/10.1016/j.bbagen.2013.10.040.
3. Shi Y, Ghosh MC, Tong W-H, Rouault TA. Human ISD11 is essential for both iron-sulfur cluster assembly and maintenance of normal cellular iron homeostasis. *Hum Mol Genet*. 2009;18:3014-3025. https://doi.org/10.1093/hmg/ddp239.
4. Lill R, Hoffmann B, Molik S, et al. The role of mitochondria in cellular iron–sulfur protein biogenesis and iron metabolism. *Biochim Biophys Acta*. 2012;1823:1491-1508. https://doi.org/10.1016/j.bbamcr.2012.05.009.
5. Maio N, Rouault TA. Iron–sulfur cluster biogenesis in mammalian cells: new insights into the molecular mechanisms of cluster delivery. *Biochim Biophys Acta*. 2015;1853:1493-1512. https://doi.org/10.1016/j.bbamcr.2014.09.009.
6. Angerer H, Schönborn S, Gorka J, et al. Acyl modification and binding of mitochondrial ACP to multiprotein complexes. *Biochim Biophys Acta*. 2017;1864:1913-1920. https://doi.org/10.1016/j.bbamcr.2017.08.006.
7. Cory SA, Van Vranken JG, Brignole EJ, et al. Structure of human Fe–S assembly subcomplex reveals unexpected cysteine desulfurase architecture and acyl-ACP–ISD11 interactions. *Proc Natl Acad Sci U S A*. 2017;114:E5325-E5334. https://doi.org/10.1073/pnas.1702849114.
8. Cai K, Tonelli M, Frederick RO, Markley JL. Human mitochondrial ferredoxin 1 (FDX1) and ferredoxin 2 (FDX2) both bind cysteine desulfurase and donate electrons for iron–sulfur cluster biosynthesis. *Biochemistry*. 2017;56:487-499. https://doi.org/10.1021/acs.biochem.6b00447.
9. Lim SC, Friemel M, Marum JE, et al. Mutations in *LYRM4*, encoding iron–sulfur cluster biogenesis factor ISD11, cause deficiency of multiple respiratory chain complexes. *Hum Mol Genet*. 2013;22:4460-4473. https://doi.org/10.1093/hmg/ddt295.
10. Vanlander AV, Van Coster R. Clinical and genetic aspects of defects in the mitochondrial iron–sulfur cluster synthesis pathway. *J Biol Inorg Chem*. 2018;23:495-506. https://doi.org/10.1007/s00775-018-1550-z.
11. Shan Y, Napoli E, Cortopassi G. Mitochondrial frataxin interacts with ISD11 of the NFS1/ISCU complex and multiple mitochondrial chaperones. *Hum Mol Genet*. 2007;16:929-941. https://doi.org/10.1093/hmg/ddm038.
12. Saha PP, Srivastava S, Praveen Kumar SK, Sinha D, D'Silva P. Mapping key residues of ISD11 critical for NFS1-ISD11 subcomplex stability: implications in the development of mitochondrial disorder, COXPD19. *J Biol Chem*. 2015;290:25876-25890. https://doi.org/10.1074/jbc.M115.678508.
13. DiMauro S, Servidei S, Zeviani M, et al. Cytochromec oxidase deficiency in leigh syndrome. *Ann Neurol*. 1987;22:498-506. https://doi.org/10.1002/ana.410220409.
14. Wortmann SB, Kluijtmans LAJ, Rodenburg RJ, et al. 3-Methylglutaconic aciduria—lessons from 50 genes and 977 patients. *J Inherit Metab Dis*. 2013;36:913-921. https://doi.org/10.1007/s10545-012-9579-6.
15. Wortmann SB, Duran M, Anikster Y, et al. Inborn errors of metabolism with 3-methylglutaconic aciduria as discriminative feature: proper classification and nomenclature. *J Inherit Metab Dis*. 2013;36:923-928. https://doi.org/10.1007/s10545-012-9580-0.
16. Ikon N, Ryan RO. On the origin of 3-methylglutaconic acid in disorders of mitochondrial energy metabolism. *J Inherit Metab Dis*. 2016;39:749-756. https://doi.org/10.1007/s10545-016-9933-1.
17. Fitzsimons PE, Alston CL, Bonnen PE, et al. Clinical, biochemical, and genetic features of four patients with short-chain enoyl-CoA hydratase (ECHS1) deficiency. *Am J Med Genet A*. 2018;176:1115-1127. https://doi.org/10.1002/ajmg.a.38658.
18. Huffnagel IC, Redeker EJW, Reneman L, Vaz FM, Ferdinandusse S, Poll-The BT. Mitochondrial encephalopathy and transient 3-methylglutaconic aciduria in ECHS1 deficiency: long-term follow-up. *JIMD Rep*. 2018;39:83-87. https://doi.org/10.1007/8904_2017_48.
19. Kovacs-Nagy R, Morin G, Nouri M, et al. HTRA2 defect: a recognizable inborn error of metabolism with 3-methylglutaconic aciduria as discriminating feature characterized by neonatal movement disorder and epilepsy—report of 11 patients. *Neuropediatrics*. 2018;49:373-378. https://doi.org/10.1055/s-0038-1667345.
20. Bartsakoulia M, Möller JS, Gomez-Duran A, Yu-Wai-Man P, Boczonadi V, Horvath R. Cysteine supplementation may be beneficial in a subgroup of mitochondrial translation deficiencies. *J Neuromuscul Dis*. 2016;3:363-379. https://doi.org/10.3233/JND-160178.

## SUPPORTING INFORMATION

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

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# Phenotyping mitochondrial glutamyl-tRNA synthetase deficiency (EARS2): A case series and systematic literature review

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# Phenotyping mitochondrial glutamyl-tRNA synthetase deficiency (*EARS2*): A case series and systematic literature review

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ABSTRACT

Mitochondrial glutamyl-aminoacyl tRNA synthetase deficiency, stemming from biallelic mutations in the *EARS2* gene, was first described in 2012. With <50 cases reported globally, this condition exhibits a distinct phenotype of neonatal or childhood-onset, often referred to as *leukoencephalopathy with thalamus and brainstem involvement and high lactate* (LTBL). It has also been one of the few reversible mitochondrial disorders described. The natural history of these patients is poorly documented, ranging from clinical and radiological improvement to early death.

Herein, we detail three cases from our centre, including follow-up on the Portuguese patient reported by Steenweg et al., These cases illustrate the phenotypic spectrum: i) rapidly progressive neonatal presentation with lactic acidemia and corpus callosum agenesis, leading to early death; ii) early onset with a severe, slowly progressive course; iii) early onset with a milder phenotype, showing some improvement and mild neurological symptoms. Additionally, we conducted a systematic literature review on cases of *EARS2*-deficient patients, focusing on clinical manifestations, laboratory findings, radiological aspects, and disease progression over time, along with respective data analysis. "Patients with EARS2 deficiency typically present within the first year of life with a well-defined neurometabolic disorder picture, often including hypotonia and/or spasticity, along with neurodevelopmental delay or regression. There are no pathognomonic features specific to EARS2 deficiency, and no genotype-phenotype correlation has been identified." Comparing to initial characterization by Steenweg et al., this analysis reveals an expanded disease spectrum. We propose a novel strategy for clustering phenotypes into severe, moderate, or mild disease based on initial presentation, seemingly correlating with disease progression. The paucity of data on the disease's natural history highlights the need for a multicentric approach to enhance understanding and management.

*Take-home message:* Analysis of all cases published with EARS2 deficiency allows for establish disease spectrum and a novel strategy for clustering phenotypes which correlate to disease progression.

## 1. Introduction

Mitochondria perform a variety of cellular functions, with oxidative phosphorylation (OXPHOS) being particularly important for the efficient production of ATP, the cell's primary energy currency (Paiva Coelho et al., 2019; DiMauro and Schon, 2003). Beyond OXPHOS, mitochondria are integral to several metabolic pathways, ion homeostasis, programmed cell death, and the handling of free radicals, complicating the pathophysiology of mitochondrial disorders (MD) beyond mere OXPHOS dysfunction (Davison and Rahman, 2017).

*Abbreviations:* aaRS, Aminoacyl-tRNA cytosolic synthetases; ADHD, Attention deficit hyperactivity disorder; ATP, Adenosine triphosphate; COXP12, Combined Oxidative phosphorylation deficiency 12; CSF, Cerebrospinal fluid; DNA, Deoxyribonucleic acid; EEG, Electroencephalography; FLAIR, Fluid attenuated inversion recovery; LTBL, Leukoencephalopathy with thalamus and brainstem involvement with high lactate; MD, mitochondrial disorders; MRI, Magnetic resonance imaging; MRS, Magnetic resonance spectroscopy; mtARS, Mitochondrial aminoacyl-tRNA synthetases; mtDNA, Mitochondrial DNA; mtGluRS, Glutamine-associated mitochondrial synthetase aminoacyl-tRNA; NR, normal range; OXPHOS, Oxidative phosphorylation; tRNA, Aminoacyl-tRNA synthetase.

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Mitochondrial functions depend on genes from both the mitochondrial and nuclear genomes, enhancing the diversity of MD (DiMauro and Schon, 2003). These organelles contain their own machinery for protein synthesis, directed by genetic codes from both mitochondrial DNA and, predominantly, nuclear DNA (Pearce et al., 2013; Diodato et al., 2014). Aminoacyl-tRNA synthetases (ARS) are enzymes for protein translation, binding amino acids to their respective tRNA molecules through a covalent bond at ATP's expense. Each ARS is specific to one amino acid and is encoded by distinct nuclear genes (Diodato et al., 2014; Al Balushi et al., 2020; Fuchs et al., 2019).

Mitochondrial ARS (mtARS or ARS2) and cytosolic ARS (aaRS or ARS1) are distinguished by their localization, with each ARS prefixed by a letter denoting its specific amino acid (e.g., *LARS* for cytosolic and *LARS2* for mitochondrial Leucyl-mtARS). mtARS synthetases are imported into mitochondria, crucial for synthesizing 13 mtDNA-encoded proteins. Deficiency in any mtARS leads to inadequate protein synthesis, causing MD due to a combined OXPHOS defect (Diodato et al., 2014; Boczonadi and Horvath, 2014a; Podmanicky et al., 2024).

The first mtARS deficiency was reported in 2007 with subsequent reports covering all mtARS, showing autosomal recessive inheritance without de novo cases (Al Balushi et al., 2020; Scheper et al., 2007; Konovalova and Tyynismaa, 2013; Fine et al., 2019). Different phenotypes have been associated with each gene, suggesting important tissue and protein specificity (Konovalova and Tyynismaa, 2013; Del Greco and Antonellis, 2022).

*EARS2* nuclear gene is responsible for encoding *mitochondrial glutamyl-tRNA synthetase* (mtGluRS), essential for protein translation. First diagnosis emerged from unbiased exome-next-generation sequencing in a patient with early-onset mitochondrial leukoencephalopathy, later supported by identifying 11 additional patients with a similar MRI pattern, all with biallelic variants in *EARS2* (Steenweg et al., 2012a; Steenweg et al., 2012b). This MRI pattern, termed 'Leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL) (OMIM#612799, COXPD12) exhibited extensive symmetrical cerebral white matter abnormalities, sparing the periventricular rim and symmetrical signal abnormalities in the thalami, midbrain, pons, *medulla oblongata*, and cerebellar white matter (Steenweg et al., 2012a). Other neuroradiological features such as basal ganglia T2 hyperintensities and cerebellar atrophy were also noted, as well as lactate peak in magnetic resonance spectroscopy (MRS) (Steenweg et al., 2012a; Steenweg et al., 2012b; Barbosa-Gouveia et al., 2020).

Steenweg et al. detailed a biphasic disease course for *EARS2* deficiency, outlining two phenotypes: *(i)* a severe form with onset under 6 months of age marked by profound neurological symptoms (absence of psychomotor development, marked hypotonia) and followed by a stable phase (spastic tetraparesis, dystonia, visual impairment, and seizures), while maintaining consistently elevated lactate levels in body fluids; (*ii*) a mild phenotype with onset between 6 and 12 months of age, characterized by normal or mildly delayed early development, spasticity, loss of milestones, seizures, extreme irritability with varying degrees of improvement noted post-first year, including amelioration of lactate levels. A similar MRI pattern was present across phenotypes and potential correlation was suggested between severe and early onset phenotype and more diffuse pattern of abnormalities and corpus callosum agenesis (Steenweg et al., 2012a; Steenweg et al., 2012b). Later described were (iii) intermediate phenotypes, manifesting within the initial 6 months of life and showing spontaneous clinical and neuroimaging recovery, thus classifying this deficiency as one of the few reversible infantile mitochondrial diseases (Boczonadi and Horvath, 2014b; Sellars et al., 2017; Biancheri et al., 2015; Hathazi et al., 2020). In fact, a recent work suggests a digenic inheritance of *EARS* heterozygous damaging variants plus a mtDNA variant (m.14674 T > C) for these reversible presentations (Hathazi et al., 2020).

Since then, disease spectrum was broadened from antenatal presentation (ranging from a(*dys*)genesia of corpus callosum to fetal akinesia) to adulthood-onset (single patient with previously unremarkable medical records) with a neurodegenerative disorder and behavioral abnormalities, pyramidal and extrapyramidal signs and progressive cognitive decline from the 7th decade of life (Pergande et al., 2020; Boutaud et al., 2023; Monfrini et al., 2020a).

### 1.1. Aims

The main goal of this work is to methodically compile information in literature pertaining to cases with mitochondrial glutamyl-tRNA synthetase deficiency (*EARS2*) and associated phenotypes, focusing on clinical manifestations, laboratory findings, radiological aspects, as well as progression over time.

## 2. Methodology

### 2.1. Case reports

We describe three cases followed at our centre with further follow-up information on the Portuguese patient reported by Steenweg et al (Steenweg et al., 2012a). Informed consent was obtained from all patient's legal representatives.

### 2.2. Literature review

A systematic and independent electronic search was pursued by one researcher for the terms '*EARS2*', 'Leukoencephalopathy with thalamus and brainstem involvement with high lactate', 'LTBL', 'Mitochondrial glutamyl-tRNA synthetase', 'Leukoencephalopathy with high lactate', 'Combined Oxidative phosphorylation deficiency 12', 'COXPD12', 'Glutamyl-tRNA synthetase 2', in PubMed, ScienceDirect and Scopus (search terms and filters can be consulted in Table SI). The search conducted in October 2023 across three databases produced a total of 318 results, according to the PRISMA 2020-compliant flow diagram depicted in Fig. 1.

Case reports, multicentric genetic studies, and/or articles on *EARS2* biallelic variants in humans were considered for inclusion. Duplicates, book sections and records with titles or frameworks deemed unrelated to topic (such as basic sciences, outside the scope of human pathology, reviews not of interest, other genes) were excluded during initial screening ($n = 247$). Additional screening of eligible reports, performed by two investigators, considered further exclusion criteria such as lacking patient referral/data, cases from unborn fetuses, digenic inheritance, monoallelic *EARS2* variants or without identified causative genes.

From the reports sought for retrieval ($n = 37$); only one report was not retrieved. Final screening of full-text articles was performed, excluding several reports ($n = 20$), resulting in the inclusion of sixteen studies in the review. Additional search methods such as citation tracking ($n = 6$) and handsearching ($n = 1$) were actively used, bringing the total studies included for review to the final number of 23 papers.

### 2.3. Data extraction

Demographics, familial history, medical history (clinical, development and growth, laboratory and radiological), therapeutic interventions and of genetic results, as outlined in Table SII.

## 3. Results

### 3.1. Part I. Case reports

**Case 1**. 34-day-old male transferred to our centre for vomiting, feeding refusal, hypotonia, poor suction, and failure to thrive, alongside lactic acidosis (pH 7.21; lactate 6.6 mmol/L, normal range (NR) <2,2 mmol/L) and elevated liver enzymes (2.5× above the upper normal

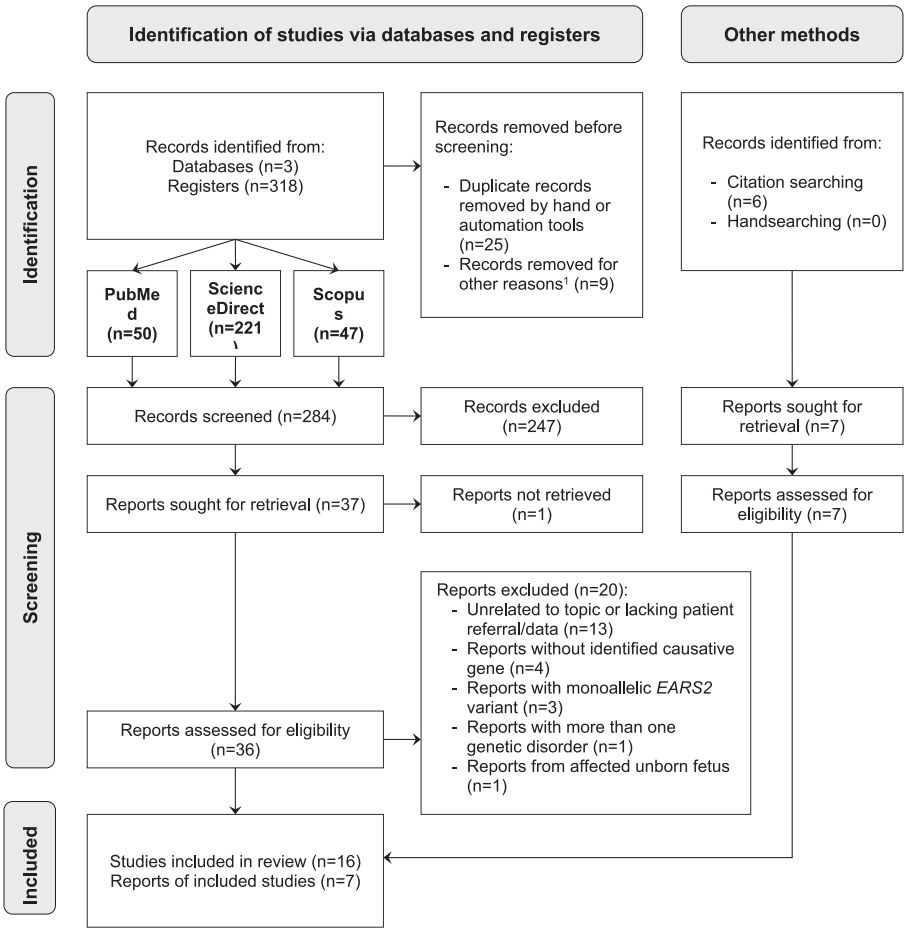


**Fig. 1.** PRISMA 2020-compliant flow diagram depicting our database search. ([1]Book sections).

limit). Persistent lactic acidosis (7.8–9.54 mmol/L), ketonuria, increased liver enzymes, and non-specific mitochondrial dysfunction markers (amino acids, urinary organic acids) were noted. Brain MRI (Fig. 2) showed corpus callosum agenesis, brainstem T1 hypointensity (hypomyelination), cerebellar T2 hyperintensity, and cerebral white matter T2 hyperintensities (subcortical parieto-occipital, anterior temporal, frontal regions, FLAIR hypointensity). MRS revealed lactate, alanine, and pyruvate peaks. Ophthalmologic and cardiac exams were normal. Nasogastric tube feeding enabled slight weight gain and home care. At 60 days, the patient presented with prostration, progressive respiratory failure, severe lactic acidosis (pH 6.9; unmeasurable HCO3 and lactate levels), leading to death within hours. Genetic testing identified biallelic the previously reported EARS2 variants: c.184 A > T, (p.Ile62Phe) and c.920 T > C, (p.Leu307Ser) (Nogueira et al., 2024).

Later pregnancies resulted in one affected fetus (terminated, normal histopathology at 20 weeks) and a healthy sibling.

**Case 2.** 10-month-old boy, born with transient icterus, hepatomegaly, and feeding difficulties, was brought to our attention for the loss of smile and speech, additional feeding difficulties, and spasticity (Steenweg et al., 2012a). In his initial months, the patient demonstrated axial hypotonia, signs of a pyramidal syndrome, nephrolithiasis, recurrent episodes of metabolic acidosis and hypoglycemia, continuous hyperlactatemia, and elevated liver enzyme levels. Brain MRI (Fig. 2) exibited bilateral symmetrical hyperintensities, predominantly affecting subcortical and periventricular white matter, with notable involvement of the corpus callosum, putaminal nuclei, thalami, mesencephalon, periaqueductal gray matter, cerebellar hemispheres' deep gray matter, and the posterior brainstem. MRS exhibited a lactate peak in cerebral cortex.

At 12 months, developmental assessment (Griffiths' scale) indicated global significant global delay. Neurological examination revealed spasticity and brisk deep tendon reflexes in upper and lower limbs. Till 18 months of age he exhibited macrocephaly, with subsequent normalizing of head circumference.

Follow-up revealed slowly progressive deterioration with epilepsy, chronic obstructive pulmonary disease and obstructive sleep apnea requiring noninvasive ventilation, constipation and neurogenic bladder dysfunction, while maintaining recurrent metabolic derangements. Follow-up revealed slowly progressive deterioration, characterized by the onset of epilepsy, chronic obstructive pulmonary disease and obstructive sleep apnea requiring noninvasive ventilation, constipation and neurogenic bladder dysfunction, while maintaining recurrent

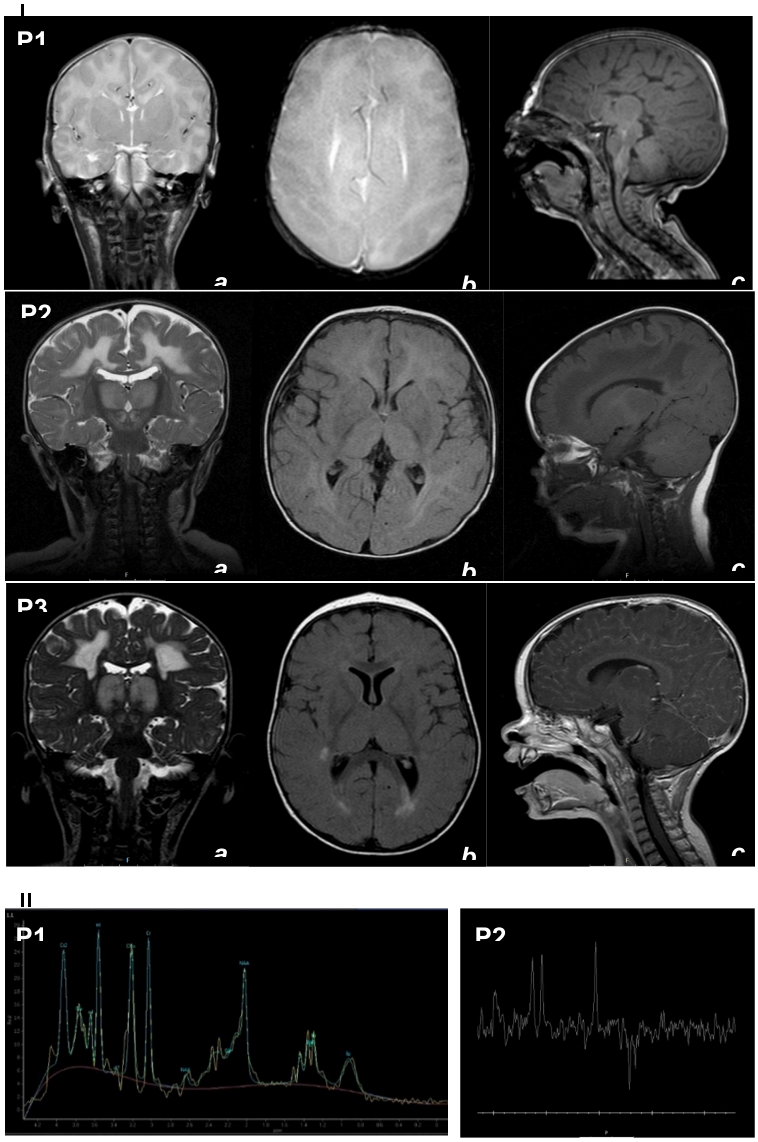


**Fig. 2.** I - First MRI images from our patients, T2 FLAIR coronal and axial and T1 sagital (c) P1. age at exam 47 days, P2. age at exam 10 months. P3. age at exam 9 months. II- H1-MRS from patients 1 and 2, showing lactate peak.

metabolic derangements. By 10 years, severe malnutrition due to feeding complications led to the placement of a gastrostomy tube. Currently, at 14 years old, he has tetrapyramidal syndrome with axial hypotonia being fully dependent on others, devoid of verbal communication or mobility. Further MRIs were refused, and he remains in domiciliary palliative care.

Molecular study revealed *EARS2* compound heterozygosity: c.1 A > G, (p.Met1?) and c.320G > A, (p.Arg107His) 16. No improvement was noted after 6 months of treatment with ubiquinone, thiamine plus biotin.

**Case 3**. 15-month-old female, delivered at full term following an uneventful pregnancy and birth, with a perfect Apgar score of 10/10 and normal birth weight. She was referred to our hospital clinic due to axial hypotonia, initially observed at 6 months. An MRI at 9 months (Fig. 2) indicated potential MD, revealing signal abnormalities in both infra- and supratentorial structures. These abnormalities presented as hyperintensity on T2 and FLAIR sequences, with a confluent pattern in the semioval centers and corona radiata but sparing the U fibers and periventricular white matter. There was no history of regression or epilepsy. She exhibited moderate global developmental delays, generalized muscle weakness, and heightened deep tendon reflexes. Laboratory investigation was largely unremarkable, including liver enzymes and creatine kinase, except for consistently elevated serum lactate levels (2.8 to 5.4 mmol/L, NR: <2,2 mmol/L), increased serum alanine, and

intermittent mild urinary excretion of glutaric acid. There was no indication of peripheral neuropathy, cardiopathy, or organomegaly. Her growth was normal and there were no feeding difficulties.

MRI at 2,5 years demonstrated extensive hyperintensities in T2 and FLAIR in the periventricular and subcortical white matter of the cerebral hemispheres, particularly in the posterior periventricular regions, bilateral involvement of basal ganglia, thalami, posterior aspect of the pons, and midbrain (especially peri-aqueductal gray matter). Areas of demyelination in the supratentorial white matter were also observed. MRS was normal.

At beginning of school, she was diagnosed with attention deficit disorder, but showed otherwise very mild development delay, learning how to read and write. Parents assure an overall improvement throughout the years. She showed static mild cerebellar syndrome, with ataxia, changes in balance and fine motor skills difficulties. Currently aged 8, her brain MRI reevaluation is pending.

She is an *EARS2* compound heterozygous for the pathogenic variants: c.1413delG, (p.Lys471Asnfs*13) and c.1235G > A, (p.Arg412His) (Nogueira et al., 2024).

Segregation studies confirmed biallelic variants of *EARS2* in all three cases.

### 3.2. Part II. Literature review

#### 3.2.1. General description of the sample

We identified 43 cases, male predominant (24/39, 62%), with a panethnic distribution of patients despite a clear European descent predominance (32/43, 74%), particularly in Portugal (6/43, 14%). Cases originated from 40 families (3 pairs of affected siblings), three of them consanguineous. One sibling's gestation was interrupted due to brain malformation.

Most patients presented within the first year of life (35/38, 92%) and, of those, several within the first month (12/38, 31%), some shortly after birth. Only one patient was assessed at late adulthood. Pre-natal findings included one case with non-neurological malformations, one with enlargement of ventricles, and another with polyhydramnios. One case experienced birth complications (preterm and needed resuscitation), and one was small for gestational age.

Follow-up information was limited: one patient for which age at diagnosis or follow-up data was entirely unknown, 1 patient for whom only the neonatal period was described; 2 cases with data till infancy (first 12 months); 7 patients till toddlerhood (from 12 to 36 months); 16 patients until childhood (from 36 months to 10 years); 6 for adolescence (10 to 19 years). The adult-onset patient had follow-up for a few years.

#### 3.2.2. Clinical manifestations

Overall clinical features (Table 1) showed a predominant neurometabolic profile for *EARS2* deficiency.

At presentation, with a median age of 4 months, the most frequent symptom was axial or generalized hypotonia (25/43, 58%), followed by developmental delay (18/43, 42%), metabolic acidosis (10/43, 23%), seizures (9/43, 21%), failure to thrive (8/43, 19%), elevation of liver enzymes (17/43, 6%), feeding difficulties (7/43, 16%), spasticity (6/43, 14%), and muscle involvement, including weakness (5/43, 12%). Regression was reported in 14% (6/43) of patients at presentation.

A significant portion of patients initially presenting with hypotonia (axial or generalized) later evolved with progressive spasticity (15/41, 37%), however both features could exist independently. Irritability during early years was common (8/41, 20%). Tetraparesis was observed in 22% patients (9/41). Epilepsy and hyperreflexia were described in 39% (16/41) and 46% (19/41), respectively. Ataxia (8/41) and movement disorders (mainly dystonia) were present in 20% of patients. The adulthood-onset patient presented with unilateral postural, intention tremor of right superior limb and neurocognitive decline. Both macro and microcephaly were described with similar frequencies [10% (4/41) and 12% (5/41), respectively].

**Table 1**
Major clinical features patients with EARS2 deficiency ([1]Excludes patients (P 14 and P 39) in whom data was insufficient or unclear to classify their clinical features).

| Clinical findings | | Prevalence at presentation ($n = 43$) | Prevalence at any age[1] ($n = 41$) | Prevalence in those who survived 1st year of life ($n = 32$) |
|---|---|---|---|---|
| Development | Developmental delay | 42% (18) | 73% (30) | 94% (30) |
| | Regression | 14% (6) | 22% (9) | 28% (9) |
| CNS | Axial or generalized hypotonia | 58% (25) | 63% (26) | 65% (21) |
| | Spasticity and/or hypertonia | 14% (6) | 49% (20) | 63% (20) |
| | Initial hypotonia with later progression to spasticity | – | 41% (17) | 47% (17) |
| | Increased deep tendon reflexes | 7% (3) | 46% (19) | 56% (18) |
| | Epilepsy/seizures | 21% (9) | 39% (16) | 50% (16) |
| | Movement disorder | 5% (2) | 19% (8) | 25% (8) |
| | Ataxia | 0% (0) | 19% (8) | 25% (8) |
| | Irritability | 2% (1) | 20% (8) | 25% (8) |
| Liver | Elevated liver enzymes | 16% (7) | 29% (12) | 25% (8) |
| Muscle | Muscle weakness and/or atrophy | 12% (5) | 27% (11) | 28% (9) |
| GI tract | Feeding difficulties | 16% (7) | 29% (12) | 25% (8) |
| Growth | Failure to thrive | 19% (8) | 24% (10) | 25% (8) |
| Metabolic | Elevated lactate levels (blood, CSF or MRS) | 39% (18) | 73% (30) | 75% (24) |

Non-neurological features include failure to thrive (10/41, 24%), isolated elevated liver enzymes (4/41, 10%), 7% elevated liver enzymes plus hepatomegaly (3/41, 7%), elevated liver enzymes plus markers of cholestasis (2/41, 5%) and liver failure (3/41, 7%). Muscle weakness and/or atrophy were common (11/41, 27%). Muscular eye involvement was present in 5% (2/41) as ptosis with ophthalmoparesis or exotropia. There were no reports of retinopathy. Unspecified visual impairment was described in 10% (4/41), with only one patient with concomitant eye muscle involvement.

Renal involvement was not reported except for a single case of nephrolithiasis (case 2). Cardiomyopathy was also observed in one patient with mild hypertrophy of cardiac interventricular septum. There were no hearing loss or endocrinological reports.

Despite its high prevalence, elevated lactate levels (blood, CSF or MRS) were not a universal finding (30/41, 73%), including in one patient with typical MRI findings without lactate peak on 1H-NMRS. Around a third of those (15/41, 37%) had persistent or recurring metabolic acidosis. Three patients developed ketosis (3/41, 7%), one of which had concurrent hyperglycemia, and another three hypoglycemia (3/41, 7%). Only one case showed hyperammonemia, while two had high alpha-fetoprotein levels (2/41, 5%). Cytopenias were not observed, and serum uric acid levels were within normal range. Acylcarnitine and urinary organic acids were uninformative. Common histological findings included ragged red fibers and COX-deficient fibers, although some patients had normal muscle histology. Analysis of OXPHOS complexes was not performed in most patients, however revealed combined complex deficiency as the most frequent finding (9/41, 22%), with a predominance of complex I and IV activity deficiencies.

#### 3.2.3. Radiological features

Neurorradiological findings at first MRI are summarized in Table 2. Three patients did not have brain MRI descriptions and age of MRI was unknown for five patients. For most patients (29/35, 83%), first MRI was performed under 2 years of age, while 9% (3/35) were performed in childhood, 6% (2/35) in adolescence and for one patient at 68 years of age.

Typical MRI presentation under two years included significant deep white matter signal abnormalities, which could present either in a diffuse or symmetrical manner, frequently associated with swelling and/or restricted diffusion. Brainstem and thalamus signal abnormalities were almost omnipresent (23/29, 79%) and basal ganglia involvement was present in 48% (14/29) of these. Despite extensive white matter involvement, periventricular rim was spared in 59% (17/29) patients. Other frequent findings were abnormalities of dentate nucleus (10/29, 34%) and delayed myelination (7/29, 24%). One patient had hypothalamus involvement. Only one patient had basal ganglia abnormalities without white matter involvement.

Corpus callosum agenesis or hypogenesis represented 65% (19/29) of patients under 2 years of age (including one cranial ultrasound description). Agenesis was more common in early onset with severe phenotypes, but thin corpus callosum (more commonly the posterior part) did not correlate with disease severity or progression. The adult-onset patient showed corpus callosum atrophy. Additionally, 4 patients had thalamus malformations unrelated to corpus callosum dysgenesis without an apparent contribution to clinical manifestations.

All patients with lactate peak on MRS or high lactate on CSF had high levels of blood lactate.

For those with follow-up MRI ($n = 15$), 67% (10/15) have reported radiological improvement and 20% (3/15) progression. Extensive review of neuroradiological features and progression elopes the scope of this work.

#### 3.2.4. Management

Mitochondrial supplementation was detailed in seven patients: all seven received CoQ10, six thiamine, five riboflavin, three L-carnitine, one received vitamin C. No information on clinical effect was provided. Several patients were prescribed chronic antiepileptic medication to manage their seizures without reference on critical adverse effects.

#### 3.2.5. Progression and outcome

Overall reported mortality was 21% (9/43) and all were patients who died within the first year of life. Causes of death included necrotizing bronchopneumonia, cardio-respiratory failure, multi-organ failure and severe lactic acidosis with progressive respiratory failure.

For those with at least two years of disease evolution, most patients (24/25, 96%) had their autonomy compromised by either global developmental delay across all fields, feeding difficulties or cognitive impairment, ranging from mild to severe (Table 3). Communication skills were severely impaired in 44% (11/25) of patients. Impaired gait was observed in 48% (12/25), with a spectrum of severity that spanned from never gaining the ability to walk (3/12, 25%) to presenting abnormal gait (9/12, 75%), such as spastic, ataxic, wide-based, or toe-walking.

Clinical and radiological disease progression based on available follow-up data (classified as stabilization, deterioration, or improvement) is presented in Tables 4 and 5. Regarding those who survived the first year of life and had available follow-up data ($n = 23$), fifteen patients showed clinical improvement (15/23, 65%), eight of which also exhibited radiological improvement reported by a second MRI after two years of age (8/10, 80%). Overall, follow-up revealed a variable degree of clinical-radiological dissociation in six patients (40%) but concordance in the rest (60%), unsupportive of previous hypothesis of clinical and radiological correlation. Overall, follow-up revealed a variable degree of clinical-radiological association, with concordance in 60% (9/15) and dissociation (i.e. a discrepancy between clinical severity and the extent of MRI lesions over time) in 40% patients (6/15). These results do not support the previous hypothesis of a consistent clinical and radiological correlation.

#### 3.2.6. Phenotype clustering

Findings at initial presentation appear to closely associate with clinical course and outcomes of the disease. Thus, we propose clustering phenotypes by severity at the time of presentation — mild, moderate, or severe — irrespective of age at onset, initial outcomes, or subsequent follow-up (Table 4 and Fig. 3A and B). Phenotype clustering was unfeasible in eight patients: seven due to lack of information and one patient exhibiting an atypical disease phenotype, with adult onset and neurodegeneration and was such an outlier that was not considered for clustering.

*EARS2* deficiency more commonly manifests as severe phenotype (20/35, 57%), primarily due to a combination of regression/significant developmental delay (either absent or severe), metabolic acidosis, failure to thrive and/or feeding difficulties. Movement disorders such as dystonia and ataxia seem to restrict to severe phenotypes and epilepsy is much more common than in milder forms. Most (16/20, 80%) presented in the first 3 months of life and all during the first year. Outcome ranged from death within the first year of life (8/20, 40%) to severe neurological disease, either slowly progressive, stable or less commonly with slow improvement (3/20, 15%). Survival was 3,52 years. One case presented with an Alpers-like syndrome with epilepsy and liver failure at 2 months of age, both of which ameliorated over time 38.

Of note, a subset of the severe patients ($n = 11$) had a catastrophic presentation, meaning, a severe and rapidly progressive presentation requiring acute hospital admission, more commonly with failure to

**Table 2**
Summary of first brain MRI findings in reported patients with *EARS2* deficiency.

| Encephalic structure | MRI findings | Prevalence ($n = 36$)* |
|---|---|---|
| Thalamus | | 64% (23) |
| Deep white matter | Overall T2 hyperintense | 78% (28) |
| | Extensive abnormalities with spared periventricular rim | 44% (16) |
| | Restricted diffusion | 36% (13) |
| | Delayed myelination | 19% (7) |
| Corpus callosum | Overall | 69% (25) |
| | Thinning | 25% (9) |
| | Hipo/Agenesia | 22% (8) |
| | Dysgenesia | 3% (1) |
| Brainstem | Overall | 53% (19) |
| | Midbrain/mesencephalon | 50% (18) |
| | Pons | 50% (18) |
| | Medulla oblongata | 50% (18) |
| Cerebellum | Overall | 47% (17) |
| | Cerebellar white matter | 42% (15) |
| | Dentate nucleus | 28% (10) |
| Hypothalamus | | 3% (1) |

* A total of 7 patients were excluded: P14, P24, P29, P30, P38, and P39 due to the absence of MRI descriptions, and P17, for whom only cranial ultrasound data were available.

**Table 3**
Functional independence (autonomy) of patients with biallelic variants of *EARS2* with at least 2 years of disease evolution.

| Function | Status | Overall prevalence ($n = 25$) |
|---|---|---|
| Locomotion | Gait impairment | 48% (12) |
| Development | Cognitive impairment | 48% (12) |
| | Communication impairment (language delay or absence) | 44% (11) |
| Feeding | Overall difficulties | 24% (6) |
| | Gastrostomy | 8% (2) |
| Breathing | Non-invasive ventilatory support | 4% (1) |

**Table 4**
Genetic results, phenotype clustering and overall disease course of *EARS2*-deficient patients (NM_001083614.2).

| P. | Ref | Variant | ACMG classification | Onset | Phenotype | Overall disease progression | Outcome death/ alive (age) |
|---|---|---|---|---|---|---|---|
| 1 | (Steenweg et al., 2012a) | c.1279_1280insTCC,p.Thr426_Arg427insLeu<br>c.502A > G, p.Arg168Gly | PM2<br>PM1, PM2, BP4 | Early infancy | Moderate | Progression | Alive (6y) |
| 2 | (Steenweg et al., 2012a) | c.1194C > G, p.Tyr398Ter<br>c.322C > T, p.Arg108Trp | PM2, PP5<br>PM1, PM2, PP5, BP4 | Infancy | Moderate | Clinical and radiological improvement | Alive (10y) |
| 3 | (Steenweg et al., 2012a) | c.322C > T, p.Arg108Trp<br>c.328G > A, p.Gly110Ser | PM1, PM2, PP5, BP4<br>PM1, PM2, PP3 | Infancy | Mild | Clinical and radiological improvement | Alive (9y) |
| 4 | (Steenweg et al., 2012a) | c.328G > A, p.Gly110Ser<br>c.610G > A, p.Gly204Ser | PM1, PM2, PP3<br>PM1, PM2, PP3 | Infancy | Severe | Clinical and radiological improvement | Alive (3,5y) |
| 5 | (Steenweg et al., 2012a) | c.286G > A, p.Glu96Lys<br>c.500G > A, p.Cys167Tyr | PM1, PM2, PP5, BP4<br>PM1, PM2, PP3, PP5 | Infancy | Mild | Clinical and radiological improvement | Alive (4y) |
| 6 | (Steenweg et al., 2012a) | c.322C > T, p.Arg108Trp<br>c.949G > T, p.Gly317Cys | PM1, PM2, PP5, BP4<br>PM1, PM2, PP3 | Infancy | Moderate | Clinical and radiological improvement | Alive (3,1y) |
| 7 | (Steenweg et al., 2012a) | c.164G > A, p.Arg55His<br>c.670G > A, p.Gly224Ser | PM1, PM2, PP3<br>PM1, BP4 | Infancy | Moderate | Improvement | Alive (1,5y) |
| 8 | (Steenweg et al., 2012a) | c.1A > G, p.Met1?<br>c.320G > A, p.Arg107His | PM2<br>PM1, PM2, PP5, BP4 | Very early onset | Severe | Slow Progression | Alive (14y) |
| 9 | (Steenweg et al., 2012a) | c.1547G > A, p.Arg516Gln<br>c.322C > T, p.Arg108Trp | PM1, PM2, PP3<br>PM1, PM2, PP5, BP4 | Early infancy | Severe, catastrophic | Stable<br>Radiological progression | Alive (6y) |
| 10 | (Steenweg et al., 2012a) | c.19A > T, p.Arg7X<br>c.322C > T, p.Arg108Trp | PM2<br>PM1, PM2, PP5, BP4 | Infancy | Severe | Stable<br>Radiological progression | Alive (2,8y) |
| 11 | (Steenweg et al., 2012a) | c.286G > A, p.Glu96Lys<br>c.500G > A, p.Cys167Tyr | PM1, PM2, PP5, BP4<br>PM1, PM2, PP3, PP5 | Early infancy | Severe | Stable | Alive (1,5y) |
| 12 | (Steenweg et al., 2012a) | c.286G > A, p.Glu96Lys<br>c.500G > A, p.Cys167Tyr | PM1, PM2, PP5, BP4<br>PM1, PM2, PP3, PP5 | Infancy | Moderate | Improvement | Alive (5y) |
| 13 | (Talim et al., 2013) | c.193A > G, p.Lys65Glu (homo) | PM1, PM2, PP3, PP5 | Early infancy | Severe, catastrophic | Rapidly progressive and fatal | Dead (6 M) |
| 14 | (Taylor et al., 2014) | c.193A > G, p.Lys65Glu (homo) | PM1, PM2, PP3, PP5 | Early infancy | Unknown | Probable rapidly progressive and fatal | Dead (3 M) |
| 15 | (Taylor et al., 2014) | c.322C > T,p.Arg108Trp<br>c.814G > A, p.Ala272Thr | PM1, PM2, PP5, BP4<br>PM1, PM2, PP5, BP4 | Early infancy | Severe, catastrophic | Probable rapidly progressive and fatal | Dead (6 M) |
| 16 | (Biancheri et al., 2015) | c.902G > C, p.Gly301Ala (homo) | PM1, PM2, PP3 | Infancy | Moderate | Improvement | Alive (6y) |
| 17 | (Danhauser et al., 2016) | c.320G > A, p.Arg107His<br>c.328G > A, p.Gly110Ser | PM1, PM2, PP5, BP4<br>PM1, PM2, PP3 | Early infancy | Severe, catastrophic | Rapidly progressive and fatal | Dead (21d) |
| 18 | (Güngör et al., 2016) | c.1234C > T, p.Arg412Cys<br>p.Lys471Asnfs*14 | PM1, PM2, PP5, BP4<br>PM2, PP5 | Infancy | Mild | Clinical and radiological improvement | Alive (3,5y) |
| 19 | (Kevelam et al., 2015) | c.454_456del, p.Lys152del (homo) | PM2 | Early infancy | Severe | Stable, radiological improvement | Alive (18y) |
| 20 | (Şahin et al., 2016) | c.1546C > T, p.Arg516Trp<br>c.319C > T, p.Arg107Cys | PM1, PM2, PP3<br>PM1, PM2, PP3, PP5, BP4 | Infancy | Severe | Unknown | Alive (8 M) |
| 21 | (Şahin et al., 2016) | c.1546C > T, p.Arg516Trp<br>c.319C > T, p.Arg107Cys | PM1, PM2, PP3<br>PM1, PM2, PP3, PP5, BP4 | Infancy | Mild | Clinical and radiological improvement | Alive (8y) |
| 22 | (Taskin et al., 2016) | c.322C > T, p.Arg108Trp (homo) | PM1, PM2, PP5, BP4 | Childhood | Mild | Improvement | Alive (3y) |
| 23 | (Pronicka et al., 2016) | c.164G > A, p.Arg55His<br>c.325G > C, p.Gly109Arg | PM1, PM2, PP3<br>PM1, PM2, PP3 | Early infancy | Severe | Unknown | Alive (10y) |
| 24 | (Pronicka et al., 2016) | c.1256C > T, p.Pro419Leu<br>c.164G > A, p.Arg55His | PM1, PM2, BP4<br>PM1, PM2, PP3 | Early infancy | Severe, catastrophic | Rapidly progressive and fatal | Dead (8 M) |
| 25 | (Kohda et al., 2016) | c.1466G > A, p.Arg489Gln<br>c.319C > T, p.Arg107Cys | PM1, PM2, PP3<br>PM1, PM2, PP3, PP5, BP4 | Early infancy | Unknown | Unknown | Unknown |
| 26 | (Oliveira et al., 2016) | c.184A > T, p.Ile62Phe<br>c.1A > G, p.Met1? | PM1, PM2, BP4<br>PM2 | Early infancy | Severe, catastrophic | Rapidly progressive and fatal | Dead (5 M) |

(*continued on next page*)

**Table 4** (*continued*)

| P. | Ref | Variant | ACMG classification | Onset | Phenotype | Overall disease progression | Outcome death/ alive (age) |
|---|---|---|---|---|---|---|---|
| 27 | (Sellars et al., 2017) | c.1394 T > C, p.Leu465Pro<br>c.322C > T, p.Arg108Trp | PM1, PM2, PP3<br>PM1, PM2, PP5, BP4 | Infancy | Severe, catastrophic | Improvement | Alive (1,5y) |
| 28 | (Prasun et al., 2019) | c.322C > T, p.Arg108Trp<br>c.328G > A, p.Gly110Ser | PM1, PM2, PP5, BP4<br>PM1, PM2, PP3 | Infancy | Severe | Improvement, radiological stable | Alive (2y) |
| 29 | (Nogueira et al., 2019) | c.1A > G, p.Met1?<br>c.286G > A, p.Glu96Lys | PM2<br>PM1, PM2, PP5, BP4 | Unknown | Moderate | Unknown | Alive (6y) |
| 30 | (Nogueira et al., 2019) | c.1A > G, p.Met1?<br>c.286G > A, p.Glu96Lys | PM2<br>PM1, PM2, PP5, BP4 | Early infancy | Severe, catastrophic | Probable rapidly progressive and fatal | Dead (2 M) |
| 31 | (Al Balushi et al., 2020) | c.293C > T, p.Ala98Val (homo) | PM1, PM2, PP5, BP4 | Early infancy | Severe, catastrophic | Progression | Alive (6y) |
| 32 | (Barbosa-Gouveia et al., 2020) | c.376C > T, p.Gln126*<br>c.670G > A, p.Gly224Ser | PM2, PP5<br>PM1, BP4 | Childhood | Moderate | Progression | Alive (19y)<br>Alive (19y) |
| 33 | (Monfrini et al., 2020b) | c.1064A > G, p.Asn355Ser<br>c.1488 + 2 T > A (r.spl) | PM1, PM2, BP4<br>PM2 | Adulthood | Late-onset | Unknown | Alive (>70y) |
| 34 | (Roux et al., 2021) | c.322C > T, p.Arg108Trp<br>c.417_418delCT, p.Cys140Phefs*24 | PM1, PM2, PP5, BP4<br>PM2, PP5 | Unknown | Unknown | Unknown | Alive (14y) |
| 35 | (Roux et al., 2021) | c.1547G > A, p.Arg516Gln<br>c.670G > A, p.Gly224Ser | PM1, PM2, PP3<br>PM1, BP4 | Early infancy | Unknown | Unknown | Alive (13y) |
| 36 | (Roux et al., 2021) | c.1413del, p.Lys471Asnfs*14<br>c.670G > A, p.Gly224Ser | PM2, PP5<br>PM1, BP4 | Early infancy | Unknown | Unknown | Alive (8y) |
| 37 | (Roux et al., 2021) | c.1A > G, p.Met1?<br>c.670G > A, p.Gly224Ser | PM2<br>PM1, BP4 | Early infancy | Unknown | Unknown | Unknown |
| 38 | (Ni et al., 2021) | c.949G > T, p.Gly317Cys (homo) | PM1, PM2, PP3 | Early infancy | Severe, catastrophic | Rapidly progressive and fatal | Dead (5d) |
| 39 | (Forny et al., 2021) | c.184A > T, p.Ile62Phe (homo) | PM1, PM2, BP4 | Early infancy | Unknown | Unknown | Unknown |
| 40 | (Sawada et al., 2021) | c.164G > T, p.Arg55Leu<br>c.484C > T, p.Arg162Trp | PM1, PM2, PP3<br>PM1, PM2, PP3 | Infancy | Moderate | Clinical and radiological improvement | Alive (13y) |
| 41 | (Gedikbasi et al., 2023) | c.1283del, p.Pro428Leufs*3<br>c.319C > T, p.Arg107Cys | PM2, PP5<br>PM1, PM2, PP3, PP5, BP4 | Early infancy | Severe | Unknown | Alive (3y) |
| 42 | (Nogueira et al., 2024) | c.1235G > A, p.Arg412His<br>p.Lys471Asnfs*14 | PM1, PM2, BP4<br>PM2, PP5 | Infancy | Mild | Clinical improvement<br>Radiological progression | Alive (8y) |
| 43 | (Nogueira et al., 2024) | c.184A > T, p.Ile62Phe<br>c.920T > C, p.Leu307Ser | PM1, PM2, PP3, PP5, BP4<br>PM1, PM2, PP3, PP5 | Early infancy | Severe, catastrophic | Rapidly progressive and fatal | Dead (2 M) |

**Table 5**
Clinical and neuroradiological follow-up by phenotype severity.

| Disease evolution | | Severe | | Moderate (n = 9) | Mild (n = 6) |
|---|---|---|---|---|---|
| | | Catastrophic (n = 11) | Non catastrophic (n = 9) | | |
| Clinical | Medium age at onset (months) | 2,5<br>1,2 | 4,2 | 5,2 | 9,5 |
| | Improvement | 15%<br>9% | 22% | 67% | 100% |
| | Stabilization | 20%<br>9% | 33% | 0% | 0% |
| | Progression | 10%<br>9% | 11% | 22% | 0% |
| | Rapidly progressive and fatal | 40% | | 0% | 0% |
| Neurorradiological | Improvement | 10%<br>0% | 22% | 44% | 67% |
| | Stabilization | 5%<br>0% | 11% | 11% | 0% |
| | Progression | 10%<br>9% | 11% | 0% | 17% |
| | Unknown | NA<br>NA | 56% | 44% | 17% |

thrive, liver involvement and metabolic acidosis as initial findings, as shown in Fig. 3C. All presented during the first 3 months of life (half as neonates) and (8/11, 73%) died within six months (median age at death 3,4 months). Those who survived ($n = 3$) had variable disease course with either slow improvement, stabilization or progression. One of these was born preterm and needed resuscitation at birth, which could negatively affect his clinical picture both at presentation and outcome (Al Balushi et al., 2020). These patients might appear to have less neurological symptoms however low survival is the probable culprit for such difference. Metabolic acidosis, but not hyperlactacidemia, appears

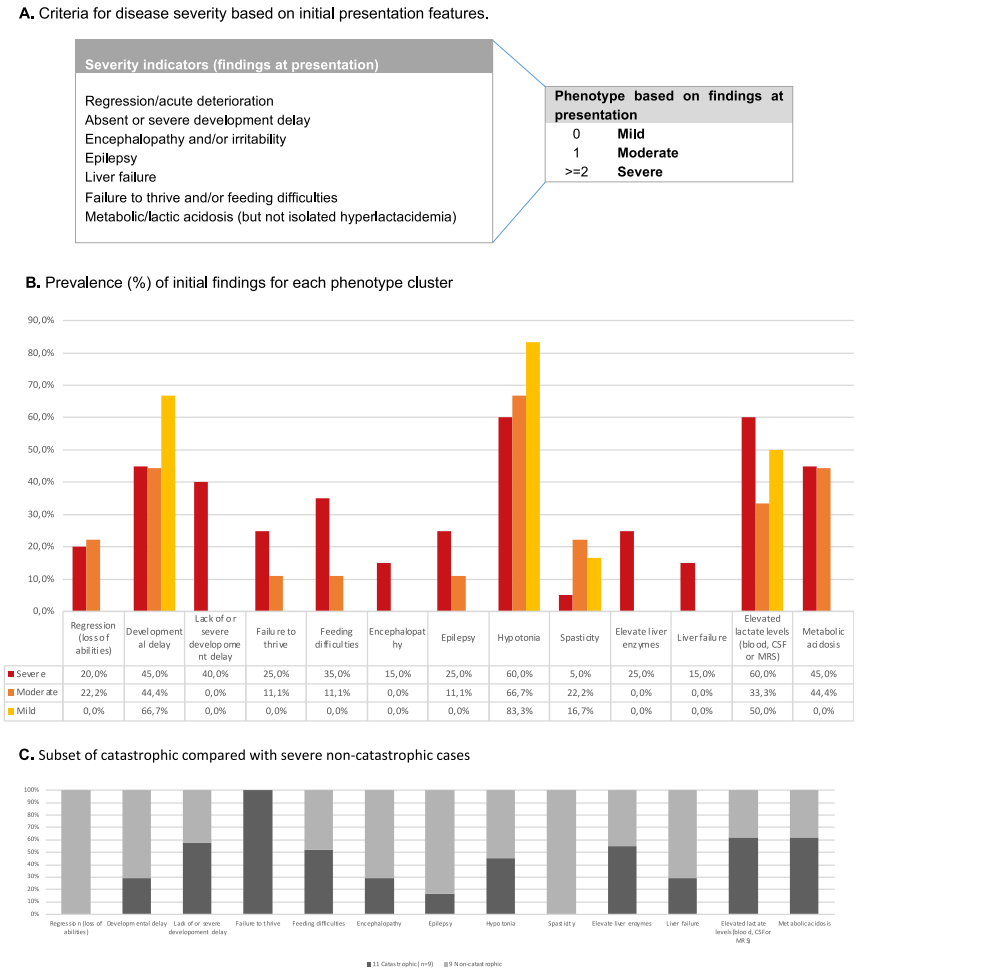


**Fig. 3.** Phenotype clustering based on clinical findings. A: criteria for disease severity; B. prevalence of clinical findings at presentation by phenotype cluster; C. Difference within severe group of patients with catastrophic presentation.

to be a predictive factor of worse presentation and outcome.

Moderate phenotype ($n = 9$) was observed more commonly with infancy to childhood onset at a median age of 5,2 months, ranging from 1 to 12 months. Most common findings at presentation were muscle tone changes (6/9, 67%), development delay (4/9, 44%) and regression (2/9, 22%). Two thirds had clinical improvement, while 22% progressed. These patients seem to hybridize between mild and severe phenotypes, as they present with severity criteria, but recovery rates more like milder forms.

Milder cases ($n = 6$) most commonly came to attention due to development delay (6/6, 100%) and hypotonia (5/6, 83%) at median age of 9,5 months. All cases with mild phenotype and known follow-up had clinical improvement, commonly with initial hypotonia that later progressed to spasticity (2/6, 33%). Non developed ataxia or movement disorder.

Muscle involvement including muscle tone alterations, muscle weakness and/or atrophy seem equally prevalent across phenotypes but might be underestimated due to semiotic and nomenclature confusion, namely with hypotonia. Liver involvement, including isolated elevation of liver enzymes, affects nearly half of severe-type patients, a feature not found in milder phenotypes.

Phenotype clustering also related with abnormalities of corpus callosum, with 55% (6/11) with either total or partial corpus callosum agenesia (4 with total agenesia) in the severe group, all in the catastrophic subset. In moderate and mild phenotypes, abnormalities of corpus callosum were more likely to be thin posterior. However, very few patients presented with follow-up MRIs.

### 3.2.7. *Genotype-phenotype correlation*

Genotype from all reported patients can be found in Table 4 as well as variant classification according to the American College of Medical Genetics and Genomics (ACGM) guidelines, age at onset, phenotype from our classification and overall disease progression and outcome.

Only eight patients were homozygous with missense variants in exon 1 or 2, across all phenotypes. The most common variant was c.322C > T, (p.Arg108Trp) ($n = 9$, one patient in homozygosity) and the second most common was c.A1 > G, (p.Met1?) in five patients, four of which were Portuguese, none in homozygous state. Schematic representation of

variants location on *EARS2* gene and attributed phenotype are represented in Fig. 4. A hotspot for pathogenic variants appears to be present in exons 1 and 2 with highly heterogenous phenotypes. Variants in mitochondrial target sequence and anticodon binding domains are less commonly reported and without homozygous cases, which might express that these domains are critical for EARS2 function and, perhaps incompatible with life.

Variant c.322C > T, (p.Arg108Trp) was present in five of the patients with total or partial corpus callosum agenesia (three with total agenesia) but not in homozygous state and with no correlation to phenotypes or outcomes. All cases with one c.328G > A, (p.Gly110Ser) variant ($n = 3$) had clinical improvement. No other variant appeared to relate to any clinical finding. We found no other phenotype-genotype correlation for exon, variant type (missense or nonsense) or affected protein domain, age at onset, severity, or outcome.

## 4. Discussion

Patients with *EARS2* deficiency usually manifest symptoms before one year of age, often exhibiting neurological signs without specific biomarkers for the disease. Despite the latter, elevated lactic acid levels in bodily fluids are a frequent finding throughout the disease, even if not initially found. Clinical presentation varies from mild to severe, leading to a wide range of clinical outcomes, either rapidly progressive and fatal or noticeable clinical recovery. While a recognized (partially) reversible syndrome, the overall impact of the disease remains significant, with patients frequently facing challenges across various aspects of life. Although symptoms can emerge from nearly all organ systems, EARS2 deficiency is particurlaly keen to the central nervous system, without a characteristic neurological phenotype but a distinct radiological syndrome (Steenweg et al., 2012b), although not highly specific or pathognomic MRI pattern of involvement. MRI typically highlights thalamus and brainstem involvement, suggesting a distinct. Early MRI may also reveal abnormalities in deep and cerebellar white matter, corpus callosum hypogenesis (thin, agenesis), and basal ganglia involvement, yet optic atrophy has not been reported. A thorough evaluation by specialists is necessary to accurately assess and compare MRI findings, as limited follow-up MRIs constrain conclusions about disease progression or genotype-phenotype links.

Clustering phenotypes according to initial presentation features has shown a meaningful link with the course of the disease: i) severe cases characterized by distinct signs (regression, profound developmental delays, encephalopathy, irritability, epilepsy, liver failure, failure to thrive, feeding difficulties, metabolic acidosis) leading to outcomes ranging from rapid, fatal progression to slow recovery or ongoing disease progression; ii) moderate cases with presentation during the first year of life but not neonatal and more likely to present with chornic symptons, such as tonus alteration and/or development delay and in whom disease evolution is more likely stable or possible improved; iii) mild cases, notably defined by changes in muscle tone and deep tendon hyperrreflexia, which is often (partially) reversible over time. This approach highlights the spectrum of the disease as a broad range, rather than confined categories, as illustrated in Fig. 5. Accordingly, we suggest moving forward with the term '*EARS2* deficiency' and phasing out 'LTBL'. It is important to note that the disease's recognized phenotype, marked by its severity, shouldn't be viewed as the sole characteristic of

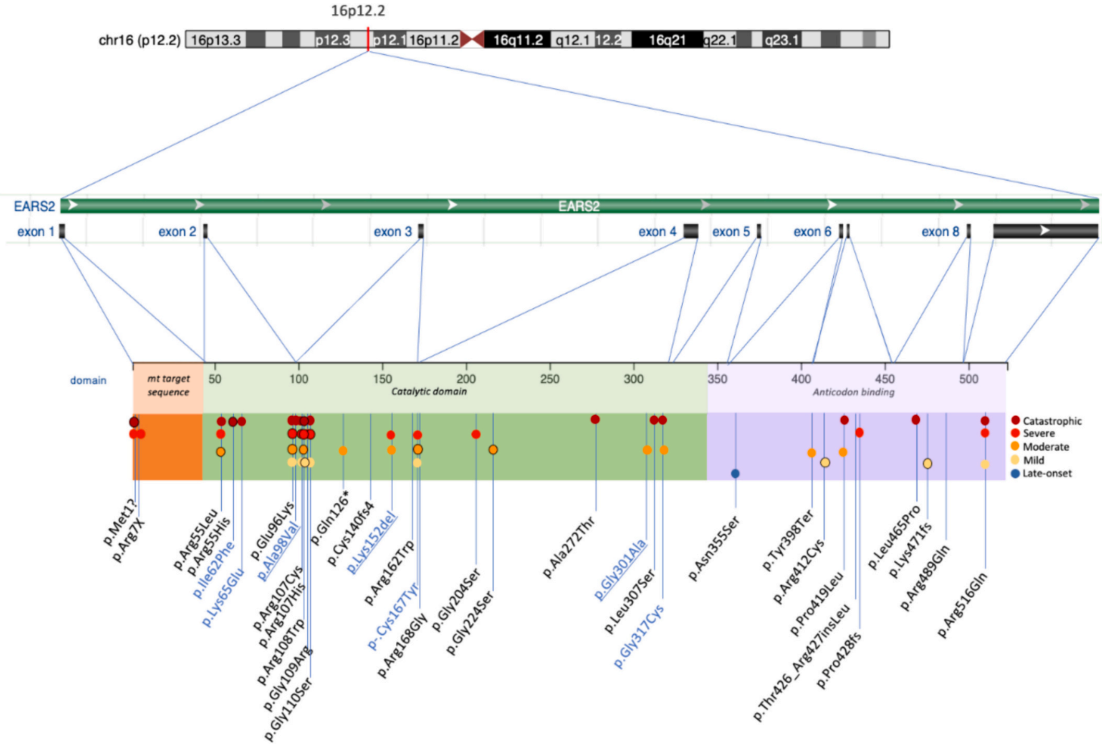


**Fig. 4.** Genotype to phenotype representation – all variants and their corresponding phenotype. Homozygous variants are highlighted in blue, underlined when described solely in homozygosity. Intronic variants are excluded (one instance). Phenotypes with multiple cases linked to the same variant are encircled in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

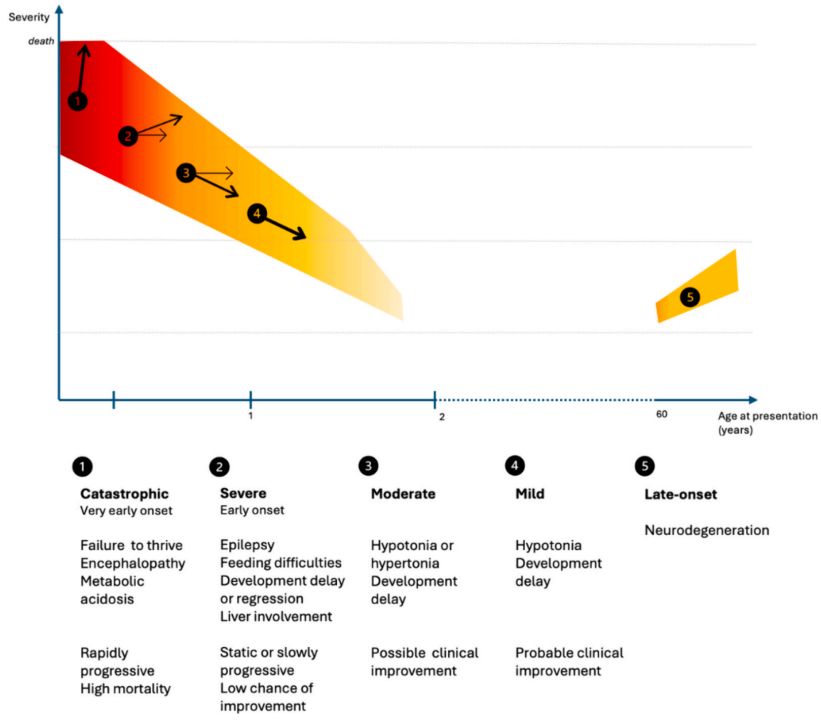


**Fig. 5.** Phenotype spectrum. Arrows represent possible evolutions.

*EARS2* deficiency, as some cases may be largely asymptomatic, as seen in a late-onset patient, or extremely severe, leading to prenatal malformations (Pergande et al., 2020; Monfrini et al., 2020a).

The underlying mechanism of mitochondrial glutamyl-tRNA synthetase (*EARS2*) deficiency is not fully understood. Anatomapathological data is anedoctal, in a catastrophic neonatal case with microvesicular steatosis, muscle biopsy with scattered multivacuolated myofibers and sarcoplasmic lipid accumulation and mild mitochondrial variations in size and christae and interestingly bilateral basal ganglia necrosis with microcalcification, consistent with Leigh syndrome (Li et al., 2017). This case was excluded from this literature review during initial screening since it was a conference abstract book.

The biphasic progression model proposed by Steenweg et al., pointing to an antenatal disruption in the structures of the central nervous system, continues to be robust hypothesis twelve years after its introduction. This disruption, commonly referred to as an 'early hit', may account for the considerable cellular damage evident at the disease's onset (Steenweg et al., 2012b). Conversely, recent findings significantly support the presence of compensatory mechanisms that safeguard mitochondrial translation during neuronal development and uphold oxidative phosphorylation components in those afflicted (Podmanicky et al., 2024). This interplay between damaging and compensatory processes could account for the observed phenotypic variation among patients, raising intriguing possibilities regarding the disease's actual prevalence. The reversibility of neurological anomalies supports the notion of an adaptative mitochondrial metabolism. The rarity of homozygosity, instances of altered prenatal neuronal development, and the association of *EARS2* with digenic inheritance highlight its crucial role in early neurodevelopment. The lack of cases in late childhood, adolescence, or early adulthood, alongside a case of late onset, prompts speculation about its significance in neurodegeneration and/or aging. Besides its obvious role in neurodevelopment, EARS2 can also have a role in neurodegeneration, a field with growing genetic evidence. ARS appear to be not only crucial for aminoacylation but also perform other noncanonical functions (Del Greco and Antonellis, 2022). In vivo effects can also relate to phenotypic diversity, e.g. in the late onset case functional essays showed normal transcription with decreased residual activity favoring a mechanism of higher enzyme degradation rather than lower expression (Monfrini et al., 2020a). Mitochondrial DNA in these patients could uncover additional variability linked to digenic (and bigenomic) inheritance patterns.

Despite the use of mitochondrial vitamins and co-factors in some patients, including ours, our search did not identify any particularly successful therapeutic option that would confer a recommendation for these patients and there are no ongoing trials or therapies under research.

Reporting bias and nomenclature inconsistencies, and variability in clinical descriptions surely compromised the results of this work. Significant efforts were made to standardize data and avoid inference. Cases reported within large cohorts using multi-gene approaches often lacked detailed clinical information and follow-up, limiting the depth of analysis. Detailed survival and disease burden analysis (such as Kaplan-Meyer curves) was not possible due to insufficient data. Nevertheless, the systematic literature review method still stands as a valuable strategy for characterizing (ultra) rare diseases.

Natural history and more detailed clinical spectrum should be further evaluate based on standardized clinical data, such as multicentric international registry and/or longitudinal studies. This would allow further information on severity scores and prognostic factors to be based on multivariate analysis. This is critical for developing and

evaluate future treatment options, providing endpoints for therapeutic trials.

## 5. Conclusions

Patients with *EARS2* deficiency typically manifest within the first year of life, displaying signs of a neurometabolic disorder alongside characteristic MRI features. Despite its rarity, the spectrum of *EARS2* deficiency is narrow but expected to broaden in the coming years.

This research underscores the critical need for systematic follow-up and thorough analysis of neuroradiological findings. Such assessments should be conducted via a natural history study, utilizing a multicentric international registry with standardized terminology, to thoroughly evaluate the disease's presentation and natural history.

## Details of ethics approval

This work was approved by the local Ethics Committee.

## Patient consent

Informed consent was obtained from all patient's legal representatives.

## Funding

None.

## CRediT authorship contribution statement

**Gonçalo Pelayo:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Margarida Paiva Coelho:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Joana Correia:** Writing – review & editing. **Anabela Bandeira:** Writing – review & editing. **Célia Nogueira:** Writing – review & editing. **Laura Vilarinho:** Supervision, Writing – review & editing. **Esmeralda Martins:** Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare that there is no conflict of interests.

## Data availability

The data that supports the findings of this study are available in the supplementary material of this article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2024.106644.

## References

Al Balushi, A., et al., 2020. Phenotypes and genotypes of mitochondrial aminoacyl-tRNA synthetase deficiencies from a single neurometabolic clinic. JIMD Rep. 51, 3–10.
Barbosa-Gouveia, S., et al., 2020. Identification of a novel variant in EARS2 associated with a severe clinical phenotype expands the clinical spectrum of LTBL. Genes (Basel) 11, 1–10.
Biancheri, R., et al., 2015. Expanding the clinical and magnetic resonance spectrum of leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL) in a patient harboring a novel EARS2 mutation. JIMD Rep. vol. 23, 85–89. Springer.
Boczonadi, V., Horvath, R., 2014a. Mitochondria: impaired mitochondrial translation in human disease. Int. J. Biochem. Cell Biol. 48, 77–84.
Boczonadi, V., Horvath, R., 2014b. Mitochondria: impaired mitochondrial translation in human disease. Int. J. Biochem. Cell Biol. 48, 77–84. https://doi.org/10.1016/j.biocel.2013.12.011. Preprint at.
Boutaud, L., et al., 2023. Neuropathological hallmarks of antenatal mitochondrial diseases with a corpus callosum defect. Brain 146, 1804–1811.
Danhauser, K., et al., 2016. EARS2 mutations cause fatal neonatal lactic acidosis, recurrent hypoglycemia and agenesis of corpus callosum. Metab. Brain Dis. 31, 717–721.
Davison, J.E., Rahman, S., 2017. Recognition, investigation and management of mitochondrial disease. Arch. Dis. Child. 102, 1082–1090. Preprint at. https://doi.org/10.1136/archdischild-2016-311370.
Del Greco, C., Antonellis, A., 2022. The role of nuclear-encoded mitochondrial tRNA charging enzymes in human inherited disease. Genes 13. https://doi.org/10.3390/genes13122319. Preprint at.
DiMauro, S., Schon, E.A., 2003. Mitochondrial respiratory-chain diseases. N. Engl. J. Med. 348, 2656–2668.
Diodato, D., Ghezzi, D., Tiranti, V., 2014. The mitochondrial aminoacyl tRNA synthetases: genes and syndromes. Int. J. Cell Biol. 2014, 1–11.
Fine, A.S., Nemeth, C.L., Kaufman, M.L., Fatemi, A., 2019. Mitochondrial aminoacyl-TRNA synthetase disorders: an emerging group of developmental disorders of myelination. J. Neurodev. Disord. 11 https://doi.org/10.1186/s11689-019-9292-y. Preprint at.
Forny, P., et al., 2021. Diagnosing mitochondrial disorders remains challenging in the omics era. Neurol. Genet. 7.
Fuchs, S.A., et al., 2019. Aminoacyl-tRNA synthetase deficiencies in search of common themes. Genet. Med. 21, 319–330.
Gedikbasi, A., et al., 2023. Clinical and bi-genomic DNA findings of patients suspected to have mitochondrial diseases. Front. Genet. 14.
Güngör, O., et al., 2016. A compound heterozygous EARS2 mutation associated with mild leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL). Brain and Development 38, 857–861.
Hathazi, D., et al., 2020. Metabolic shift underlies recovery in reversible infantile respiratory chain deficiency. EMBO J. 39.
Kevelam, S.H., et al., 2015. Absent thalami caused by a homozygous EARS2 mutation: expanding disease spectrum of LTBL. Neuropediatrics 47, 64–67.
Kohda, M., et al., 2016. A comprehensive genomic analysis reveals the genetic landscape of mitochondrial respiratory chain complex deficiencies. PLoS Genet. 12.
Konovalova, S., Tyynismaa, H., 2013. Mitochondrial aminoacyl-tRNA synthetases in human disease. Mol. Genet. Metab. 108, 206–211.
Li, L., Deisch, J., Zuppan, C., 2017. Autopsy findings in Leigh-like syndrome associated with a novel EARS2 mutation [abstract] presented at the United States & Canadian Academy of Pathology annual meeting, Texas, 4-10 March, 2017. Lab. Investig. 97, 4–11.
Monfrini, E., et al., 2020a. Late-onset leukoencephalopathy in a patient with recessive EARS2 mutations. Neurol. Genet. 6.
Monfrini, E., et al., 2020b. Late-onset leukoencephalopathy in a patient with recessive EARS2 mutations. Neurol. Genet. 6.
Ni, M., et al., 2021. Metabolic impact of pathogenic variants in the mitochondrial glutamyl-tRNA synthetase EARS2. J. Inherit. Metab. Dis. 44, 949–960.
Nogueira, C., et al., 2019. Targeted next generation sequencing identifies novel pathogenic variants and provides molecular diagnoses in a cohort of pediatric and adult patients with unexplained mitochondrial dysfunction. Mitochondrion 47, 309–317.
Nogueira, C., et al., 2024. The genetic landscape of mitochondrial diseases in the next-generation sequencing era: a Portuguese cohort study. Front. Cell Dev. Biol. 12.
Oliveira, R., et al., 2016. Lethal neonatal LTBL associated with biallelic EARS2 variants: case report and review of the reported neuroradiological features. JIMD Rep. 4, 61–68.
Paiva Coelho, M., Martins, E., Vilarinho, L., 2019. Diagnosis, management, and follow-up of mitochondrial disorders in childhood: a personalized medicine in the new era of genome sequence. Eur. J. Pediatr. 178, 21–32. https://doi.org/10.1007/s00431-018-3292-x. Preprint at.
Pearce, S., Nezich, C.L., Spinazzola, A., 2013. Mitochondrial diseases: translation matters. Mol. Cell. Neurosci. 55, 1–12.
Pergande, M., et al., 2020. The genomic and clinical landscape of fetal akinesia. Genet. Med. 22, 511–523.
Podmanicky, O., et al., 2024. Mitochondrial aminoacyl-tRNA synthetases trigger unique compensatory mechanisms in neurons. Hum. Mol. Genet. 33, 435–447.
Prasun, P., Mintz, C., Cork, E., Naidich, T.P., Webb, B.D., 2019. Broad spectrum of clinical presentation in EARS2 beyond typical "leukoencephalopathy with thalamus and brain stem involvement". J. Neurol. Sci. 406, 116448.
Pronicka, E., et al., 2016. New perspective in diagnostics of mitochondrial disorders: two years' experience with whole-exome sequencing at a national paediatric centre. J. Transl. Med. 14.
Roux, C.J., et al., 2021. Phenotypic diversity of brain MRI patterns in mitochondrial aminoacyl-tRNA synthetase mutations. Mol. Genet. Metab. 133, 222–229.
Şahin, S., et al., 2016. Leukoencephalopathy with thalamus and brainstem involvement and high lactate caused by novel mutations in the EARS2 gene in two siblings. J. Neurol. Sci. 365, 54–58.
Sawada, D., et al., 2021. Remitting and exacerbating white matter lesions in leukoencephalopathy with thalamus and brainstem involvement and high lactate. Brain and Development 43, 798–803.

Scheper, G.C., et al., 2007. Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. Nat. Genet. 39, 534–539.
Sellars, E.A., et al., 2017. Severe metabolic acidosis and hepatopathy due to leukoencephalopathy with thalamus and brainstem involvement and high lactate. Neuropediatrics 48, 108–110.
Steenweg, M.E., et al., 2012a. Leukoencephalopathy with thalamus and brainstem involvement and high lactate 'LTBL' caused by EARS2 mutations. Brain 135, 1387–1394.
Steenweg, M.E., et al., 2012b. Novel infantile-onset leukoencephalopathy with high lactate level and slow improvement. Arch. Neurol. 69, 718–722.
Talim, B., et al., 2013. Multisystem fatal infantile disease caused by a novel homozygous EARS2 mutation. Brain 136. https://doi.org/10.1093/brain/aws197. Preprint at.
Taskin, B.D., et al., 2016. Early-onset mild type leukoencephalopathy caused by a homozygous EARS2 mutation. J. Child Neurol. 31, 938–941.
Taylor, R.W., et al., 2014. Use of whole-exome sequencing to determine the genetic basis of multiple mitochondrial respiratory chain complex deficiencies. JAMA 312, 68–77.

# NMR-based Urinary Biomarkers in Pediatric Primary Mitochondrial Disorders and Chronic Kidney Disease: Shared Mitochondrial Dysfunction, Diverging Biosignatures

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ORIGINAL ARTICLE

# NMR-based urinary biomarkers in pediatric primary mitochondrial disorders and chronic kidney disease: shared mitochondrial dysfunction, diverging biosignatures

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## Abstract

**Background** Renal involvement is a recognized feature of primary mitochondrial disorders (PMD), either at presentation or during the disease course. Simultaneously, the metabolomic fingerprint of chronic kidney disease (CKD) is often associated with underlying mitochondrial dysfunction. This study aimed to characterize urinary metabolic signatures in genetically confirmed paediatric PMD without chronic kidney disease, comparing them to healthy controls, suspected (unconfirmed) mitochondrial disease (SMD), and non-mitochondrial CKD.

**Methods** We performed untargeted $^{1}$H NMR metabolomic profiling of 76 urine samples from 51 paediatric patients and 10 healthy controls. PMD patients in acute decompensation or known CKD and statistical outlier samples were excluded. Final comparisons included genetically confirmed PMD without CKD ($n=13$), SMD ($n=10$), non-mitochondrial CKD ($n=28$; 17 at stages 1–2 and 9 at stages 3–5), and healthy controls ($n=10$). Spectral data were analyzed using multivariate statistical approaches—including principal component analysis (PCA) and partial least squares–discriminant analysis (PLS-DA)—as well as univariate methods with Mann-Whitney U for pairwise group metabolite comparison.

**Results** Urinary metabolic profiles of PMD patients differed from healthy controls and CKD patients. Multivariate analysis revealed a strong discriminative ability between PMD and controls ($Q^2 = 0.53$) and advanced CKD ($Q^2=0.78$). Compared to controls, PMD patients had increased levels of Krebs cycle intermediates (*cis*-aconitate, fumarate and succinate), creatine, tryptophan, homovanillate (HVA) and hypoxanthine, as well as decreased histidine. All, except fumarate and histidine, remained discriminative when comparing PMD to CKD. CKD patients showed a diverging metabolomic fingerprint with 1-methylnicotinamide (MNA) and 2-hydroxyisobutyrate emerging as potential CKD-specific biomarkers, effectively discriminating between CKD stage 3–5 from earlier stages and controls. A five-metabolite panel comprising *cis*-aconitate, fumarate, HVA, tryptophan and histidine achieved high diagnostic performance for identifying PMD, with an area under the curve (AUC) of 0.836 (PMD vs. controls) and AUC = 0.783 across all groups. This biosignature integrates metabolites involved in distinct functional domains including energy metabolism, neurotransmitter turnover and amino acid metabolism and renal handling.

**Conclusion** Urinary metabolomic profiling by NMR revealed a distinct biosignature in pediatric PMD patients without renal involvement, characterized by elevated levels of tryptophan, HVA, and Krebs cycle intermediates, and diminished histidine. The divergent changes in tryptophan, histidine and HVA, suggest a mitochondria-specific metabolic phenotype in PMD. These findings support the use of urinary NMR metabolomics as a non-invasive tool for biomarker discovery in PMD and highlight the potential of integrated, multiparametric metabolic fingerprints for diagnostic refinement and patient stratification.

**Keywords** Primary mitochondrial disorders · Pediatric CKD · Metabolomics · Urine · NMR · Biomarkers

Extended author information available on the last page of the article

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**Abbreviations**

| | |
|---|---|
| $^{1}$H NMR | Proton nuclear magnetic resonance spectroscopy |
| ACR | Albumin-to-creatinine ratio |
| AKI | Acute kidney injury |
| AUC | Area under the curve |
| CAKUT | Congenital anomalies of the kidney and urinary tract |
| CKD | Chronic kidney disease |
| eGFR | Estimated glomerular filtration rate |
| FC | Fold change |
| GC-MS | Gas chromatography–mass spectrometry |
| HVA | Homovanillic acid (or homovanillate) |
| KEGG | Kyoto encyclopedia of genes and genomes |
| MA | Microalbuminuria |
| MNA | 1-methylnicotinamide |
| MVA | Multivariate statistical analysis |
| PCA | Principal component analysis |
| PLS-DA | Partial least squares–discriminant analysis |
| PMD | Primary mitochondrial disorder |
| SMD | Suspected mitochondrial disorder |
| SMPDB | Small molecule pathway database |
| UTI | Urinary tract infection |
| VIP | Variable importance in projection |
| VUR | Vesicoureteral reflux |

## 1 Introduction

Renal involvement is a recognized and common feature of primary mitochondrial disorders (PMD), either as an initial manifestation or later in the disease course (Finsterer, 2022; Finsterer & Scorza, 2017; Hall et al., 2015; Parasyri et al., 2022; Roper et al., 2020). The most recognized renal phenotypes in PMD include proximal tubulopathy, nephrotic syndrome, focal segmental glomerulosclerosis and end-stage chronic kidney disease (CKD). Kidney involvement is also observed in conditions with secondary mitochondrial dysfunction conditions, such as Prader-Willi syndrome (Martín-Hernández et al., 2005).

Variants in both mitochondrial and nuclear-encoded mitochondrial genes are an established risk factors for kidney disease susceptibility and progression of CKD (Jotwani et al., 2024). Mitochondria play a central role in the pathophysiology of non-mitochondrial CKD and acute kidney injury, in an interplay between mitochondrial function and renal homeostasis (Bhatia et al., 2020; Duann & Lin, 2017; Galvan et al., 2017; Hu et al., 2018; Kazubek-Zemke et al., 2014; Kuhn et al., 2024; Rao et al., 2018). Metabolic profiling in CKD research has been extensively studied, having revealed impaired energy metabolism illustrated by elevated levels of Krebs cycle metabolites and diminished mitochondrial function and biogenesis, despite different CKD etiologies (Hao et al., 2013; Sharma et al., 2013; Posada-Ayala et al., 2014; Hong et al., 2019; Lucio-Gutiérrez et al., 2022). These profiles correlate with CKD stage and progression, underscoring the importance of mitochondrial dysfunction in renal pathophysiology (Kobayashi et al., 2014; Kimura et al., 2016; Khan et al., 2020; Lucio-Gutiérrez et al., 2022).

Urinary metabolite profiling, particularly through techniques such as $^{1}$H NMR or mass spectrometry (MS), has become an interesting non-invasive method for assessing mitochondrial dysfunction (Boenzi & Diodato, 2018). Only a few studies have comprehensively characterized the metabolomic profile of PMD patients, particularly in pediatric cohorts (Thompson Legault et al., 2015), most studies reporting targeted organic acid analyses or plasma-based metabolomics. Furthermore, until recently, some studies lacked genetic confirmation of diagnosis, and their findings must therefore be interpreted with caution (Smuts et al., 2010; Venter et al., 2015). More frequently, studies compared disease groups with healthy controls and less often with pre-symptomatic carriers, disease control groups (myopathy) or clinical control group (suspected not confirmed disease) (Barshop, 2004; Buzkova et al., 2018; Esterhuizen et al., 2017; Hall et al., 2015; Venter et al., 2015). For instance, the urine metabolome of adult mitochondrial patients with the m.3243 A>G variant showed significant differences from healthy controls, even in the absence of known renal involvement or relation to heteroplasmy levels, showing impaired redox balance, beta-oxidation, one-carbon metabolism, but also an altered trans-sulfuration pathway and methylation cycle(Esterhuizen et al., 2019; Hall et al., 2015).

To date, no studies have directly compared the urinary metabolomes of PMD and CKD patients, despite the growing evidence that renal dysfunction in mitochondrial disorders can mimic and coexist with CKD. In this context, untargeted $^{1}$H NMR metabolomics of urine may offer novel insight into disease-specific metabolic pathways and early renal involvement in PMD. This study aims to contribute to filling that knowledge gap by systematically comparing the urinary metabolomic profiles of pediatric patients with PMD, CKD, and SMD.

## 2 Methods

### 2.1 Subject recruitment and clinical and laboratory data collection

Patients were selected by the metabolic and nephrology pediatric teams based on availability and willingness to

participate, without age or sex matching between groups. This study was approved by the local Ethics Committee, reference 82018.162(139-DEFI/138-CES) and written informed consent was obtained from all participants or their legal guardians, in accordance with the Declaration of Helsinki. As an observational study, no modifications to clinical management were made. This study analyzed urinary samples collected from pediatric patients followed at our center for suspected or confirmed mitochondrial or renal pathology, as well as healthy controls, between February 2020 and July 2024. A 2-year gap in recruitment occurred between March 2020 and September 2022 due to pandemic constraints. Inclusion and exclusion criteria, along with the recruitment process and sample numbers per group, are detailed in Table S1 and Figure S1.

Data extracted from clinical records included family history and detailed clinical presentation, encompassing neuromuscular symptoms, renal involvement, and other organ-specific manifestations. Regular medication use was also documented. Laboratory evaluation comprised blood counts, blood urea nitrogen (BUN), plasma and urinary creatinine, urea, and serum electrolytes (sodium, potassium, chloride, calcium). Urinary analyses included total proteinuria, microalbuminuria, α-1 and β-2 microglobulins, fractional excretion of sodium (FENa), tubular phosphate reabsorption, calciuria, glycosuria, urine pH, and specific gravity. Renal ultrasonography was performed in all patients with suspected or confirmed kidney disease. Patients were further classified according to the KDIGO 2024 guidelines, with glomerular filtration rate (GFR) estimated using the creatinine-based CKiD U25 Equation (or the average- creatinine-cystatin based U25 Equation when available), except for patients younger than 12 months, where estimated GFR (eGFR) was calculated using the Schwartz formula (Levin et al., 2024), and for patients older than 18 years, where eGFR was based on CKD-EPI 2021 equation. CKD severity was staged according to the 2024 KDIGO guidelines (Levin et al., 2024) and patients were stratified into two subgroups: stages 1–2 (mild CKD) and stages 3–5 (moderate to advanced CKD).

We recruited children from 2 months to 18 years of age into four groups (Table S2): (*i*) patients with genetically confirmed primary mitochondrial disorders including mutations in mitochondrial DNA (mtDNA) or nuclear genes (PMD, $n=15$); (*ii*) individuals with clinical suspicion of mitochondrial disease but no established molecular diagnosis (SMD, $n=10$); (*iii*) patients with chronic kidney disease (CKD, any stage, $n=29$); and (*iv*) an opportunistic control group ($n=10$) comprising otherwise healthy individuals followed in the Nephrology outpatient clinic, with no evidence of kidney or metabolic disease (e.g., mild vesicoureteral reflux or post-infectious follow-up). Regarding SMD, cases were selected based on suggestive clinical and biochemical features (e.g., elevated lactate and/or alanine, neuromuscular symptoms, developmental delay, radiological findings compatible with mitochondrial dysfunction) but without a confirmed genetic diagnosis. Only individuals with persistent suspicion of PMD and no alternative diagnosis after genetic testing were retained. In patients with chronic renal impairment of unknown etiology, mtDNA sequencing and whole exome sequencing (WES) was performed to exclude PMD. One patient with steroid-dependent nephrotic syndrome was reclassified as having a mitochondrial disorder related to *RRM2B*-related mitochondrial disorder (P25). Patients with PMD and known CKD phenotype were excluded (P25 and P15). Samples collected during acute decompensations or intercurrent infections were excluded based on clinical metadata and multivariate outlier detection, ensuring that profiles reflected patients' baseline metabolic state. Out of the 64 patients initially screened, 58 were retained for final analysis (13 PMD, 10 SMD, 26 CKD patients and 10 controls) (Figure S1).

### 2.2 Urine sampling

Casual urine samples were obtained during routine outpatient visits (therefore, irrespective of fasting period), promptly refrigerated, and aliquots were frozen (-80 °C) within 24 h. Clinical and general laboratory evaluations were performed on the same day. A single sample was collected per patient, except for 5 primary mitochondrial patients with known abnormal urinary metabolite excretion, for whom 2–5 samples were collected for time-variability assessment. In these cases, a single sample per patient was selected, minimizing factors such as acute exacerbations or disease progression (out of scope in this work).

### 2.3 NMR spectroscopy

Urinary $^1$H NMR analysis was performed on samples thawed at room temperature, after centrifugation, buffered with a phosphate solution ($KH_2PO_4$ in $D_2O$ with 0.1% sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-$d_4$ acid, TSP), and pH adjusted to 7.40 (±0.02) using KOH (potassium hydroxide, in $D_2O$) or HCl (hydrochloric acid, in $D_2O$). $^1$H NMR spectra were acquired on a Bruker AVANCE III spectrometer operating at a frequency of 500 MHz for proton, using a standard NOESY-1D pulse sequence with pH adjustment and TSP calibration. Peak assignment was performed using Chenomx NMR Suite©, with the aid of 2D NMR spectra (total correlation spectroscopy- TOCSY and heteronuclear single quantum correlation- HSQC for selected samples) and of internal and public spectral libraries. A list of identified metabolites can be found in Table S3 (including some

putative assignments, which would require additional spiking experiments, not carried out here). In some cases, statistical total correlation spectroscopy (STOCSY) (Cloarec et al., 2005) was used to aid metabolite assignment. Given the inclusion of patients with *TMEM70* and *TAZ*-related disease, typically associated with 3-methylglutaconic (3MGA) aciduria, we specifically searched for $^{1}$H NMR signals corresponding to previously reported 3MGA resonances (Engelke et al., 2006). No compatible resonance pattern was detected in our cohort, suggesting that 3MGA was either absent, below detection threshold, or not consistently elevated in basal conditions.

### 2.4 Data preprocessing and statistical analysis

Spectral region exclusion was applied to eliminate regions corresponding to the water peak and the highly variable urea signal, which could introduce artefacts in multivariate analysis. Spectral alignment (Matlab R2014a, The MathWorks Inc., Natick, MA, USA) was performed prior to normalization through Probabilistic Quotient Normalization (PQN). PQN was chosen instead of total area normalization due to the large creatinine peak variations in several samples (reaching up to 12% of total spectral area in some cases). This rendered spectral total area not solely a function of sample dilution and, thus, not adequate for normalization in this study. Multivariate analysis (MVA) trough Principal component analysis (PCA) and partial-least-squares discriminant analysis (PLS-DA) models (SIMCA-P 11.5, Umetrics, Umeå, Sweden), were performed on full resolution $^{1}$H NMR spectra, after unit variance (UV) scaling of the spectra. MVA results were visualized through factorial coordinates ('scores plots') and factorial contributions ('loadings plots'). The corresponding PLS-DA loading weights plots were back-transformed by multiplying each variable by its standard deviation and colored according to variable importance to the projection (VIP) (Matlab 8.3.0, The MathWorks Inc., Natick, Massachusetts, USA). PLS-DA models with predictive power ($Q^2$) values $\geq 0.5$ were considered of satisfactory or good robustness. Peak integration was performed after manual peak selection from the original spectra (Amix 3.9.5, Bruker BioSpin, Rheinstetten, Germany) and normalized using PQN. For comparison, normalization to creatinine was also carried out, due to still frequent use of this metabolite for concentration ratioing in the clinic, although acknowledging the inadequacy of this metabolite as a reference.

Individual metabolites´ variations were assessed using the Kruskal-Wallis test (for multiple group comparisons) and the Mann-Whitney U test (for pairwise comparisons), with a significance threshold of $p < 0.05$. Statistical analyses were performed using R software (version 4.3.2) and IBM SPSS Statistics (version 30.0). Metabolite variations were considered relevant if they exhibited a fold change (FC) $\leq 0.8$ or $\geq 1.2$ with a $p$-value $< 0.05$, or if they presented a significant effect size (Hedge's g $\geq 0.2$) with statistical significance. PLS-DA biplots for quantified peaks and corresponding VIP scores were generated using MetaboAnalyst 5.0, as well as, metabolites correlations, assessed with Spearman's rank correlation coefficient. Significance Analysis of Metabolomics (SAM) and Empirical Bayesian Analysis of Metabolomics (EBAM) were also used to identify discriminant metabolites (supplementary material). Metabolic pathway analyses were based on the list of significantly altered metabolites, annotated using the KEGG database and literature review. Pathway enrichment and topology analyses were performed in MetaboAnalyst 5.0 using auto-scale data normalized to total spectral area. Analyses included KEGG pathway impact (topology and enrichment analysis) and metabolite set enrichment based on the SMPDB library.

To explore the diagnostic potential of urinary metabolites for PMD, we performed receiver operating characteristic (ROC) curve analysis and calculated the area under the curve (AUC) for each compound. A subset of candidate metabolites was then selected based on statistical significance (univariate tests), AUC thresholds, and multivariate variable importance. These selected metabolites were used to construct classification models—including logistic regression, PLS-DA, random forest, and linear support vector machines (SVM)—to assess their discriminative performance across diagnostic groups.

## 3 Results

### 3.1 Patients and group characteristics

Demographic and clinical characteristics across the five study groups are summarized in Table 1, including age and sex distribution, estimated glomerular filtration rate (eGFR), and microalbuminuria stages. The PMD group ($n = 13$) comprised patients with mtDNA mutations ($n = 4$), single mtDNA deletion ($n = 1$), and nuclear gene defects ($n = 8$), while all SMD cases ($n = 10$) remained undiagnosed. CKD groups were stratified into stages 1–2 ($n = 17$) and 3–5 ($n = 9$), with underlying diagnoses including glomerular disease, CAKUT, tubulopathies, and nephronophthisis. Significant albuminuria, according to KDIGO guidelines (A2: 30–300 mg/g; A3: >300 mg/g) were only observed in CKD groups, with A2 and A3 present in 59% and 41% of CKD1–2, and in 33% and 44% of CKD3–5, respectively. Detailed patient data are provided in Table S2.

For group validation we performed supervised multivariate analysis (PLS-DA models) with full normalized spectra.

**Table 1** Group characteristics including sex and age distribution, estimated glomerular filtration rate (eGFR) and microalbuminuria categories, according to KDIGO guidelines (A2: ACR 30–300 mg/g; A3: ACR > 300 mg/g)

| Group | *n* | Median Age [Range] | Sex (M/F) | eGFR mean ± SD | ACR stage A2 (%) | ACR stage 3 (%) | Diagnoses |
|---|---|---|---|---|---|---|---|
| Primary mitochondrial disease (PMD) | 13 | 9.5 [1.1–18.8] | 6/7 | 153.4 ± 63.2 | 0% | 0% | mtDNA point mutations (*n* = 4)<br>Single mtDNA deletion (*n* = 1)<br>Nuclear gene defects (*n* = 8) |
| Suspected mitochondrial disease (SMD) | 10 | 4.1 [0.2–18.7] | 3/7 | 111.0 ± 18.7 | 0% | 0% | Undiagnosed (*n* = 10) |
| Control | 10 | 11.2 [2.7–18.2] | 7/10 | 110.4 ± 9.2 | 0% | 0% | CAKUT (isolated urogenital tract malformations) (*n* = 5)<br>Mild vesicoureteral reflux (*n* = 2)<br>Other (*n* = 3) |
| CKD stages 1–2 | 17 | 12.1 [2.8–17.9] | 12/5 | 97.8 ± 15.3 | 59% | 41% | Glomerular (*n* = 4)<br>CAKUT (*n* = 3)<br>Tubulopathies (*n* = 2)<br>Other (*n* = 8) |
| CKD stages 3–5 | **9** | 12.9 [4.7–15.8] | 6/3 | 20.3 ± 7.0 | 33% | 44% | Glomerular (*n* = 3)<br>CAKUT (*n* = 2)<br>Nephronophthisis (*n* = 2)<br>Other (*n* = 2) |

Diagnoses were grouped into clinically relevant categories

*ACR* albumin-to-creatinine ratio, *eGFR* estimated glomerular filtration rate, *CKD* chronic kidney disease, *SD* standard deviation, *MA*, *mtDNA* mitochondrial DNA, *CAKUT* Congenital Anomalies of the Kidney and Urinary Tract

PLS-DA was unable to discriminate between controls and the CKD stage 1–2 group ($Q^2 < 0$) (Table S4), as anticipated by similar eGFR between groups and the recruitment strategy (opportunistic control group of children followed in the Nephrology clinic). To explore the impact of microalbuminuria as potential sources of variability within this cohort, we carried out additional subgroup comparisons comparing CKD stages 1–2 patients with and without significant microalbuminuria (ACR > 30 mg/g, regardless of its etiology). Discrimination was not significant ($Q^2 = 0.29$) (Table S4), supporting the clustering of all CKD stages 1–2 patients into a single group for downstream comparisons.

### 3.2 General overview

A comparative overview of the urinary metabolomic profile of average $^1$H NMR spectra of controls and PMD patients is shown in Fig. 1, highlighted for regions 3.5–0.5 ppm and 9.5–6.0 ppm, revealed marked apparent differences in several chemical shift regions (Fig. 1). For instance, PMD samples seem to show, on average, higher levels of lactate, acetate, succinate, creatine, hippurate and formate, and possible lower levels of two unassigned resonances at 2.16 and 2.18 ppm. The latter may be putatively assigned to acetaminophen (either in its glucuronide and/or sulfate forms) methyls, as suggested by their strong statistical correlations (through STOCSY, results not shown) with several aromatic resonances (6.90, 7.10, 7.32, 7.42) as well as with a 5.14 ppm anomeric resonance confirming the glucuronide from. However, an unambiguous assignment would require further research, while at this stage no clear biological relevance to the study groups is identified. These visual changes refer only to average spectra and thus require further careful analysis through multivariate analysis and subsequent peak integration and univariate analysis.

Identified metabolites are summarized In Table S3. Overall, 57 metabolites were identified (4 of them still corresponding to putative assignments, requiring additional investigation to validate their identification). The identified metabolites are consistent with the literature for NMR urine metabolomics studies(Bouatra et al., 2013).

Unsupervised multivariate analysis performed by Principal Component Analysis (PCA) using all samples (Fig. 2A) highlighted distinct metabolic fingerprint in CKD patients compared to other groups. PLS-DA scores plot (Fig. 2B) illustrated a diverging tendency between groups, with the observation of three clusters: PMD and suspected mitochondrial disorders (SMD), CKD1-2 and Control, and CKD3-5.

Pairwise comparisons between groups (Fig. 3 and Table S4) confirmed the overall observed tendencies, with robust discrimination ($Q^2 = 0.52$) between PMD and controls (Fig. 3A). The corresponding loadings plot (Fig. 3E) revealed several resonances with high VIP values, suggesting higher amounts of organic acids (acetate, lactate, formate, fumarate and succinate), 3-HBA, alanine, creatine, hippurate and TMAO in PMD samples, and decreased quantities of 4-HPA, creatinine, *N*-PhAcGly and taurine, as well as two still unassigned peaks at 2.16 (U2.16, singlet) and 2.18 (U2.18, singlet), but possibly arising from acetaminophen forms, as mentioned earlier.

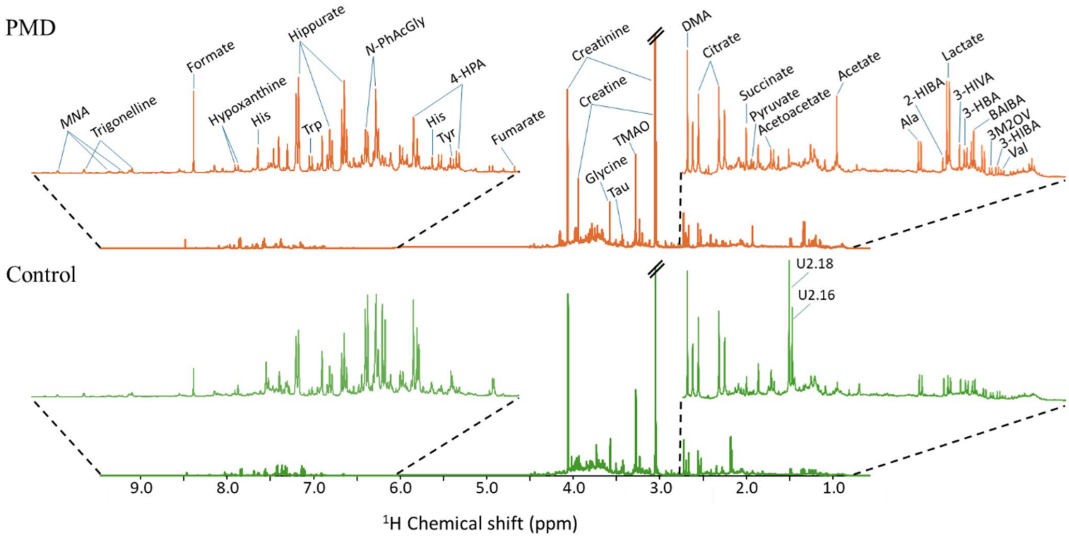


**Fig. 1** Average urinary ¹H NMR spectra of controls and patients with primary mitochondrial disease (PMD). Representative comparison between the average spectra of control subjects (green) and PMD patients (orange). Insets show expanded aliphatic (δ 0.8–2.8) and aromatic (δ 6.0–9.5) regions. Three-letter code used for amino acids. Abbreviations: 2-HIBA: 2-hydroxyisobutyrate; 3-HBA: 3-hydroxybutyrate; 3-HIBA: 3-hydroxyisobutyrate; 3-HIVA: 3-hydroxyisovalerate; 3M2OV: 3-methyl-2-oxovalerate; 4-HPA: 4-hydroxyphenylacetate; BAIBA: 3 (or beta)-aminoisobutyrate; DMA: dimethylamine; His: histidine; MNA: *N*-methylnicotinamide; *N*-PhAcGly: *N*-phenylacetylglycine; Tau: taurine; TMAO: trimethylamine *N*-oxide, Trp: tryptophan; Tyr: tyrosine. Compounds remaining unknown or not unambiguously assigned are represented by U followed by the corresponding ppm value

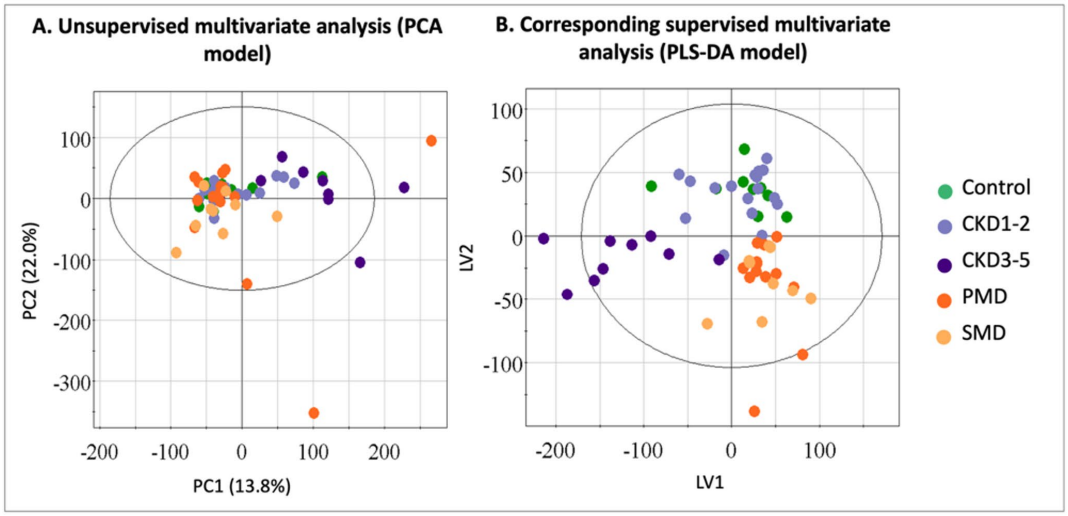


**Fig. 2** Multivariate analysis of urinary ¹H NMR metabolomic profiles across groups: Primary Mitochondrial Disease (PMD, orange), Suspected Mitochondrial Disease (SMD, yellow), Control individuals (green), Chronic Kidney Disease (CKD) stages 1–2 (lilac) and stages 3–5 (purple). The ellipses represent 95% confidence intervals. **A** Principal Component Analysis (PCA) highlights distinct metabolic fingerprint in CKD patients compared to other groups. **B** Corresponding supervised model using partial least squares discriminant analysis (PLS-DA) shows metabolic divergence between groups, particularly between PMD, CKD3-5, and controls. *PMD* primary mitochondrial disease, *SMD* suspected mitochondrial dysfunction, *CKD* chronic kidney disease

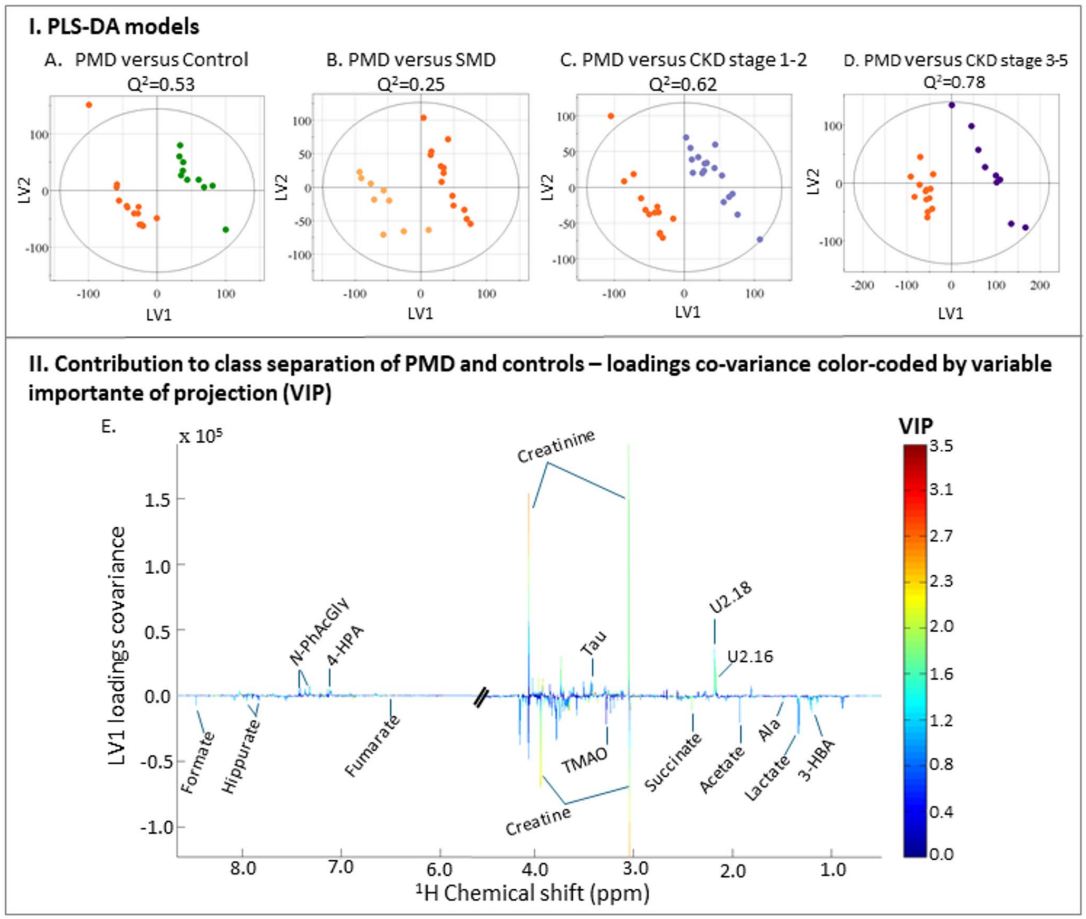


**Fig. 3** Supervised multivariate models and spectral features associated with mitochondrial disorders. I. Partial Least Squares Discriminant Analysis (PLS-DA) models were generated to compare patients with PMD to: **A** controls, **B** SMD, **C**. CKD stages 1–2, and **D**. CKD stages 3–5. The corresponding $Q^2$ values, indicate the predictive performance of each model. Clear discrimination was observed between PMD and controls ($Q^2$ = 0.53), and both CKD subgroups ($Q^2$ = 0.62 and 0.78). In contrast, the model comparing PMD to SMD showed lower predictive performance ($Q^2$ = 0.25), reflecting significant metabolic overlap. II. Corresponding loadings plot of model A, color-coded by variable importance in projection (VIP) scores for the first latent variable (LV1) highlighting spectral regions that contribute most to the separation between PMD and controls. (regions around 1.2–2.5 ppm and 3.0–4.5 ppm, consistent with metabolites involved in amino acid metabolism, organic acids, and energy-related pathways). Three-letter code used for amino acids. Abbreviations: 3-HBA: 3-hydroxybutyrate; 4-HPA: 4-hydroxyphenylacetate; *N*-PhAcGly: *N*-phenylacetylglycine. Ui: resonances at chemical shift i, which remain unassigned (or with tentative assignment)

Despite the clear metabolic separation between PMD patients and healthy controls, multivariate analysis failed to discriminate PMD patients from individuals with SMD ($Q^2$ = 0.32). Regarding the other comparisons for PMD against the CDK group, vs. CKD1-2 (Fig. 3C) and vs. CKD3-5 (Fig. 3D), the PLS-DA models revealed a high predictive power ($Q^2$=0.62 and $Q^2$=0.78, respectively), reflecting strong metabolic differences between these groups.

To validate these findings, peak integration and corresponding statistical significance of metabolites variations we performed systematic pairwise comparisons using the Mann–Whitney U test for each metabolite. Quantified peaks are discriminated in Table S4. This approach was necessary given that omnibus tests (e.g., Kruskal–Wallis) may underestimate group-specific differences in large, heterogeneous datasets. Table 2 summarizes the urinary metabolites showing significant differences between PMD, CKD stage 3–5,

**Table 2** Metabolite comparison across primary mitochondrial disease (PMD), control and chronic kidney disease (CKD) stages 3–5

| Compound name | ppm | FC | Hedge's (g) | *p*-value | Sign.[1] |
|---|---|---|---|---|---|
| PMD vs. Control | | | | | |
| *cis*-Aconitate | 3.11 | 1.48 | 0.63 | 0.020 | * |
| Creatine | 3.93 | 1.84 | 0.95 | 0.020 | * |
| Fumarate | 6.52 | 2.22 | 0.88 | 0.006 | ** |
| Histidine | 7.10 | 0.36 | -0.62 | 0.038 | * |
| Homovanillate (HVA) | 6.94 | 1.70 | 0.99 | 0.020 | * |
| Hypoxanthine | 8.19 | 1.56 | 1.13 | 0.020 | * |
| Succinate | 2.41 | 1.52 | 0.93 | 0.010 | * |
| Tryptophan | 7.75 | 2.02 | 1.34 | 0.002 | ** |
| PMD vs. CKD3-5 | | | | | |
| 1-Methylnicotinamide (MNA) | 9.29 | 2.96 | -0.95 | 0.001 | *** |
| 3-Aminoisobutyrate | 2.20 | 1.42 | -0.94 | 0.019 | * |
| 2-Hydroxyisobutyrate | 1.36 | 1.30 | -0.76 | 0.033 | * |
| 3-Hydroxybutyrate | 1.23 | 1.59 | -1.01 | 0.011 | * |
| 3-Hydroxyisovalerate | 1.27 | 2.08 | -1.98 | 0.002 | ** |
| 4-Hydroxyphenylacetate | 7.17 | 1.34 | -0.39 | 0.045 | * |
| *cis*-Aconitate | 3.11 | 1.68 | -0.81 | 0.019 | * |
| Creatine | 3.93 | 1.80 | -1.01 | 0.033 | * |
| Formate | 8.46 | 2.87 | -0.62 | 0.006 | ** |
| Glucuronate | 5.27 | 0.09 | 1.23 | 0.003 | ** |
| Glycolate | 3.96 | 1.37 | -0.83 | 0.038 | * |
| Homovanillate (HVA) | 6.94 | 2.53 | -1.79 | 0.001 | *** |
| Hypoxanthine | 8.19 | 1.46 | -0.53 | 0.005 | ** |
| Propylene glycol | 1.14 | 2.31 | -1.25 | 0.006 | ** |
| Succinate | 2.41 | 1.39 | -0.86 | 0.045 | * |
| Tryptophan | 7.75 | 1.73 | -1.23 | 0.009 | ** |
| Tyrosine | 6.90 | 2.10 | -1.26 | 0.001 | *** |
| CKD3-5 vs. Control | | | | | |
| 1-Methylnicotinamide (MNA) | 9.29 | 0.35 | -1.25 | 0.010 | * |
| 3-Hydroxyisovalerate | 1.27 | 0.42 | -1.01 | 0.037 | * |
| Acetate | 1.93 | 1.83 | 1.12 | 0.030 | * |
| Citrate | 2.66 | 0.59 | -0.71 | 0.004 | ** |
| Fumarate | 6.53 | 2.42 | 1.44 | 0.030 | * |
| Glucuronate | 5.27 | 24.19 | 9.21 | 0.002 | ** |
| Lactate | 1.34 | 2.36 | 1.53 | 0.020 | * |

Statistical comparisons were performed using Mann-Whitney U tests, normalized to PQN. Features with $p<0.05$ but minimal variation (FC between 0.8 and 1.2) and negligible effect size (Hedge's g between −0.2 and 0.2) were excluded to avoid overinterpretation of biologically irrelevant findings

Shown chemical shifts correspond to the metabolite peaks used for integration. The table includes representative metabolites from amino acid metabolism, organic acids, and energy-related pathways, particularly those showing statistically significant differences between PMD and other groups, including controls and CKD stages 3–5

[1] Statistical significance (Sign.): *$p<0.05$, **$p<0.01$, ***$p<0.001$

*PMD* primary mitochondrial disease, *SMD* suspected mitochondrial dysfunction, *CKD* chronic kidney disease

SMD and control groups (Table S5 for remaining group comparisons). Fold changes (FC) and Hedge's effect size (g) were calculated to assess group differences.

### 3.3 Urinary metabolomic of primary mitochondrial disease differs from controls with minor differences to suspected mitochondrial disease

Several metabolites displayed moderate to large effect sizes in the comparison between patients with PMD and controls. Krebs cycle metabolites (*cis*-aconitate, succinate and fumarate) were significantly elevated in the PMD group (FC 1.48–2.22, $p<0.02$), with moderate to large effect sizes (Hedges' g = 0.63–0.93). Tryptophan, hypoxanthine, and homovanillate (HVA) were significantly increased in PMD compared to controls (fold change 1.56–2.02), all showing large effect sizes (Hedges' g = 0.99–1.34), supporting their potential as discriminant metabolic markers. For the latter, considering the existence of a significant outlier, we repeated the Mann–Whitney U test after excluding it, and

the difference remained statistically significant ($p=0.030$), confirming that the result was not driven solely by a single outlier. Histidine was the only significantly reduced metabolite in PMD (FC = 0.36, g = −0.62). The EBAM analysis identified tryptophan, fumarate, succinate, hypoxanthine, and *cis*-aconitate as the most statistically robust metabolites distinguishing PMD from controls, based on the lowest local false discovery rates and highest posterior probabilities. These findings corroborate the Mann–Whitney results and highlight the biological relevance of these metabolites.

PLS-DA biplot and Volcano Plot based on quantified metabolites (one peak per metabolite) between PMD patients and controls, allows a visualization of the direction and relative contribution of individual metabolites to group discrimination and corresponding VIP plot listing the top discriminating metabolites per group (Figure S2). Correlation analysis revealed multiple clusters of positively correlated metabolites with shared biochemical roles (Figures S3). Fumarate was most strongly correlated with other Krebs cycles intermediates but also with HVA, tryptophan, isoleucine and tyrosine. *cis*-Aconitate displayed strong associations with HVA, 3-hydroxyisobutirate and 2-hydroxyisovalerate. Histidine showed weak to moderate negative associations with hypoxanthine and tryptophan. These findings suggest coordinated metabolic responses within mitochondrial-related pathways, with specific signatures centered around Krebs cycle intermediates and amino acid derivatives.

Pathway enrichment analysis (SMPDB and KEGG-based analysis) revealed multiple significant perturbations in mitochondrial-related and amino acid metabolism pathways in PMD patients compared to controls (Figures S3 and S4). The most relevant pathways included the as expected the energy metabolism and redox balance - pyruvate metabolism, Krebs cycle and mitochondrial electron transport chain and beta-oxidation. Additional relevant pathways included oxidation of branched-chain fatty acids, glutamate, arginine, proline, tryptophan metabolism, and glutathione metabolism.

While Krebs cycle intermediates, such as *cis*-aconitate, fumarate, and succinate, and creatine were elevated in both PMD and SMD relative to controls, the associated fold changes and effect sizes were consistently lower in the SMD group (e.g., FC for fumarate: 1.99 in SMD vs. 2.22 in PMD). These metabolites did not differ significantly between PMD and SMD, indicating overlapping metabolic profiles. In addition, 3-hydroxybutyrate (FC = 1.23, $p=0.045$) and propylene glycol (FC = 1.69, $p=0.028$) were significantly increased in PMD compared to SMD but did not differ significantly from controls in either group.

Tryptophan was significantly increased in PMD compared to controls (FC = 2.02, $p=0.002$), while no significant change was observed in SMD (FC = 0.91, $p=0.055$); the PMD and SMD levels were similar (FC 1.18) yielding a non-significant difference. Histidine was significantly reduced in PMD relative to both controls (FC = 0.36, $p=0.038$) and SMD (FC = 0.50, $p=0.005$), with no significant difference between SMD and controls, suggesting a discriminatory role for this metabolite.

### 3.4 Diverging urinary metabolomic signatures between chronic kidney disease and mitochondrial disorders

The urinary metabolome of advanced CKD (stages 3–5) differed significantly from controls and early CKD (stages 1–2). Significant increases were observed for lactate, fumarate and acetate, while citrate and 1-methylnicotinamide (MNA) were reduced. Glucuronate levels were markedly elevated when compared to controls and CKD1-2 (FC = 24.19 and 6.69, respectively).

However, the urinary metabolome of PMD differed markedly from patients with CKD across stages ($Q^2 > 0.62$), particularly with advanced CKD (stages 3–5). Seven teen urinary metabolites (Table 2) showed significant differences between PMD and CKD stages 3–5, including Krebs cycle intermediates (*cis*-aconitate and succinate), as well as 3-hydroxyisovalerate, 2-hydroxyisobutyrate, tryptophan, tyrosine, and HVA, all showing large effect sizes (Hedges' g > 1), consistent with strong discriminatory potential. Additionally, the levels of formate, MNA, and propylene glycol were elevated in PMD, whereas that of glucuronate was markedly reduced (FC = 0.09, g = 1.23).

In fact, several metabolites showed with divergent profiles between PMD and CKD3-5 with opposite directions of change relative to controls and significant differences between the two groups (Fig. 4). Among divergent metabolites, HVA, hypoxanthine, succinate, tryptophan, were significantly elevated in PMD compared to both CKD3-5 and controls and histidine was selectively decreased in PMD, with no significant change in CKD3-5. Conversely, MNA and 3-hydroxyisovaleric acid were significantly reduced, and glucuronate strongly increased, in CKD3-5 but not in PMD, pointing to renal-specific metabolic alterations likely reflecting impaired excretion or tubular dysfunction.

Decreased MNA and 2-hydroxyisovalerate consistently distinguished CKD from controls and further discriminated CKD3–5 from CKD1–2 (Fig. 4), showing progressive depletion with advancing renal dysfunction, which is according to previous studies(Kwan et al., 2020; Lucio-Gutiérrez et al., 2022). These patterns support their potential as specific biomarkers markers of CKD disease and potential correlation with stage severity.

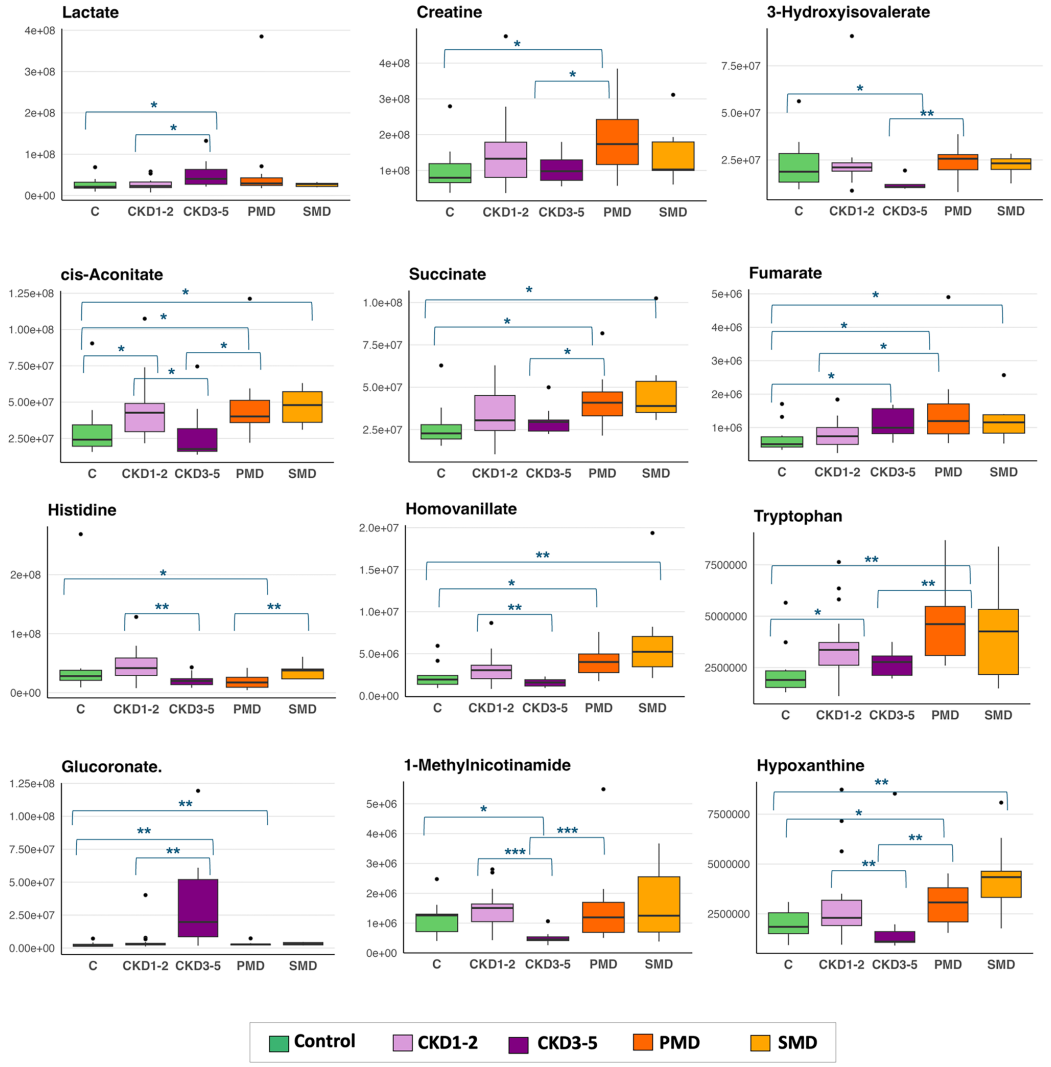


**Fig. 4** Boxplots of selected urinary metabolites showing statistically significant differences across primary mitochondrial disease (PMD), control, suspected mitochondrial dysfunction (SMD), and chronic kidney disease (CKD) groups at stages 1–2 and 3–5. Metabolites were selected based on biological relevance and statistical significance (non-parametric Mann-Whitney U test for each comparison). Significance thresholds are indicated as $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Corresponding statistical results are presented in Table 2 (main group comparisons) and Table S5 (remaining group comparisons). *PMD*, primary mitochondrial disease; *SMD*, suspected mitochondrial dysfunction; *CKD*, chronic kidney disease

### 3.5 Impact of normalization strategy on urinary metabolomic profiles

To assess the impact of normalization strategies on metabolomic data, both PQN and to urinary creatinine (a common strategy in clinical setting) were applied and their consistency was computed by Spearman's rank correlation coefficients for each quantified metabolite (Table S6). Overall, the correlation of 38 metabolites between the two approaches was moderate (median $r_s = 0.48$), with only 5 metabolites exhibiting strong or very strong monotonic relationships ($r_s \geq 0.7$) while 10 had no correlation ($r_s < 0.3$).

However, a 10% of analytes showed weak ($p = 0.1–0.3$) or no correlation ($p < 0.1$), including among others, urea, inosine, glycolate and DMA. For CKD-related univariate comparisons (114 pairwise metabolite comparisons) 75% showed concordant statistical significance under both normalization strategies. However, 20 comparisons were statistically discordant − 15 of which emerged when comparing CKD stages. Only a small subset of metabolites (including acetate, pyruvate, trigonelline, TMAO, and MNA) reached statistical significance exclusively under creatinine normalization. In our study this impact was most pronounced in comparisons involving CKD groups, particularly stages 3–5, where markedly reduced urinary creatinine concentrations contributed to a loss of statistical significance for several metabolites under creatinine normalization (such as 3-hydroxybutyrate, histidine, HVA, tyrosine, formate, hypoxanthine or tryptophan).

While normalization to urinary creatinine is widely used in clinical practice to adjust for variations in sample concentration, these results show that the method may introduce systematic *bias* in pediatric or pathophysiological settings where creatinine excretion is altered, such as CKD and myopathy (common in PMD). In contrast, PQN-normalized NMR data offer a data-driven alternative based on the overall spectral profile and is less influenced by single-analyte variability. In any case, in our cohort, measured urinary creatinine (by photometric assays) was significantly decreased only in the CKD3–5 group, which would attenuate group differences specifically in this subset.

### 3.6 Candidate biomarkers for PMD

ROC analysis supported the discriminative potential of several urinary metabolites for PMD. Histidine (AUC = 0.78, log2FC = − 1.15) and tryptophan (AUC = 0.74, log2FC = + 0.56) showed the highest specificity for PMD versus other groups, aligned with univariate findings. Propylene glycol, creatine, and fumarate also showed good performance (AUC ≥ 0.71), reflecting mitochondrial dysfunction, although with less specificity.

A targeted five-metabolite panel consisting of *cis*-aconitate, fumarate, HVA, tryptophan and histidine achieved significant separation across all five diagnostic groups (PMD, SMD, CKD1–2, CKD3–5, controls) in one-way ANOVA and displayed strong classification performance in Random Forest analysis. These metabolites contributed consistently to group discrimination, supporting their potential as a biosignature for diagnostic stratification.

To further assess the potential clinical applicability, of the above metabolite signature, we constructed classification models using the most discriminative compounds. The same, five-metabolite panel achieved excellent accuracy in distinguishing PMD from controls, with AUCs of 0.949 (linear SVM), 0.938 (PLS-DA), 0.88 (random forest), and 0.836 (logistic regression). When applied to a broader comparison of PMD versus all others, it maintained strong discriminative power (logistic regression AUC = 0.783), highlighting its robustness in non-binary diagnostic classification.

## 4 Discussion

To our knowledge, no previous metabolomic studies have directly compared urinary profiles between PMD and CKD. The use of disease-specific control groups enables a more precise attribution of metabolic changes to disease-specific mechanisms, reducing confounding from overlapping or nonspecific differences. This comparison is particularly important because renal dysfunction can mimic or coexist with mitochondrial phenotypes, independently affecting the excretion of metabolites typically interpreted as mitochondrial markers. Focusing on younger patients minimizes the influence of age-related comorbidities.

The normalization strategy is a critical step in urinary metabolomics to ensure comparability across samples. Normalization to urinary creatinine, a conventional approach in clinical setting, assumes stable endogenous creatinine excretion. However, creatinine is lower during infancy (under one year of age, as in patients from our SMD group) and in patients with myopathy, or impaired renal function (due to reduced glomerular filtration). In our study this impact was most pronounced in comparisons involving CKD groups, particularly stages 3–5, where markedly reduced urinary creatinine concentrations tends to underestimated changes.

### 4.1 Distinct metabolomic signatures in PMD patients

Our findings reveal that the urinary metabolome of pediatric PMD patients is significantly different from healthy controls, even under basal clinical conditions. Differences were consistently observed in multivariate and univariate analyses, supporting the existence of a mitochondrial disease-specific metabolic footprint.

Key metabolic alterations in PMD included increased urinary levels of HVA, *cis*-aconitate and fumarate and decreased histidine with moderate-to-large effect sizes compared to controls and CKD stage 3–5 patients, revealing how differently PMD and CKD impact the urinary metabolome. Pathway enrichment analysis identified significant involvement of energy metabolism and redox balance, including glutathione metabolism, as well as pathways related to amino acid turnover—specifically glutamate, arginine, proline, and tryptophan metabolism—and the oxidation

of branched-chain fatty acids. These findings support the biological consistency of the urinary metabolomic profile in PMD and underscore the contribution of mitochondrial energy production, amino acid catabolism, and oxidative stress response to its pathophysiology.

Substantial overlap was observed between PMD and SMD and effect sizes were generally small. These findings reflect the heterogeneity of the group and probably reflect selection bias for patients with biochemical evidence of mitochondrial dysfunction to be considered in this group. It most likely includes both true mitochondrial disorders and "mitochondrial mimics".

### 4.2 Mitochondrial impairment in renal disease differs from primary mitochondrial dysfunction

Despite the shared involvement of mitochondrial pathways in both PMD and CKD, our data support the existence of distinct urinary metabolomic signatures. In PMD patients compared to controls, elevated levels of fumarate, *cis*-aconitate tryptophan and HVA were consistently observed, even in the absence of renal impairment. In contrast, these metabolites were either unchanged or variably modulated in CKD, with *cis*-aconitate showing an inverse trend with CKD progression. CKD3–5 patients exhibited increased urinary excretion of MNA, 3-hydroxybutyrate, and other branched-chain organic acids, alongside reductions in HVA and histidine.

These findings emphasize that while mitochondrial dysfunction is a recognized feature of CKD pathophysiology, the metabolic footprint of CKD differs significantly from that of primary mitochondrial disease. Notably, many of the altered metabolites are linked to mitochondrial energy metabolism (e.g., succinate, formate, 3-hydroxybutyrate), amino acid turnover (e.g., tyrosine, tryptophan, hypoxanthine), or renal handling of organic acids (e.g., glycolate, glucuronate). Among Krebs cycle intermediates, fumarate was the only metabolite significantly increased in CKD3-5, suggesting an altered mitochondrial or renal tubular handling. In contrast, citrate and *cis*-aconitate levels were markedly reduced, and succinate showed only non-significant trends toward elevation, reinforcing fumarate's potential as a CKD-associated metabolite with preserved detectability in urine.

Figure 5 illustrates the putative metabolic distinctions observed between PMD and CKD, highlighting key urinary metabolites altered in each condition and their integration within mitochondrial metabolic pathways.

These findings reinforce the pathobiological divergence between primary mitochondrial dysfunction and advanced CKD and reinforce the utility of urinary metabolomics in discriminating disease-specific biochemical signatures. Simultaneous, these results reinforce the value of disease-specific control groups in metabolomic studies as an opportunity for better understanding of disrupted metabolic pathways.

### 4.3 Candidate biomarkers for CKD

Two potential biomarkers specific for CKD (but not PMD) were decreased MNA, and 3-hydroxyisovalerate, both of which effectively discriminated between CKD stage 3–5 and earlier stages and controls.

Previous studies that proposed MNA as a proxy for proximal tubular secretion (Maïza et al., 1992) and also demonstrated its reduction in tubulointerstitial disorders, such as Focal Segmental Glomerulosclerosis (Hao et al., 2013). Our findings with preserved MNA levels in the PMD group, align with the mechanistic model proposed by Takahashi et al. (2022) (Takahashi et al., 2022), in which reduced MNA levels reflect significant tubular damage and fibrosis, rather than early dysfunction.

Although 2-hydroxyisobutyrate pathways and renal metabolism are less well characterized, a previous metabolomic study associated its urinary excretion with early but not advanced diabetes-related CKD (Lucio-Gutiérrez et al., 2022). In our data, 2-hydroxyisobutyrate distinguished between early and advanced CKD, but not between CKD and control groups. Additionally, citrate excretion was significantly reduced only in advanced CKD (stages 3–5) as previously reported for diabetes-CKD(Kwan et al., 2020), supporting a progressive decline in urinary citrate with progressive renal impairment, possibly reflecting impaired distal nephron acid–base regulation in advanced CKD. MNA although not consistently altered in PMD versus controls, was strongly decreased in CKD3–5, contributing to discrimination between renal and mitochondrial signatures. These findings highlight the critical need to differentiate mitochondrial-specific metabolic derangements from renal-related metabolic changes when interpreting urinary biomarkers in clinical and research settings.

### 4.4 Candidate biomarkers for PMD

Several metabolites emerged as potential urinary biomarkers for PMD, based on consistent alterations across multiple comparisons and their metabolic relevance. Increased tryptophan and decreased histidine emerged as the most specific discriminators of PMD versus both SMD) and controls. Succinate and fumarate, both Krebs cycle intermediates, were consistently elevated in PMD and SMD compared to controls, supporting their utility as sensitive—though not specific—markers of mitochondrial dysfunction. In contrast, cis-aconitate was significantly increased in PMD

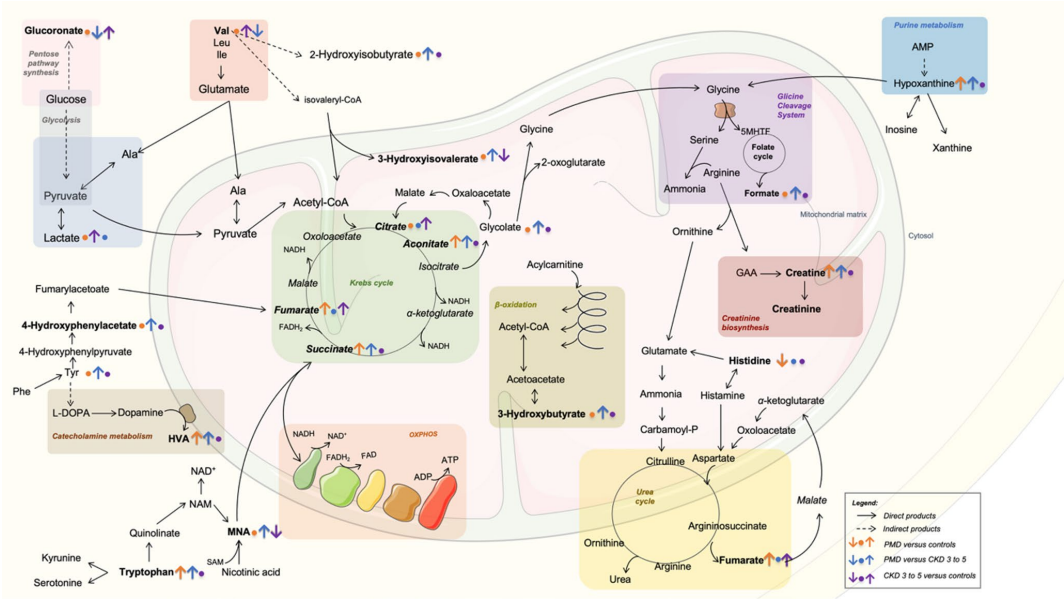


**Fig. 5** Overview of diverging metabolomic signatures between primary mitochondrial disease (PMD) and chronic kidney disease (CKD) and controls, based on significant urinary metabolites identified by $^{1}$H NMR spectroscopy. Significance is based on univariate analysis. Arrows indicate the direction of change (↑ or ↓) or non-significant change (circle) in urinary levels in PMD versus controls (orange), PMD versus CKD stages 3–5 (blue), and CKD stages 3–5 versus controls (purple): This schematic representation of how pathways interact does not account for tissue variability (for example, urea cycle occurring predominantly in hepatocytes while creatine metabolism happens mainly in muscle and brain). Three-letter code used for amino acids. Adapted with partial use of graphical elements from Servier Medical Art (https://smart.servier.com), licensed under Creative Commons Attribution 3.0 *CKD* chronic kidney disease, *FAD* flavin adenine dinucleotide, *HVA* homovanillate, *L-DOPA* L-3,4-dihydroxyphenylalanine, *NAD*$^{+}$*/NADH* nicotinamide adenine dinucleotide (oxidized/reduced), *OXPHOS* oxidative phosphorylation, *PMD* primary mitochondrial disease

but reduced in advanced CKD, highlighting its potential to differentiate mitochondrial from renal dysfunction. Similarly, HVA was increased in both PMD and SMD and may reflect broader neuro-metabolic perturbations rather than PMD-specific alterations. Notably, the consistent decrease in urinary histidine in PMD, despite normal levels in SMD and early CKD, and its further reduction in advanced CKD, raises the hypothesis of subclinical renal involvement in PMD patients. This possibility warrants further investigation, as early mitochondrial-related renal dysfunction may contribute to altered amino acid excretion patterns.

HVA, the major dopamine catabolite, is generated via sequential metabolism by mitochondrial monoamine oxidase (MAO) and cytosolic catechol-O-methyltransferase (COMT), establishing a link between mitochondrial redox function and dopamine turnover (Naoi & Maruyama, 2010). HVA is excreted by glomerular filtration and tubular secretion, predominantly via OAT1 and OAT3 (Burckhardt et al., 2001; You et al., 2025) Urinary HVA has been validated as a useful surrogate of central dopaminergic metabolism in selected monogenic conditions, even when measured by non-targeted $^{1}$H NMR approaches (Brennenstuhl et al., 2020). In our cohort, urinary HVA was elevated in PMD, suggesting alterations in mitochondrial-dependent monoamine metabolism or energy-coupled transport. Alterations in cerebrospinal HVA have been reported across several mitochondrial phenotypes and interpreted as secondary to impaired oxidative metabolism and MAO-dependent dopamine catabolism (Batllori et al., 2018; Papandreou et al., 2018). HVA was particularly elevated (>2SD above control mean) in the patient with *TMEM70*-related disease, presenting with mild movement disorder sequelae following severe neonatal-onset. A 1.5-fold increase was also observed in patients with *m.8993T>G* (P11), *LYRM4* (P14), and *m.3271T>C* (P05) mutations. While the first has Leigh syndrome the others have no neurological or cognitive features. These findings raise the possibility of subclinical alterations in dopaminergic turnover or tissue-level oxidative metabolism, and underscore the potential of HVA as a sensitive, though nonspecific, marker of mitochondrial dysfunction even in the absence of a recognized neurological phenotype, as previously suggested in POLG-related disease and other

mitochondrial disorders (Batllori et al., 2018; Papandreou et al., 2018).

Histidine was consistently decreased in PMD patients when compared to controls, suggesting a possible depletion or altered reabsorption in the proximal tubule. The reduction was more pronounced in PMD compared to CKD3-5, where a mild reduction was observed. Reduction of histidine in CKD rats was previously established (Zhang et al., 2015). As an essential amino acid, histidine participates in multiple mitochondrial-dependent processes, including oxidative stress defense, acid–base buffering, and the biosynthesis of carnosine and histamine (Brosnan & Brosnan, 2020; Li & Hoppe, 2023). Histidine catabolism occurs via mitochondrial pathways, involving its conversion to glutamate and subsequently to $\alpha$-ketoglutarate. This conversion requires tetrahydrofolate (THF), directly linking histidine metabolism to one-carbon metabolism and mitochondrial energy production (Brosnan & Brosnan, 2020). Thus, the consistent reduction in urinary histidine in PMD may reflect increased mitochondrial utilization to replenish $\alpha$-ketoglutarate pools or impaired energy-dependent renal reabsorption.

The consistent elevation of metabolites such as tryptophan and hypoxanthine in PMD patients, divergent from both controls and CKD, strongly suggests a distinct, mitochondria-driven metabolic phenotype rather than subclinical renal damage. Further studies are needed to determine whether these metabolomic signatures predict future renal involvement or represent stable, disease-specific traits associated with mitochondrial dysfunction.

Our findings underscore the potential of specific urinary metabolites, such as tryptophan and histidine, as novel candidate biomarkers for primary mitochondrial disease. Given the increasing number of clinical trials targeting mitochondrial dysfunction, the identification of robust and non-invasive biomarkers is essential to support patient stratification and treatment monitoring.

Building upon the consistent differences across pairwise comparisons, we identified a five-metabolite panel (*cis*-aconitate, fumarate, HVA, tryptophan and histidine) with high discriminatory power for PMD, which were subsequently validated trough multivariate modeling. These metabolites capture both PMD-specific alterations—notably increased tryptophan and reduced histidine—and broader mitochondrial dysfunction signatures, including elevated fumarate and cis-aconitate, which also contributed to separation from CKD patients. HVA further strengthened the panel's ability to delineate PMD from overlapping phenotypes. Overall, this metabolic signature integrates specificity and sensitivity, supporting its potential use in clinical stratification and diagnostic workflows for suspected mitochondrial disease.

However, as previously highlighted, the development of such biomarkers remains a major challenge due to clinical and genetic heterogeneity, lack of standardization, and limited size of patient cohorts (Rahman & Rahman, 2018). Further validation in larger, longitudinal studies is needed to determine whether these metabolites can serve as biomarkers for supporting diagnosis or even surrogate endpoints in future therapeutic trials.

### 4.5 Study limitations

Methodological limitations include a relatively small sample size (for instance adequate external validation of statistical models), absence of strict age and sex matching between groups, and significant genetic and clinical heterogeneity, particularly within the SMD and CKD cohorts. The cross-sectional design, based on single spot urine samples and non-standardized dietary conditions, may introduce biological and environmental variability. Although there were age differences between groups, previous studies suggest that age exerts limited impact on urinary metabolomic profiles in pediatric cohorts.

Similarly, while the absence of standardized fasting protocols introduces potential variability, it reflects real-world clinical practice. In this context, the metabolic signatures observed are likely to be persistent and disease-driven rather than transient nutritional artifacts. As previously recognized, fasting state represents a potential pre-analytical confounder in urinary metabolomic studies, however, while physiological variability was moderate among controls, greater dispersion was observed in PMD and CKD patients, likely reflecting disease-modified metabolic responses rather than nutritional variability.

Although $^{1}$H NMR is a robust and reproducible technique for untargeted analysis, it has lower sensitivity for detecting low abundance metabolites.

## 5 Conclusions

This study demonstrates that untargeted urinary $^{1}$H NMR metabolomics is a feasible and informative approach for detecting metabolic alterations in pediatric primary mitochondrial disease We identified consistent disease-specific metabolic signatures, with significantly increased urinary levels of fumarate, *cis*-aconitate, HVA, hypoxanthine, tryptophan, and succinate, and decreased histidine in PMD compared to both controls and patients with CKD, even in the absence of renal impairment .

These findings support the translational potential of $^{1}$H NMR-based urinary metabolomics as a non-invasive platform for biomarker discovery for diagnostic and monitoring,

patient stratification, and assessment of renal involvement in mitochondrial disease.

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**Author contributions** MPC : Conceptualization, Funding, Investigation, Data curation, Formal analysis, Statistical Analysis, Visualization, Writing – original draft. JER : Methodology, Software, Investigation, Resources, Supervision, Writing - review & editing. TC : Conceptualization, Investigation, Resources, Data curation. AD : Resources, Investigation, Data curation. ICRG: Software, Visualization, Writing - review & editing. HR : Conceptualization, Formal analysis, Writing - review & editing. LV : Supervision, Writing - review & editing. EM : Supervision, Writing - review & editing. AMG : Conceptualization, Methodology, Supervision, Writing - review & editing. All authors read and approved the manuscript.

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**Data availability** The full processed dataset and raw 1 H NMR spectra have been deposited at the Metabolomics Workbench under Study ID ST003924 (Project DOI: http://dx.doi.org/10.21228/M81Z64).

## Declarations

**Competing interests** The authors declare no competing interests.

**Ethical approval** All procedures involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committees and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written informed consent was obtained from all individual participants (or their legal guardians) prior to inclusion in the study.

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## References

Barshop, B. A. (2004). Metabolomic approaches to mitochondrial disease: Correlation of urine organic acids. *Mitochondrion*, *4*(5–6), 521–527. https://doi.org/10.1016/j.mito.2004.07.010

Batllori, M., Molero-Luis, M., Ormazabal, A., Montero, R., Sierra, C., Ribes, A., et al. (2018). Cerebrospinal fluid monoamines, pterins, and folate in patients with mitochondrial diseases: Systematic review and hospital experience. *Journal of Inherited Metabolic Disease*, *41*(6), 1147–1158. https://doi.org/10.1007/s10545-018-0224-x

Bhatia, D., Capili, A., & Choi, M. E. (2020). Mitochondrial dysfunction in kidney injury, inflammation, and disease: Potential therapeutic approaches. *Kidney Research and Clinical Practice*, *39*(3), 244–258. https://doi.org/10.23876/j.krcp.20.082

Boenzi, S., & Diodato, D. (2018). Biomarkers for mitochondrial energy metabolism diseases. *Essays in Biochemistry*, *62*(3), 443–454. https://doi.org/10.1042/EBC20170111

Bouatra, S., Aziat, F., Mandal, R., Guo, A. C., Wilson, M. R., Knox, C., et al. (2013). The human urine metabolome. *Plos One*, *8*(9), e73076. https://doi.org/10.1371/journal.pone.0073076

Brennenstuhl, H., Garbade, S. F., Okun, J. G., Feyh, P., Hoffmann, G. F., Langhans, C. D., & Opladen, T. (2020). Semi-quantitative detection of a vanillactic acid/vanillylmandelic acid ratio in urine is a reliable diagnostic marker for aromatic L-amino acid decarboxylase deficiency. *Molecular Genetics and Metabolism*, *131*(1–2), 163–170. https://doi.org/10.1016/j.ymgme.2020.07.001

Brosnan, M. E., & Brosnan, J. T. (2020). Histidine metabolism and function. *The Journal of Nutrition*, *150*, 2570S–2575S. https://doi.org/10.1093/jn/nxaa079

Burckhardt, G., Bahn, A., & Wolff, N. A. (2001). Molecular physiology of renal *p* -Aminohippurate secretion. *Physiology*, *16*(3), 114–118. https://doi.org/10.1152/physiologyonline.2001.16.3.114

Buzkova, J., Nikkanen, J., Ahola, S., Hakonen, A. H., Sevastianova, K., Hovinen, T., et al. (2018). Metabolomes of mitochondrial diseases and inclusion body myositis patients: Treatment targets and biomarkers. *EMBO Molecular Medicine*, *10*(12), e9091. https://doi.org/10.15252/emmm.201809091

Cloarec, O., Dumas, M. E., Craig, A., Barton, R. H., Trygg, J., Hudson, J., et al. (2005). Statistical total correlation spectroscopy: Na exploratory approach for latent biomarker identification from metabolic $^{1}$H NMR data sets. *Analytical Chemistry*, *77*(5), 1282–1289. https://doi.org/10.1021/ac048630x

Duann, P., & Lin, P. H. (2017). Mitochondria damage and kidney disease. *Mitochondrial Dynamics in Cardiovascular Medicine*, *982*, 529–551. https://doi.org/10.1007/978-3-319-55330-6_27

Engelke, U. F. H., Kremer, B., Kluijtmans, L. A. J., Van Der Graaf, M., Morava, E., Loupatty, F. J., et al. (2006). NMR spectroscopic studies on the late onset form of 3-methylglutaconic aciduria type I and other defects in leucine metabolism. *NMR in Biomedicine*, *19*(2), 271–278. https://doi.org/10.1002/nbm.1018

Esterhuizen, K., Van Der Westhuizen, F. H., & Louw, R. (2017). Metabolomics of mitochondrial disease. *Mitochondrion*, *35*, 97–110. https://doi.org/10.1016/j.mito.2017.05.012

Esterhuizen, K., Lindeque, J. Z., Mason, S., Van Der Westhuizen, F. H., Suomalainen, A., Hakonen, A. H., et al. (2019). A urinary biosignature for mitochondrial myopathy, encephalopathy, lactic acidosis and stroke like episodes (MELAS). *Mitochondrion*, *45*, 38–45. https://doi.org/10.1016/j.mito.2018.02.003

Finsterer, J. (2022). The spectrum of renal abnormalities in mitochondrial disorders is broad. *Kidney International Reports*, *7*(7), 1722. https://doi.org/10.1016/j.ekir.2022.05.014

Finsterer, J., & Scorza, F. (2017). Renal manifestations of primary mitochondrial disorders. *Biomedical Reports*, *6*(5), 487–494. https://doi.org/10.3892/br.2017.892

Galvan, D. L., Green, N. H., & Danesh, F. R. (2017). The hallmarks of mitochondrial dysfunction in chronic kidney disease. *Kidney International*, *92*(5), 1051–1057. https://doi.org/10.1016/j.kint.2017.05.034

Hall, A. M., Vilasi, A., Garcia-Perez, I., Lapsley, M., Alston, C. L., Pitceathly, R. D. S., et al. (2015). The urinary proteome and metabonome differ from normal in adults with mitochondrial disease. *Kidney International*, *87*(3), 610–622. https://doi.org/10.1038/ki.2014.297

Hao, X., Liu, X., Wang, W., Ren, H., Xie, J., Shen, P., et al. (2013). Distinct metabolic profile of primary focal segmental glomerulosclerosis revealed by NMR-Based metabolomics. *Plos One*, *8*(11), e78531. https://doi.org/10.1371/journal.pone.0078531

Hong, J., Shao, X., Jia, S., Qu, L., Weng, C., Shen, X., et al. (2019). The Mitochondria-Targeted metabolic tubular injury in diabetic kidney disease. *Cellular Physiology and Biochemistry*, *52*(2), 156–171. https://doi.org/10.33594/000000011

Hu, Q., Ren, J., Ren, H., Wu, J., Wu, X., Liu, S., et al. (2018). Urinary mitochondrial DNA identifies renal dysfunction and mitochondrial damage in Sepsis-Induced acute kidney injury. *Oxidative Medicine and Cellular Longevity*, *2018*(1), 8074936. https://doi.org/10.1155/2018/8074936

Jotwani, V., Yang, S. Y., Thiessen-Philbrook, H., Parikh, C. R., Katz, R., Tranah, G. J., et al. (2024). Mitochondrial genetic variation and risk of chronic kidney disease and acute kidney injury in UK biobank participants. *Human Genetics*, *143*(2), 151–157. https://doi.org/10.1007/s00439-023-02615-4

Kazubek-Zemke, M., Rybka, J., Marchewka, Z., Rybka, W., Pawlik, K., & Długosz, A. (2014). Preliminary study on application of urine amino acids profiling for monitoring of renal tubular injury using GLC-MS. *Postępy Higieny I Medycyny Doświadczalnej*, *68*, 1299–1311. https://doi.org/10.5604/17322693.1128846

Khan, S., Ince-Dunn, G., Suomalainen, A., & Elo, L. L. (2020). Integrative omics approaches provide biological and clinical insights: Examples from mitochondrial diseases. *Journal of Clinical Investigation*, *130*(1), 20–28. https://doi.org/10.1172/JCI129202

Kimura, T., Yasuda, K., Yamamoto, R., Soga, T., Rakugi, H., Hayashi, T., & Isaka, Y. (2016). Identification of biomarkers for development of end-stage kidney disease in chronic kidney disease by metabolomic profiling. *Scientific Reports*, *6*(1), 26138. https://doi.org/10.1038/srep26138

Kobayashi, T., Yoshida, T., Fujisawa, T., Matsumura, Y., Ozawa, T., Yanai, H., et al. (2014). A metabolomics-based approach for predicting stages of chronic kidney disease. *Biochemical and Biophysical Research Communications*, *445*(2), 412–416. https://doi.org/10.1016/j.bbrc.2014.02.021

Kuhn, C., Mohebbi, N., & Ritter, A. (2024). Metabolic acidosis in chronic kidney disease: Mere consequence or also culprit? *Pflügers Archiv - European Journal of Physiology*, *476*(4), 579–592. https://doi.org/10.1007/s00424-024-02912-5

Kwan, B., Fuhrer, T., Zhang, J., Darshi, M., Van Espen, B., Montemayor, D., et al. (2020). Metabolomic markers of kidney function decline in patients with diabetes: Evidence from the chronic renal insufficiency cohort (CRIC) study. *American Journal of Kidney Diseases*, *76*(4), 511–520. https://doi.org/10.1053/j.ajkd.2020.01.019

Levin, A., Ahmed, S. B., Carrero, J. J., Foster, B., Francis, A., Hall, R. K., et al. (2024). Executive summary of the KDIGO 2024 clinical practice guideline for the evaluation and management of chronic kidney disease: Known Knowns and Known unknowns. *Kidney International*, *105*(4), 684–701. https://doi.org/10.1016/j.kint.2023.10.016

Li, Q., & Hoppe, T. (2023). Role of amino acid metabolism in mitochondrial homeostasis. *Frontiers in Cell and Developmental Biology*, *11*, 1127618. https://doi.org/10.3389/fcell.2023.1127618

Lucio-Gutiérrez, J. R., Cordero-Pérez, P., Farías-Navarro, I. C., Tijerina-Marquez, R., Sánchez-Martínez, C., Ávila-Velázquez, J. L., et al. (2022). Using nuclear magnetic resonance urine metabolomics to develop a prediction model of early stages of renal disease in subjects with type 2 diabetes. *Journal of Pharmaceutical and Biomedical Analysis*, *219*, 114885. https://doi.org/10.1016/j.jpba.2022.114885

Maïza, A., Waldek, S., Ballardie, F. W., & Daley-Yates, P. T. (1992). Estimation of renal tubular secretion in Man, in health and Disease, using endogenous N-1-Methylnicotinamide. *Nephron*, *60*(1), 12–16. https://doi.org/10.1159/000186698

Martín-Hernández, E., García-Silva, M. T., Vara, J., Campos, Y., Cabello, A., Muley, R., et al. (2005). Renal pathology in children with mitochondrial diseases. *Pediatric Nephrology*, *20*(9), 1299–1305. https://doi.org/10.1007/s00467-005-1948-z

Naoi, M., & Maruyama, W. (2010). Monoamine oxidase inhibitors as neuroprotective agents in Age-Dependent neurodegenerative disorders. *Current Pharmaceutical Design*, *16*, 2799–2817. https://doi.org/10.2174/138161210793176527

Papandreou, A., Rahman, S., Fratter, C., Ng, J., Meyer, E., Carr, L. J., et al. (2018). Spectrum of movement disorders and neurotransmitter abnormalities in paediatric *POLG* disease. *Journal of Inherited Metabolic Disease*, *41*(6), 1275–1283. https://doi.org/10.1007/s10545-018-0227-7

Parasyri, M., Brandström, P., Uusimaa, J., Ostergaard, E., Hikmat, O., Isohanni, P., et al. (2022). Renal phenotype in mitochondrial diseases: A multicenter study. *Kidney Diseases*, *8*(2), 148–159. https://doi.org/10.1159/000521148

Posada-Ayala, M., Zubiri, I., Martin-Lorenzo, M., Sanz-Maroto, A., Molero, D., Gonzalez-Calero, L., et al. (2014). Identification of a urine metabolomic signature in patients with advanced-stage chronic kidney disease. *Kidney International*, *85*(1), 103–111. https://doi.org/10.1038/ki.2013.328

Rahman, J., & Rahman, S. (2018). Mitochondrial medicine in the omics era. *The Lancet*, *391*(10139), 2560–2574. https://doi.org/10.1016/S0140-6736(18)30727-X

Rao, M., Jaber, B. L., & Balakrishnan, V. S. (2018). Chronic kidney disease and acquired mitochondrial myopathy. *Current Opinion in Nephrology and Hypertension*, *27*(2), 113–120. https://doi.org/10.1097/MNH.0000000000000393

Roper, T., Harber, M., Jones, G., Pitceathly, R. D. S., & Salama, A. D. (2020). Delayed diagnoses of mitochondrial cytopathies in patients presenting with end stage kidney disease: Two case reports. *BMC Nephrology*, *21*(1), 361. https://doi.org/10.1186/s12882-020-02002-5

Sharma, K., Karl, B., Mathew, A. V., Gangoiti, J. A., Wassel, C. L., Saito, R., et al. (2013). Metabolomics reveals signature of mitochondrial dysfunction in diabetic kidney disease. *Journal of the American Society of Nephrology*, *24*(11), 1901–1912. https://doi.org/10.1681/ASN.2013020126

Smuts, I., Louw, R., Toit, D., Klopper, H., Mienie, B., L. J., & Van Der Westhuizen, F. H. (2010). An overview of a cohort of South African patients with mitochondrial disorders. *Journal of Inherited Metabolic Disease*, *33*(S3), 95–104. https://doi.org/10.1007/s10545-009-9031-8

Takahashi, R., Kanda, T., Komatsu, M., Itoh, T., Minakuchi, H., Urai, H., et al. (2022). The significance of NAD+metabolites and nicotinamide N-methyltransferase in chronic kidney disease. *Scientific Reports*, *12*(1), 6398. https://doi.org/10.1038/s41598-022-10476-6

Thompson Legault, J., Strittmatter, L., Tardif, J., Sharma, R., Tremblay-Vaillancourt, V., Aubut, C., et al. (2015). A metabolic signature of mitochondrial dysfunction revealed through a Monogenic form of Leigh syndrome. *Cell Reports*, *13*(5), 981–989. https://doi.org/10.1016/j.celrep.2015.09.054

Venter, L., Lindeque, Z., Van Rensburg, J., Van Der Westhuizen, P., Smuts, F., I., & Louw, R. (2015). Untargeted urine metabolomics reveals a biosignature for muscle respiratory chain deficiencies. *Metabolomics*, *11*(1), 111–121. https://doi.org/10.1007/s11306-014-0675-5

You, C., Guo, J., & Xun, Y. (2025). Renal organic anion transporter 1: Clinical relevance and the underlying mechanisms in chronic kidney disease. *BMC Nephrology*, *26*(1), 93. https://doi.org/10.1186/s12882-025-03974-y

Zhang, Z. H., Wei, F., Vaziri, N. D., Cheng, X. L., Bai, X., Lin, R. C., & Zhao, Y. Y. (2015). Metabolomics insights into chronic kidney disease and modulatory effect of rhubarb against tubulointerstitial fibrosis. *Scientific Reports*, *5*(1), 14472. https://doi.org/10.1038/srep14472

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# *Supplemental material*

## S1. Ethics Committee approval

centro hospitalar do Porto

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ASSUNTO: Projeto de Investigação - **"Defeitos genéticos das doenças mitocondriais: abordagem por sequenciação de nova geração"** - N/ REF.ª 2016.194(164-DEFI/153-CES)

O Conselho de Administração do CHP autoriza a realização do estudo acima mencionado a realizar no Serviço de Pediatria, desta Instituição e tendo como Investigador Principal a Prof.ª Doutora Laura Vilarinho.
O estudo foi previamente analisado pela Comissão de Ética para a Saúde, pelo Gabinete Coordenador de Investigação, pela Direção do Departamento de Ensino, Formação e Investigação e pelo Presidente do Conselho de Administração tendo obtido parecer favorável.

Cumprimentos,

CONSELHO DE ADMINISTRAÇÃO 19 ABR. 2017

Dr. PAULO BARBOSA — Presidente
Prof. Doutor JOSÉ BARROS — Diretor Clínico
Dr.ª ÉLIA GOMES — Vogal Executiva
Dr. RUI PEDROSO — Vogal Executivo
Enf.º EDUARDO ALVES — Enfermeiro Diretor

* Em todas as eventuais comunicações posteriores sobre este estudo é indispensável indicar a nossa ref.ª.

centro hospitalar do Porto

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APRECIAÇÃO E PARECER PARA A REALIZAÇÃO DE **PROJETO DE INVESTIGAÇÃO**

| | | |
|---|---|---|
| Título: **"Defeitos genéticos das doenças mitocondriais: abordagem por sequenciação de nova geração"** | | Ref.ª: **2016.194(164-DEFI/153-CES)** |
| | | Investigador:<br>**Prof.ª Doutora Laura Vilarinho**<br>**Instituto Nacional Ricardo Jorge** |

| | |
|---|---|
| **DIREÇÃO DE ENFERMAGEM:**<br><br>☒ **NÃO SE APLICA**<br><br>☐ **PARECER FAVORÁVEL**<br><br>☐ **PARECER NÃO FAVORÁVEL**<br><br>Data:<br><br>_____ | **PRESIDENTE DO CONSELHO DE ADMINISTRAÇÃO:**<br><br>☒ **PARECER FAVORÁVEL**<br><br>☐ **PARECER NÃO FAVORÁVEL**<br><br>Data: 18/4/2017<br><br>**DR. PAULO BARBOSA**<br>Presidente do Conselho de Administração do CHP |

Em conformidade. Pode ser autorizado

Diretora do DEFI 24/03/2017

Prof.ª Doutora Luísa Lobato
Diretora do DEFI

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COMISSÃO DE ÉTICA PARA A SAÚDE

## APRECIAÇÃO E VOTAÇÃO DO PARECER

| Deliberação | Data: 15.2.2017 | Órgão: Reunião Plenária |
|---|---|---|

| Título: **"Defeitos genéticos das doenças mitocondriais: abordagem por sequenciação de nova geração"** | | Ref.ª: **2016.194(164-DEFI/153-CES)** |
|---|---|---|
| Protocolo/Versão:<br>**PI** | Promotor:<br>**o(a) próprio(a)** | Investigador:<br>**Dra Laura Vilarinho – Farmacêutica, Investigadora Auxiliar, Instituto Nacional de Saúde Dr. Ricardo Jorge (INSRJ).** |

A Comissão de Ética para a Saúde – CES do CHP, ao abrigo do disposto no Decreto-Lei n.º 97/95, de 10 de Maio, em reunião realizada nesta data, apreciou a fundamentação do relator sobre o pedido de parecer para a realização de **PI** acima referenciado:

Ouvido o Relator, o processo foi votado pelos Membros da CES presentes:

Presidente: Dr.ª Luisa Bernardo
Vice-Presidente: Dr.ª Paulina Aguiar

Dr.ª Fernanda Manuela, Enf.ª Paula Duarte, Prof.ª Doutora Carla Teixeira, Prof.ª Doutora Maria Manuel Araújo Jorge, Dr. Gonçalo Senhorães Senra.
Resultado da votação:

**PARECER FAVORÁVEL**

A deliberação foi aprovada por unanimidade.

Pelo que se submete à consideração superior.

PARECER FAVORÁVEL
18/4/017
Dr. SEVERO TORRES
Assessor do Presidente do Conselho de Administração

Data 15.2.2017

A Presidente da CES

Dr.ª Luisa Bernardo

Imp.10/2009

EA 11/4/017

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## S3. List of genes included in the NGS targeted gene panel

*AARS2; ABCB7; ACAD9; ACO2; ADCK3; AGK; AIFM1; ALAS2; ALG13; APTX; ATP5A1; ATP5E; ATPAF2; AUH; BCS1L; BOLA3; C10ORF2; C12ORF65; C19ORF12; C9ORF116; CARS2; CHKB; CISD2; CLN3; CLN5; CLN6; CLN8; CLPB; COA3; COA5; COQ2; COQ4; COQ6; COQ9; COX10; COX14; COX15; COX20; COX4I2; COX5A; COX5B; COX6A1; COX6B1; COX7B; CTSD; CWF19L1; CYC1; DARS2; DGUOK; DLAT; DLD; DNA2; DNAJC19; DNAJC5; DNM1L; DOLK; EARS2; ECHS1; ELAC2; ETFA; ETFB; ETFDH; ETHE1; FARS2; FASTKD2; FBXL4; FDX1L; FLAD1; FOXRED1; GDAP1; GFER; GFM1; GLRX5; GTPBP3; HACE1; HARS2; HCCS; HIBCH; IARS2; IBA57; ISCU; KARS; KCTD7; KIF5A; LARS; LARS2; LIAS; LIPT1; LRPPRC; LYRM4; LYRM7; MARS2; MEGF10; MFF; MFN1; MFN2; MFSD8; MGME1; MICU1; MPC1; MPI; MPV17; MRPL23; MRPL3; MRPL44; MRPL50; MRPL57; MRPS16; MRPS22; MTFMT; MTO1; MTPAP; NARS2; NDUFA1; NDUFA10; NDUFA11; NDUFA12; NDUFA2; NDUFA4; NDUFA9; NDUFAF1; NDUFAF2; NDUFAF3; NDUFAF4; NDUFAF5; NDUFAF6; NDUFB3; NDUFB9; NDUFS1; NDUFS2; NDUFS3; NDUFS4; NDUFS6; NDUFS7; NDUFS8; NDUFV1; NDUFV2; NFU1; NOTCH3; NUBPL; OPA1; OPA3; PARS2; PC; PCK2; PDHA1; PDHB; PDHX; PDP1; PDSS1; PDSS2; PLD1; PMM2; PNPT1; POLG; POLG2; PPT1; PUS1; RARS2; RMND1; RNASEH1; RRM2B; SARS2; SCO1; SCO2; SDHA; SDHAF1; SDHAF2; SDHB; SDHC; SDHD; SERAC1; SLC19A3; SLC25A22; SLC25A3; SLC25A4; SLC25A42; SLC4A3; SLC52A1; SLC52A2; SLC52A3; SPATA5; SUCLA2; SUCLG1; SURF1; TACO1; TARS2; TAZ; TIMM8A; TK2; TMEM126A; TMEM126B; TMEM70; TPK1; TPP1; TRMU; TRNT1; TSFM; TTC19; TUFM; TYMP; UQCRB; UQCRC2; UQCRQ; VARS2; WARS2; WDR45; WFS1; YARS2.*

## S4. Summary Description of Patients Included in This Thesis

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## S4A. Patients with Primary Mitochondrial Disorders

Patients P34, P38, P40, P44, P47–49, P51, P53, P56–57, P61, P68 and P69 were previously reported by our group in Nogueira et al., 2019, and subsequently revisited in Nogueira et al., 2024[143,303]. P57 was also included in Baldo et al., 2023[304]; P44 was originally described in Pereira et al., 2007; patient P69 (*LYRM4*) was first reported in this thesis and in Coelho et al., 2019, then re-included in Nogueira et al., 2024[143,340]; and one EARS2 case (P64) had also been part of the original LTBL cohort reported by Steenweg et al., 2012[176]. Patient P63 had been functionally characterised by another group, demonstrating reduced *FASTKD2* protein expression; to our knowledge, this case has not been published as a full peer-reviewed report[386].

***Table S1. Clinical, biochemical, imaging, and genetic characteristics of patients with a confirmed diagnosis of primary mitochondrial disease (PMD) in our cohort (n=38).***

| P # | O | Sex | Age | Description | Metabolic | Muscle biopsy | MRI | MDC | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|
| **P33** | 3.0 | F | 24† | DD, Severe cognitive impairment, absent speech. ASD. Epilepsy and ataxia. Myopathy with recurrent rhabdomyolysis. NSHL. Retinitis pigmentosa. CKD (on dialysis). Anaemia (N/N). Hypoventilation. CMP, 2nd degree AV block (pacemaker). Hyperinsulinism, DM, hypothyroidism. FTT, short stature. Feeding difficulties. Hepatic steatosis, ↑ transaminases. | ↑ L | Fibber size variability. Type 2 fibber atrophy<br>RC: multiple | T2 hyperintensity involving the cerebellar white matter, peduncles, pons, and midbrain, with signs suggestive of progression towards cavitation<br>MRS: lactate peak | 8 | Kerne-Sayre syndrome<br>Single large scale mtDNA deletion |
| **P34** | 0.6 | F | 5 | DD. Myopathy, ptosis. FTT. Short stature. Hypothyroidism. IGF1 deficiency. Anaemia. ↑ transaminases. Episodes of ketoacidosis and metabolic acidosis. | ↑ L, AOs: ketonuria | NP | NP | 6 | Kerne-Sayre syndrome<br>Single large scale mtDNA deletion |
| **P35** | 7.0 | F | 26† | DD/cognitive impairment with regression. Dementia. Epilepsy; Regression episode at 4 years, with hemiparesis, dystonia, dysarthria, ataxia, dysphagia. Strabismus. WPW syndrome. FTT. Feeding difficulties. Proteinuria. CKD (on dialysis). | ↑ L | Normal, Q10 normal<br>OXPHOX: IV (34%) | Left lenticulostriate ischaemic lesion. | 8 | MELAS/LS<br>*MT-ND5*: m.13513G>A, heteroplasmy NA |
| **P36** | 3.0 | F | 19 | DD with cognitive impairment. Ataxia, dystonia, dysarthria, spasticity. Myopathy with muscle weakness and gait disturbance. Strabismus. FTT, short stature. | ↑ L | Normal<br>RC: multiple | Basal ganglia T2 hyperintensities and atrophy (++ putamina and caudate nuclei). Follow-up MRI: plus the globi pallidi. Neuronal loss and calcifications. Mild cerebellar atrophy.<br>MRS: lactate peak | 8 | mtDNA-related LS<br>m.10191T>C |
| **P37** | 4.0 | M | 13 | Cognitive impairment. Myopathy with muscle weakness. Hypertrophic CMP. NSHL. FTT, | ↑ L, AOs: ketonuria, 3- | | Normal | 7 | Mitochondrial myopathy and multisystemic disease |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | short stature. IGF1 deficiency. ↑ transaminases, amylase, and LDH. HyperCKemia | MGA, 3HO-glutraric | | | | *MT-TL1*: m.3271T>C, heteroplasmy 65% blood |
| **P38** | 2.0 | M | 11 | DD with cognitive impairment. Intermittent ataxia. Central hypoventilation. Feeding difficulties with dysphagia and vomiting. Visual impairment. Pancytopenia. Precocious pubarche. IgG deficiency. | ↑ L, L/P 9 | Type 1 fibber predominance, Lipid droplet accumulation<br>RC: NP | Cerebellar atrophy with T2 hyperintensities in the medulla, cerebellum (WM), and left thalamus. | 8 | mtDNA-related LS<br>*MT-ND5*: m.13513G>A, heteroplasmy 58% blood, 80% muscle |
| **P39** | 0.5 | F | 29 | DD with cognitive impairment and absent speech. Epilepsy. Ataxia, tremor, hypotonia, peripheral axonal sensory neuropathy. Repolarisation abnormalities. Retinitis pigmentosa, NSHL. FTT. Glomerular proteinuria. | ↑ L, L/P 18↑ Ala, , ↑ Lys, ↓ Cit | NP | Basal ganglia T2 hyperintensity and atrophy. Cerebellar and brainstem atrophy. Follow-up MRI: T2/FLAIR hyperintensity and atrophy of the striatum, brainstem and cerebellum, cavitated lesion in putamen | 8 | NARP syndrome<br>*MT-ATP6*: m.8993T>G, heteroplasmy 95% blood |
| **P40** | 2.0 | F | 17 | DD with cognitive impairment and absent speech. Ataxia. Pyramidal signs. NSHL. Non-compaction of the left ventricle. WPW syndrome. FTT. Short stature. | ↑ L, L/P 20, ↑ Ala | Normal<br>RC: normal, CS↑ | Bilateral T2 hyperintensity and atrophy in the putamina. Non-acute stroke-like lesion in cerebellar hemisphere. Cerebellar atrophy. Follow-up MRI: T2 hyperintensity in the putamina and right caudate nucleus, bilateral necrotic lesions of the striatum. MRS: lactate peak | 8 | mtDNA-related LS<br>*MT-ND1*: m.3946G>A, heteroplasmy 70% blood |
| **P41** | 10.0 | M | 17 | Epilepsy. Mild cognitive impairment. Myopathy with hiperCKemia. Short stature. Myopia. Diagnosed presymptomatically (family screening). | | ↑ Fibber size variability. Subsarcolemmal glycogen accumulation<br>RC: normal | Normal | 3 | Mitochondrial encephalomyopathy<br>*MT-TK*: m.8344A>G, heteroplasmy >95% blood |
| **P42** | 0.3 | M | 8† | DD, severe cognitive impairment with absent speech. Epilepsy. Early hypotonia with developmental regression. Myopathy. Hypertrophic CMP. Feeding difficulties. | ↑ L, LP 37, ↑ Ala, AOs: Krebs metabolites, 4-OH-phenyl-lactic | NP | T2 hyperintensity in the striatum, sublenticular and subthalamic nuclei, hypothalamus, thalami, cerebral peduncles, midbrain, pons, and cerebellum. MRS: lactate peak | 8 | mtDNA-related LS<br>*MT-ATP6*: m.8993T>G, heteroplasmy >95% blood |
| **P43** | 2.0 | F | 21 | DD with moderate cognitive impairment and absent speech. Ataxia. Myopathy with myalgias and hyperCKemia. Spasticity. Hepatic steatosis. Respiratory failure with metabolic acidosis after infectious trigger. | ↑ L, L/P 9 | Normal<br>RC: normal | Bilateral putaminal atrophy with T2 hyperintensity. MRS: lactate peak | 7 | mtDNA-related LS<br>*MT-ATP6*: m.8993T>G, heteroplasmy >95% blood |
| **P44** | 1.0 | M | 16† | DD with severe cognitive impairment. Progressive neurological disease with ataxia, dystonia, tremor, dysarthria, and spasticity. | ↑ L, L/P 24 | RRF<br>RC: IV (23%) | Global cerebral atrophy with striatal necrosis involving putamina and caudate nuclei. | 8 | mtDNA-related LS<br>*MT-CYB*: m.14724G>A, 94% (muscle), 62% (blood) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Myopathy. NSHL. Left ventricular dysfunction. FTT, short stature. IGF1 deficiency. | | | MRS: lactate peak | | |
| **P45** | 2.0 | M | 10 | DD with regression triggered by infection at 18 months. ASD. Epilepsy. Absent speech. Astigmatism. | | RC: normal | Normal | 2 | Mitochondrial encephalopathy<br>*MT-TL1*: m.3243A>G, 56% |
| **P46** | 1.0 | F | 11 | DD with cognitive impairment and language delay. Early hypotonia. Ataxia. Encephalopathy with regression after sedation. Epilepsy. Myopathy. HyperCKemia. Glomerular proteinuria. | ↑ L, L/P 23, ↑ Ala, ↑ Gly, ↓ Cit | NP | Leigh | 7 | mtDNA-related LS<br>*MT-ATP6*: m.8993T>G >C, heteroplasmy >90% blood |
| **P47** | 0.5 | F | 7 | DD with severe cognitive impairment and absent language. Epilepsy. Congenital ataxia. Dystonia. Apraxia. Ophthalmoparesis. Microcephaly. ↑ transaminases. | ↑ L, L/P 22, ↑ Ala | | T2 hyperintensity and calcifications in the globi pallidi | 8 | Pantothenate kinase-associated neurodegeneration (PKAN)<br>*PANK2*: c.1537-3C>G, (p.?) (homozygous) |
| **P48** | 0.4 | F | 25† | Cognitive impairment. Epilepsy. Hypotonia. Congenital cerebellar ataxia. Slowly progressive sensorimotor neuropathy. Divergent strabismus and nystagmus. Oculomotor apraxia. NSHL. Dysphagia. | | Normal<br>RC: normal | Cerebellar atrophy, optic nerve atrophy | 4 | Mitochondrial RNA import protein deficiency<br>*PNPT1*: c.1177-2A>C, (p.?) and c.1519G>T, p.(Ala507Ser) |
| **P49** | 0.5 | F | 10 | DD with mild cognitive impairment. Hypotonia, hemiparesis, dysmetria, dysarthria, and ataxia. Myopathy with elevated CK. | ↑ L, ↑ Ala | Type 2 fibber atrophy<br>RC: II partial | T2/FLAIR hyperintensity in periventricular and subcortical WM, basal ganglia, thalami and brainstem. Mild supratentorial WM rarefaction. Follow-up MRI: New foci in the thalami and posterior midbrain grey matter. | 7 | EARS2-deficiency<br>*EARS2*: c.1413delG; p.(Lys471Asnfs*14) and c.1235G>A; p.(Arg412His) |
| **P50** | 5.0 | M | 23 | DD. Ataxia, myoclonus, cervical dystonia and generalized tremor, dysmetria, and clonus. Myopathy. Decreased visual acuity. | ↑ L, L/P 27, ↑ Lys, AOs: ketonuria and aminobutyric acid | NP<br>RC: II partial | Cerebellar atrophy | 4 | Primary Coenzyme Q10 deficiency<br>*COQ8A*: c.1042C>T p.(Arg348*) and c.785G>A p.(Arg262Gln) |
| **P51** | 0.0 | F | 0.1 † | Neonatal seizures. Axial hypotonia. Microcephaly. Hypertrophic CMP. DM. Anaemia and thrombocytopenia. Proximal tubulopathy. Omphalocele. Metabolic lactic acidosis. | ↑ L | Normal<br>RC: NP | NP | 6 | Mitochondrial asparaginyl-tRNA synthetase deficiency<br>*NARS2* c.500A>G (p.His167Arg), c.690-?_959+?del (p.Ala231_Ile320del) |
| **P52** | 0.5 | M | 17 | DD. Hypotonia. Non-progressive cerebellar syndrome with gait imbalance. Peripheral neuropathy. Ophthalmoparesis without nystagmus. Lymphopenia, thrombocytopenia. Low IgG. Mixed ventilatory impairment. FTT. | | Type 2 fibber atrophy. Mild fibber atrophy<br>RC: normal, CS↑ | Initial MRI (age 3) Normal. Follow-up MRI (2024): T2/FLAIR hyperintensity in the cerebellum, globi pallidi hypointensity, probable abducens nerve hypoplasia.Cerebellar atrophy. | 4 | VARS2 deficiency<br>*VARS2*: c.1100C>T, p.(Thr367Ile) and c.1258G>A, p.(Ala420Thr) |
| **P53** | 1.5 | F | 11 | DD with language delay. Ataxia. Recurrent episodes of encephalopathy. | ↑ L | NP<br>RC: NP | T2 hyperintensity in the caudate nuclei, lentiform nuclei, | 6 | Thiamine-biotin responsive basal ganglia disease |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | thalami, and periaqueductal gray matter. Follow-up MRI: Cortical T2 hyperintensity with diffusion restriction in cerebral and vermis of the cerebellum. More T2 hyperintense foci in the basal ganglia with atrophic aspect of the lentiform nuclei. | | *SLC19A3*: c.980-14A>G (splicing) and c.117G>A, p.(Trp59) |
| **P54** | 5.0 | M | 11 | Progressive myopathy with ptosis and ophthalmoparesis. NSHL. Vomiting. Nephrotic syndrome, CKD (on dialysis). Anemia. Hypogammaglobulinemia. HyperCKemia. | ↑ L | Fibber atrophy. Multiple COX- and SDH+ fibbers. Numerous RRR. ↑ lipid droplets. RC: normal | NP | 6 | Mitochondrial DNA depletion syndrome *RRM2B*: c.146A>G, p.(Tyr49Cys)) and c.550+3A>G, p.? |
| **P55** | 0.0 | M | 0.0 † | Severe neonatal metabolic (lactic) acidosis. Glomerulocystic kidney disease (post-mortem finding). | ↑ L, AOs: DC | | NP | 4 | HSD10 disease *HSD17B10* c.284C>A p.Ala95Glu (hemizygous) |
| **P56** | 0.5 | M | 22 | DD. Paroxysmal dystonia. Ataxia. | ↑ L, AOs: DC | | NP | 5 | Pyruvate dehydrogenase deficiency *PDHX*: c.850C>T, p.(Arg284*), homozygous |
| **P57** | 0.8 | M | 1.7 † | DD. Epilepsy. Nystagmus. Optic atrophy. Hypotonia. Myopathy with muscle atrophy. FTT. Feeding difficulties. Neutropenia and lymphopenia. Metabolic acidosis. | ↑ L, AOs: ketonuria | NP RC: I 38% | T2 hyperintensities in cerebral peduncles (predominantly the substantia nigra), tegmental tracts of the pons, and medulla oblongata, extending into the cervical spinal cord. | 8 | LS (nuclear encoded) *NDUFS1*: c.470A>T; p.(Lys157Met) and c.1798G>C; p.(Glu600Gln) |
| **P58** | 0.0 | M | 6 | Neonatal hypoglycemia and metabolic acidosis. Myopathy. Dilated CMP. Repolarisation abnormalities. Neutropenia. ↑ transaminases. FTT. | ↑ L, AOS: 3-MGA | NP RC: V 31% | NP | 6 | Barth syndrome *TAZ:* c.280C>T (p.Arg94Cys) |
| **P59** | 0.0 | M | 2 | Neonatal metabolic acidosis with hyperammonemia and hyperlactacidemia. DD. Hypotonia. Myopathy. Hypertrophic CMP. Repolarisation abnormalities. NSHL. ↑ transaminases. FTT. Short stature. | ↑ L, L/P 36, ↑ Ala, ↑ Lys, AOs: 3-MGA, Krebs metabolites | Normal RC: II 37% | Normal (1 month of age) | 8 | TMEM70 deficiency *TMEM70*: c.317-1A>G, p.? (homo) |
| **P60** | 0.0 | F | 16 | Proximal tubulopathy (Fanconi) with neonatal onset with CKD and glomerular proteinuria. Ataxia. Myopathy. Vomiting. FTT. Short stature. IGF1 deficiency. Hypogonadism. Anemia. | ↑ L, AOs: ketonuria, Krebs metabolites, DC, 4-OH-phenyl-lactic and -pyruvic | Normal RC: I (12%) | Normal | 7 | GRACILE syndrome *BCS1L*: c.-147A>G p.? and c.166C>T, p.(Arg56Ter) |
| **P61** | 0.0 | F | 27 | DD. Ataxia, dystonia, pyramidal signs. Myopathy. Ventricular repolarization abnormalities. | ↑ L (blood and CSF), ↑ Ala | Normal RC: normal | T2/FLAIR hyperintensity of the cerebral WM | 5 | Mitochondrial encephalomyopathy *NDUFV2*: c.748T>C, p.(250Glnext34), homozygous |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **P62** | 4.0 | F | 24 | Congenital lactic acidosis. DD with cognitive impairment and regression. Epilepsy. Spastic tetraparesis, dystonia, hypertonia. Myopathy and fatigue. Optic atrophy. Loss of language. Feeding difficulties. FTT. Elevated transaminases. IgA and IgG deficiency. Neonatal hypoglycemia. | ↑ L, ↑ Ala, ↓ Cit AOs: DC, 3-MGA | Normal RC: multiple | T2/FLAIR hyperintensity in pons, cerebral peduncles, medial thalami, globi pallidi, and putamina. Follow-up MRI: Similar | 8 | Dihydrolipoamide dehydrogenase deficiency *DLD* c.803_804del, p.(Gln268Argfs*3) and c.259C>T, p.(Pro87Ser) |
| **P63** | 3.0 | M | 16 | Episodic neuroregression. DD with cognitive impairment and absent language. Epilepsy. Spastic tetraparesis. Dystonia. Choreoathetosis of the right upper limb. Hypotonia during crisis. FTT. Elevated transaminases. Microcytic hypochromic anemia. left palate. Worsening with infections. | ↑ L | Metabolic myopathy with prominent lipid accumulation. RC: normal | Extensive cortical and subcortical atrophy, cavitation of the basal ganglia (lenticular and striatum). Follow-up MRIs: Progressive and marked atrophy of the globi pallidi and putamina./ New lesion in the caudate nucleus and cerebellar atrophy. | 8 | LS (nuclear encoded) *FASTKD2* c.882-1G>A (p.?), homozygous |
| **P64** | 0.3 | M | 15 | DD with severe cognitive impairment, absent speech. Epilepsy. Hypotonia. FTT. Hypoventilation. Feeding difficulties. Hepatomegaly with ↑transaminases. IGF1 deficiency. Renal lithiasis. | ↑ L, AOs: DC | Frequent fibres with reduced COX activity and apparent glycogen deposition. RC: NP | T2/FLAIR hyperintensity of the white matter with involvement of the thalami, cerebellum, pons, and *medulla oblongata*. | 8 | EARS2-deficiency *EARS2* c.1A>G, (p.Met1?) and c.320G >A, (p.Arg107His) |
| **P65** | 0.0 | M | 0.8 † | DD. Hypotonia. Myopathy. Epilepsy. Liver failure, hepatomegaly. Dilated CMP. FTT. Short stature. Microcephaly. Glomerular proteinuria. Metabolic acidosis. | ↑ L, AOs: ethylmalonic aciduria, 4-OH-phenyl-lactic and -pyruvic | NP RC: NP | NP | 7 | Mitochondrial DNA depletion syndrome *DGUOK*: c.677A>G, p.(His226Arg) and c.749T>C, p.(Leu250Ser) |
| **P66** | 0.5 | F | 2 | Acute *status epilepticus*. Regression. DD with absent speech. Hypotonia. Myopathy. Cortical blindness. ↑ transaminases (post valproate). Feeding difficulties, vomiting. FTT. Short stature. IGF1 deficiency. | ↑ L, ↑ Ala | Normal RC: normal | Restricted diffusion in the perirolandic region. | 7 | Alpers–Huttenlocher syndrome *POLG*: c.911T>G, p.(Leuc304Arg) and c.2542G>A p.(Gly848Ser) |
| **P67** | 0.3 | F | 24 | Degenerative disease with cognitive deterioration. Myoclonic epilepsy. Ataxia. Dystonia. Choreoathetosis. | ↑ L, AOs: ketonuria, DC | Presence of some atrophic fibres, and predominance of type 1 fibres. RC: normal | T2 hyperintensity in the basal ganglia and cerebellum. | 6 | GABA-transaminase deficiency *ABAT*: c.275G>A, p.(Arg92Gln), and c.491G>C, p.(Gly164Ala) |
| **P68** | 0.0 | M | 0.2† | Severe DD. Hypotonia. Elevated transaminases. Feeding difficulties. FTT. Metabolic lactic acidosis. | ↑ L, L/P 23, ↑ Ala, ↑ Gly, ↓ Cit, AOs: ketonuria, Krebs metabolites, DC, 3-OH- | Normal RC: normal | Agenesis of the corpus callosum. T2/FLAIR hyperintensity of the dentate nuclei and white matter. | 8 | EARS2-deficiency *EARS2* c.184 A >T, (p.Ile62Phe) and c.920 T >C, (p.Leu307Ser) |

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|---|---|---|---|---|---|---|---|---|---|
| | | | | | propionic, 4-OH-phenyl-lactic | | | | |
| **P69** | 3.0 | F | 11 | Two episodes of cardiorespiratory arrest triggered by respiratory infections, ↑ transaminases and CK. Thrombocytopenia | ↑ L, ↑ Ala, ↑ Lys, ↑ Gly, 3-MGA | Mild myogenic changes, abnormal lipid accumulation<br>RC: multiple | NP | 6 | Multisystem mitochondrial disorder<br>*LYRM4* c.A92G, (p.Y31C) (homo) |
| **P179** | 0.5 | F | 21 | Moderate-to-severe cognitive impairment. Behavioural disturbances. Cerebellar syndrome and dysarthria. Strabismus and ophthalmoparesis. | ↑ L | NP<br>RC: NP | Not available | 6 | Pyruvate dehydrogenase deficiency<br>*PDHX*: c.160+1G>A, homozygous |

*[1]Age at onset (years); [2]sex [3]Last known age (years); † deceased patients. Abbreviations: 3-MGA: 3-methylglutaconic acid; ASD: autism spectrum disorder; AV: atrioventricular; CS: citrate synthase; DC: dicarboxylic aciduria; DD: developmental delay; DM: diabetes mellitus; F: female; FTT: failure to thrive; ICR1: imprinting control region; LS: Leigh syndrome; M: male; MDC: Nijmegen mitochondrial disease criteria score (maximum 8 points); MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MRI: magnetic resonance imaging; MRS: magnetic resonance spectroscopy; N: normal; NARP: neuropathy, ataxia, and retinitis pigmentosa; NG: nasogastric; NP: not performed; NSHL: neurosensory hearing loss; RC: respiratory chain; RRF: ragged-red fibbers; WM: white matter; WPW: Wolff–Parkinson–White syndrome.*

## S4B. Patients with non-mitochondrial disorders

*Table S2. Clinical, biochemical, histological, neuroimaging and genetic findings in 28 patients with confirmed non-mitochondrial genetic diagnoses.*

| P # | O (y)[1] | S[2] | Age[3] | Description | Metabolic | Muscle biopsy | MRI | MDC | Diagnosis |
|---|---|---|---|---|---|---|---|---|---|
| **P03** | 0.3 | M | 15 | Severe DD, absent language. Hypotonia. Myoclonic epilepsy, early-onset encephalopathy. Ataxic gait. Bifid uvula, submucous cleft palate, cryptorchidism, convergent strabismus. one episode of regression | ↑ L | Normal<br>RC: multiple | Normal | 5 | Jeffries-Lakhani neurodevelopmental syndrome (JELANS)<br>*CRELD1* c.575G>A and c.303_318del |
| **P04** | 0.5 | F | 26 | Severe DD, epilepsy, axial hypotonia, arreflexia, muscle atrophy, dystonia, ataxia,optic atrophy, interventricular septal hypertrophy, repolarisation abnormalities, FTT, IGF1 deficiency, feeding difficulties, elevated transaminases, neonatal hypoglycaemia. | ↑↑↑ L, L/P 17, ↑ CK | Abnormal mitochondria structure<br>RC: multiple | Delayed myelination | 6 | Megaconial-type congenital muscular dystrophy<br>*CHKB* c.754dup (p.(Ser252Phefs*7)) and c.1031+3G>C (r.spl) |
| **P05** | 1 | M | 17† | DD, epilepsy, hyperreflexia without spasticity, hypertonia, behavioural disorder/agitation, congenital cataracts, NSHL, dysmorphic features, microcytic anaemia unresponsive to iron, lymphopenia, short stature | ↑ L, ↑ Ala, ↑ CK | Normal<br>RC: multiple | Cerebral atrophy; encephaloclastic changes in the right inferior temporal region, pineal cyst | 6 | Ayme-Gripp syndrome<br>*MAF* c.172A>G (p.(Thr58Ala)) (*de novo*) |
| **P06** | 14 | F | 21 | Progressive hypotonia, proximal tetraparesis, ataxia, dystonia, dysarthria, muscle contractures, muscle weakness. Atrioventricular block requiring pacemaker. | ↑ L, ↑ CK | Fiber atrophy and necrosis, lipidic deposition and RRF<br>RC: multiple | NP | 6 | Emery-Dreifuss muscular dystrophy<br>*LMNA* c.1357C>T (p.(Arg453Trp)) |
| **P07** | 6 | F | 13 | Neonatal hypotonia, developmental delay, osteopenia, dysmorphic syndrome. | ↑ L, ↑ CK | type 2 fiber atrophy<br>RC: normal | Normal | 4 | ZC4H2-Associated Rare Disorder<br>*ZC4H2* c.450dup (p.(Ile151Hisfs*3)) |
| **P08** | 0.1 | M | 5 | DD, epilepsy, choreoathetosis, irritability. FTT. Glomerular and tubular proteinuria, suspected tubulopathy. | ↑ L, ↑ Ala, OAs:Krebs metabolites, DC | NP<br>RC: NP | Normal | 7 | Angelman syndrome del15q11-q13 |
| **P09** | 6 | M | 20 | DD with moderate cognitive impairment. First-degree AV block. | ↑ L and P | Normal<br>RC: II partial | NP | 3 | 22q11.2 duplication syndrome dup22q11.22q11.23 |
| **P10** | 3 | M | 12 | Non-progressive DD, ASD, hypotonia (axial > appendicular), ataxia, and bradykinesia. Muscle atrophy, joint hyperlaxity, ophthalmoparesis, and optic atrophy. FTT. Precocious adrenarche. One episode of regression. Recurrent marked ketonuria with altered organic acid excretion. | ↑ L, OAs: ketonuria, Krebs metabolites, DC | Normal RC: normal | Normal | 7 | GRIN2B-Related Neurodevelopmental Disorder<br>*GRIN2B* c.1821G>T, (p.(Trp607Cys)) |
| **P11** | 0.6 | M | 11 | DD, one episode of motor regression. Early hypotonia, distal muscle atrophy, ataxia, | ↑ L, OAs: ketonuria, DC | type 2 fiber atrophy<br>RC: NP | NP | 4 | Ehlers–Danlos syndrome<br>*COL5A1* (genotype not available) |

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| | | | | nystagmus, dystonia, and pyramidal signs. Feeding difficulties. | | | | |
| **P12** | 2 | M | 14 | Severe DD, macrocephaly, early hypotonia, epilepsy (including status epilepticus), cerebellar syndrome, horizontal nystagmus, hemiplegic migraine, and peripheral neuropathy | ↑ L, ↑ Ala | Normal<br>RC: multiple | 1st MRI: normal. Follow-up MRI: Global brain atrophy with predominant cerebellar involvement. | 5 | Spinocerebellar Ataxia Type 6<br>*CACNA1A* c.4052G>A, (p.(Arg1351Gln)) (de novo) |
| **P13** | 2 | F | 18 | Severe DD, ataxia, NSHL, strabismus, neonatal hypoglycaemia, short stature, and spastic tetraparesis. Worsening with infection. | ↑ L | NP<br>RC: II partial | Progressive cerebellar atrophy | 3 | Ataxia espinocerebelar tipo 20 - SNX14<br>*SNX14* c.331C>T p.(Arg111*), homozygous |
| **P14** | 1 | M | 17 | Global developmental delay, speech delay, spastic tetraparesis (predominantly lower limbs), pyramidal signs, ataxia, oculomotor apraxia, paroxysmal gait disturbances triggered by fever, and dysarthria. | - | Normal<br>RC: normal | NP | 2 | TANGO2 deficiency *TANGO2* *c*.[(56+1_57-1), (p.(*1_?)del) and c.605+1G>A, (p.?) |
| **P15** | 0.8 | M | 11 | Early-onset hypotonia with severe DD, progressive early-onset myopathy, and possible ataxia. | ↑ L | Dystrinophopathy<br>RC: II 23% | NP | 3 | Duchenne muscular dystrophy<br>DMD del exons 46-47 |
| **P16** | 0.6 | M | 4 | Intrauterine growth restriction, macrocrania, DD, axial hypotonia, joint hyperlaxity, FTT, and short stature. | ↑ L, Oas: MMA and glutharic acid | type 2 fiber atrophy, fibers with ↑oxidative activity<br>RC: V partial | NP | 5 | Silver-Russell syndrome<br>Hypomethylation of the imprinting control region 1 (ICR1) at 11p15.5 |
| **P17** | 0 | M | 11 | Neonatal distress, hypotonia, and early-onset Hypertrophic interventricular septum. | ↑ L,↑ CK | fiber atrophy, some necrotic fibers<br>RC: ↑ CS, N | NP | 5 | Duchenne muscular dystrophy<br>*DMD* del exon 53 |
| **P18** | 2 | M | 22 | Myopathy, café-au-lait spots. Hepatomegaly with hepatic steatosis and mildly elevated transaminases. Hypothyroidism. | ↑ L | Normal<br>RC: IV partial | T2-FLAIR hyperintensities in thalami, left corona radiata, bilateral subcortical WM, cerebellar peduncle, pontocerebellar and bilateral cerebellar WM | 6 | Constitutional mismatch repair-deficiency syndrome<br>*PMS2* del exon 14_homozygous |
| **P19** | 0 | F | 14 | DD, early-onset hypotonia, FTT, feeding difficulties (requiring NG tube), recurrent hypoglycaemia and asymmetric ventriculomegaly. | ↑ L | type 2 fiber atrophy, congenital myopathy<br>RC: multiple | NP | 2 | Congenital Myasthenic Syndrome type 22<br>*PREPL* c.883C>T, p.(Arg295*) |
| **P20** | 1.8 | M | 8 | Cerebellar ataxia, pyramidal signs, apraxia, dystonia, and chorea. | ↑ L, ↑ Ala | NP<br>RC: NP | Normal | 4 | Ataxia-oculomotor apraxia 4<br>*PNKP* c.1221_1223del (p.(Thr408del)) and c.1315_1330delinsGGGGACG(.pArg439_Pro444delinsGlyAspAla) |
| **P21** | 2 | M | 19 | Hemidystonia and left-sided hemiparesis, generalized dystonia. Dilated CMP. Intermittent proteinuria, short stature. Clinical fluctuations triggered by infections | ↑ L | Normal<br>RC: normal | Cerebellar atrophy, dentate nucleus T2 hyperintensity; follow-up MRI: T2 | 6 | Aicardi-Goutieres syndrome type 6<br>*ADAR* c.3116A>G (p.(Lys1039Arg) (LP) and c.577C>G (p.(Pro193Ala)) |

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|---|---|---|---|---|---|---|---|---|
| | | | | | | hyperintensity and atrophy of putamen | | |
| **P22** | 9 | M | 21 | DD, ASD, myopathy, and FTT. Easy fatigability and exercise intolerance. Recurrent vomiting with ketosis | ↑ L | type 1 fiber atrophy<br>RC: normal | Abnormal hippocampal morphology with atypical gyration pattern | 4 | KBG syndrome<br>*ANKRD11* c.3770_3771del, p.(Lys1257Argfs*25), de novo |
| **P23** | 5 | M | 20 | DD, epilepsy, myopathy,ptosis, pectus excavatum, asymmetric scapulae and iliac crests, microcephaly, strabismus. Low forehead, high nasal bridge, mild alar hypoplasia, left preauricular pit, low-set auricles, enlarged central incisors, brachydactyly | ↑ L | Normal<br>RC: II 18% | Cerebellar atrophy and cortical atrophy | 4 | 3q29 microdeletion syndrome<br>del3q29 |
| **P24** | 4 | M | 13 | DD, hypotonia, myopathy with muscle weakness. Hypertrophic CMP. | ↑ L, ↑ CK, Oas: phenyllactic and phenylpyruvic | lipid deposits, type 2 fibers atrophy<br>RC: normal | Normal | 4 | Danon Disease<br>*LAMP2* g.(?_119602944)_(119603191_?)del |
| **P25** | 0 | M | 16 | DD, epilepsy, and hypotonia. Hypothyroidism | ↑ L | Normal<br>RC: normal | T2 hyperintensity (globus pallidus, periventricular, pons, midbrain); delayed myelination. 2nd MRI with normalization of basal ganglia | 6 | STXBP1 encephalopathy with epilepsy<br>*STXBP1* c.875G>A (p.Arg292His)_heterozygous |
| **P26** | 0 | M | 2† | Epilepsy, encephalopathy, and hypothyroidism. Suspected salt-wasting tubulopathy. Worsening with infection. | - | NP<br>RC: NP | NP | 3 | *HCN1*-Epileptic encephalopathy<br>*HCN1 c*.1172G>A, (p.Gly391Asp), *de novo* |
| **P27** | 3 | M | 20 | Severe DD, ASD, ataxia, bradykinesia, and pyramidal signs in the lower limbs. Hyperuricaemia (> age 8). Elevated transaminases and hepatomegaly. | ↑ L, ↑ CK, ↑ Ala | Normal<br>RC: I 23% | Normal | 4 | Rauch-Steindl syndrome<br>*NSD2* c.1589del p.(Lys530Argfs*68), *de novo* |
| **P28** | 2 | M | 10 | Congenital ataxia, severe DD, hypotonia, microcephaly, and dystonia. FTT, short stature. | ↑ L, OAs: phenylpyruvic | Unspecific findings<br>RC: normal | NP | 5 | Spinocerebellar ataxia 42<br>*CACNA1G* c.3792G>T, (p.Arg1264Ser) |
| **P29** | 1.5 | F | 9 | DD, epilepsy (including status epilepticus) ataxia, and dystonia. Recurrent severe infections. Restrictive muscular ventricular septal defect, mild aortic regurgitation. Acute encephalopathy with 3-MGA (infection with need of intensive care). | ↑ L, OAs: 3-MGA | NP<br>RC: NP | Extensive subependymal heterotopias, thin corpus callosum, and mega cisterna magna | 4 | Huttenlocher syndrome<br>*FLNA* c.7792del, (p.Arg2598Glyfs*9) (mat) |
| **P32** | 0.1 | F | 2 | DD, Early-onset epilepsy | ↑ Ala, ↑ Lys | NP<br>RC: NP | NP | 4 | CDKL5-Epileptic Encephalopathy<br>*CDKL5* c.448A>G, (p.Lys150Glu), *de novo* |

*[1]Age at onset (years); [2]sex [3]Last known age (years); † deceased patients. Abbreviations: 3-MGA, 3-methylglutaconic acid; ASD, autism spectrum disorder; AV, atrioventricular; CS, citrate synthase; DC, dicarboxylic aciduria; DD, developmental delay; FTT: Failure to Thrive; F, female; ICR1, imprinting control region; M, male; MDC, Nijmegen mitochondrial disease criteria score (maximum 8 points); MRI, magnetic resonance imaging; N, normal; NG, nasogastric; NSHL: Neurosensory Hearing Loss ; NP, not performed; RRF, ragged-red fibers; WM, white matter.*

## S4C. Patients' description of subjects included in the Metabolomics study

***Table S3. Clinical and Genetic Characterization of Groups in the Metabolomics Study.***

| ID | Age (y) | Sex | Phenotype | Genetic basis* | Usual medication | eGFR * | U25-Cr | U25-CisC | U25 Mean eGFR | ACR | CKD stage** | Prot (u) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PMD** | | | | | | | | | | | | |
| P02 | 8.7 | F | Hypotonia; GDD; basal ganglia lesions | *EARS2* | Q10, thiamine, riboflavine | 194 | 165.1 | x | x | x | G1A1 | 0.187 |
| P03 | 18.8 | M | Primary Q10 deficiency | *COQ8A* | Clonazepam, ibedenone, Q10 | 105 | 82.42 | x | x | no | G1A1 | 0.036 |
| P04 | 14 | F | LS | m.10191T>C | Q10, thiamine, riboflavine | 124.8 | 116.6 | x | x | no | | 0.09 |
| P05 | 10.5 | M | Cardiomiopathy, myopathy, NSHL, FFT | m.3271T>C | Q10, thiamine, riboflavine | 159.6 | 148.9 | x | x | no | G1A1 | 0.147 |
| P06 | 10.4 | M | LS | m.8993T>G | Q10, thiamine, riboflavine, arginine | 158.9 | 148.2 | x | x | 12.8 | G1A1 | 0.124 |
| P07 | 4.0 | F | FFT, Low stature, miopathy, muscle weakness, ptosis | SLSMD | Levotiroxine, Q10, thiamine, riboflavine, L-carnitine | 156.2 | 103.1 | x | x | x | G1A1 | x |
| P08 | 15.5 | F | LS | m.3946G>A | Q10, Thiamine, riboflavine | 91.36 | 86.47 | x | x | x | G1A1 | 0,072 |
| P09 | 4.8 | M | Cardiomiopathy intermittent 3-MGA | *TAZ* | Captopril, spironolactone, carvedilol | 129.1 | 115 | x | x | x | G1A1 | 0.172 |
| P10 | 1.0 | M | Neonatal severe metabolic acidosis, high Lactate, CMP, 3-MGA-uria | *TMEM70* | Q10, thiamine, riboflavine, arginine, $NaHCO_3$ | 91.63 | 79.27 | x | x | 19.2 | G1A1 | 0.392 |
| P11 | 16.9 | F | LS | m.8993T>G | Q10, thiamine, riboflavine | 135.5 | 132.4 | x | x | x | G1A1 | in. |
| P12 | 1.1 | F | Alpers syndrome | *POLG* | Clonazepam, phenobarbital, lacosamide, Q10, thiamine, riboflavin | 334.5 | 268.1 | x | x | 26.1 | G1A1 | 0.522 |
| P13 | 8.8 | M | Epilepsy and ASD | m.8993T>C | Risperidone, atomexetine, valproate | 128.2 | 118 | x | x | x | G1A1 | 0.118 |
| P14 | 9.4 | F | Cardiorrespiratory arrests, 3-MGA-uria | *LYRM4* | None | 185.9 | 159.1 | x | x | 7.1 | G1A1 | x |
| P15 | 10.5 | F | GRACILE | *BCSL1* | Q10, thiamine, $NaHCO_3$, phosphate , enalapril, esomeprazol, $VitD_3$ | 88.8 | 68.07 | 87.8 | 77.9 | 278.9 | G2A3 | x |
| P25 | 9.8 | M | NS, NSHL and myopathy | *RR2MB* | Q10, thiamine, steroids, micophenate mofetil, pravastatine | 83.25 | 77.37 | 41.7 | 59.5 | 9175 | G2A3 | 11,6 |
| **SMD** | | | | | | | | | | | | |
| P16 | 0.3 | M | ALTE, metabolic acidosis, ketosis, high alanine, low arginine. | - | Arginine | 75.4 | x | x | x | x | G1A1 | x |
| P17 | 16.8 | F | Recurrent myositis, easy fatigability; CV 11%. | - | Q10 | 122 | | x | x | x | G1A1 | x |
| P18 | 0.9 | F | Neonatal distress with hypoglycemia; early onset hypotonia; spasticity, assimetric ptosis, sensory disturbance; GDD. | - | None | 109 | | x | x | x | G1A1 | x |
| P19 | 0.2 | F | Aregenerative anemia, neutropenia, ethylmalonic aciduria and 3-methylglutaconic aciduria (3MGA); neonatal distress without sentinel event. | - | Phenobarbital | 82.8 | | x | x | x | G1A1 | x |

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| P20 | 18.7 | F | FTT; LV hypertrophy cardiomiopathy without heart failure; muscle biopsy with multiple RC deficiencies. | - | Q10, thiamine, riboflavine | 136 | 118.5 | x | x | x | G1A1 | x |
| P21 | 3.7 | M | Progressive spasticity (++ lower limb), GDD. | - | Q10, thiamine, riboflavine | 125.3 | | x | x | 13.8 | G1A1 | 0.13 |
| P22 | 4.5 | F | Episodic ataxia and sonnomlence; low stature; intermittent high Lactate and alanine; hyperintense cerebellar lesions. Krebs metabolites in Oas. | - | Q10, thiamine, riboflavine | 123.3 | 101.8 | x | x | 0 | G1A1 | 0 |
| P23 | 19.5 | F | Epileptic encephalopathy, absent language, GDD/ID. | - | Vigabatrine, zonisamide, lacosamide, ketogenic diet | 132 | 108.3 | x | x | 0 | G1A1 | 0 |
| P24 | 2.2 | F | FFT, low stature, muscle weakness, paradoxal ketosis, persistent high Lactate and alanine, distal tubular acidosis, Oas Krebs metabolites. | - | Q10, thiamine, riboflavine | 118.4 | | x | x | <9 | G1A1 | 0.24 |
| **C** | | | | | | | | | | | | |
| P26 | 2.7 | M | Renal duplication with bilateral pyelectasis | - | None | 122 | 108.1 | 96.6 | 102.4 | 8.3 | G1A1 | 0.19 |
| P28 | 3.1 | F | Previous UTI | - | None | 162.7 | 132.4 | 92.9 | 112.7 | 13.8 | G1A1 | 0.23 |
| P29 | 9.8 | M | Crossed renal ectopia | - | None | 129.1 | 111.9 | 106.1 | 109 | 9.9 | G1A1 | 0.1 |
| P30 | 13.3 | M | Ureterohydronephrosis | - | None | 102.9 | 106.1 | 91.8 | 98.9 | 6.3 | G1A1 | 0.11 |
| P31 | 12.4 | F | Renal duplication with bilateral pyelectasis | - | None | 109.7 | 100.4 | | | <3,4 | G1A1 | 0.06 |
| P32 | 8.1 | F | Vesicoureteral reflux | - | None | 122.8 | 104.8 | 80.6 | 92.7 | <3,4 | G1A1 | 0.13 |
| P33 | 13.5 | M | Vesicoureteral reflux with spontaneous regressions | - | None | 110.8 | 119.4 | 89.9 | 104.7 | 26.3 | G1A1 | 0.15 |
| P34 | 13.9 | M | Transient creatinine elevation | - | None | 100.1 | 107.9 | 90.8 | 99.4 | 7.4 | G1A1 | 0.06 |
| P35 | 18.2 | M | Posterior urethral valve (corrected) | - | None | 130 | 107.4 | 90.5 | 98.9 | 9.5 | G1A1 | 0.05 |
| P36 | 16.8 | M | Solitary kidney | - | None | 85.66 | 105.4 | 89.7 | 97.6 | 11.9 | G1A1 | 0.06 |
| **CKD1-2** | | | | | | | | | | | | |
| P37 | 6.6 | M | *Ataxia Telangiectasia* | *ATM* | None | 112.5 | 102.9 | | | 16.9 | G1A1 | 0.15 |
| P38 | 15.8 | M | Polymalformative syndrome with CAKUT | - | None | 70.45 | 82.9 | 73.1 | 78 | 17.3 | G2A1 | 0.05 |
| P39 | 17.9 | F | CAKUT with multicystic kidneys | - | None | 113 | 83.3 | 84.1 | 83.7 | 3.1 | G2A1 | 0.05 |
| P40 | 14.3 | M | Renal Agenesia and ASD | - | None | 79.85 | 86 | 89.9 | 87.9 | 2.3 | G2A1 | 0.04 |
| P41 | 8.1 | F | PKD | - | None | 129.1 | 112.8 | 90.7 | 101.8 | 7.2 | G1A1 | 0.16 |
| P42 | 17.8 | M | Steroid-dependent NS | - | Steroids, Mycophenolate mofetil | 138 | 127 | 96.4 | 111.7 | 4.5 | G1A1 | 0.04 |
| P43 | 11.1 | M | X-L Hypophosphatemic Rickets (PHEX) | *PHEX* | Burosumab | 134 | 117.1 | 70.7 | 93.9 | 16.5 | G1A1 | 0.13 |
| P44 | 7.2 | M | X-L Hypophosphatemic Rickets | *PHEX* | Burosumab | 163 | 149.1 | 120.6 | 134.9 | 5.4 | G1A1 | 0.14 |
| P45 | 17.1 | M | Alport Syndrome | *COL4A3* | None | 88.87 | 113.2 | 87.6 | 100.4 | 14.4 | G1A1 | 0.06 |
| P46 | 4.8 | M | Steroid-sensitive NS; ASD | - | Steroids, Mycophenolate mofetil | 259.9 | 234 | X | X | 516 | G1A3 | 0.86 |
| P47 | 2.8 | F | VUR and UTIs ; DM type 1 | - | Insuline | 207.4 | 101.3 | X | X | 2742 | G1A3 | 5.33 |
| P48 | 16.9 | M | Nutcracker Syndrome | - | 0 | 82.65 | 101.7 | x | X | 158.7 | G1A2 | 0.26 |
| P49 | 11.4 | M | Steroid-dependent NS | - | Steroids, cyclosporine, ramipril | 150.4 | 148.4 | X | X | 2020 | G1A3 | 2.42 |
| P50 | 14.8 | F | PKD | - | None | 105.2 | 100.8 | 73 | 86.9 | 79.3 | G1A2 | 0.16 |
| P51 | 16.2 | M | Probable IgA Nephropathy | - | Ramipril | 97.62 | 114.9 | 101.7 | 108.3 | 782.3 | G1A3 | 1.08 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P52 | 7.8 | M | Glomerulonephritis - IgA Nephropathy | - | Finerenone | 146.8 | 135.4 | X | x | 3174 | G1A3 | 4.11 |
| P53 | 17.3 | M | Urethral stricture | - | None | 87.58 | 107.7 | 87.6 | 97.65 | 148 | G1A2 | 0.39 |
| **CKD3-5** | | | | | | | | | | | | |
| P54 | 12.9 | F | Cystinosis | *CTNS* | Cysteamine, phosphate, $NaHCO_3$, sodium citrate, potassium citrate, KCl, paricalcitol, $CaCO_3$, $VitD_3$, iron | 26.12 | 23.9 | 43.8 | 33.85 | 3983 | G4A3 | 6.24 |
| P55 | 4.7 | M | CAKUT with dysplastic and cystic kidneys | - | $NaHCO_3$, epoetin α, paricalcitol, $VitD_3$, folic acid | 13.99 | 12.6 | 18.3 | 15.45 | 2650 | G5A3 | x |
| P56 | 15.7 | M | Ochoa syndrome with CAKUT | *HNF1B* | $CaCO_3$, amlodipine, $VitD_3$, silodosin, resin, αcalcidol, iron | 43.27 | 50.9 | | | 13.4 | G3aA1 | x |
| P57 | 9.5 | M | Joubert syndrome w/ nephronophthisis | *TCTN2* | $NaHCO_3$, iron, calcitriol, allopurinol, epoetin α, amlodipine, esomeprazole | 10.42 | 9.8 | 17.6 | 13.7 | 194.6 | G5A2 | x |
| P58 | 15.8 | M | CAKUT | - | $NaHCO_3$, calcitriol, enalapril, oxybutynin, tamsulosin, resin | 18.95 | 22.3 | 22.1 | 22.2 | 46.3 | G4A2 | x |
| P59 | 14.6 | M | AR-PKD and liver fibrosis | *PKHD1* | $NaHCO_3$, epoetin α, $VitD_3$, paricalcitol, iron | 23.53 | 26.5 | 26.4 | 26.45 | 15.7 | G4A1 | x |
| P60 | 8.2 | M | Scarring nephropathy; VUR | - | $NaHCO_3$, epoetin α, $VitD_3$, paricalcitol, enalapril, oxybutynin, somatotropin | 16.24 | 15.2 | 26.1 | 20.65 | 76.4 | G4A2 | x |
| P61 | 15.3 | F | MMC; VUR; neurogenic bladder | - | $NaHCO_3$, epoetin α, $VitD_3$, paricalcitol, iron, $CaCO_3$ | 11.42 | 10.9 | 17.9 | 14.4 | 3063 | G5A3 | x |
| P62 | 10.8 | F | AR-PKD | *PKHD1* | $NaHCO_3$, iron, αcalcidol, sevelamer, epoetin α, propranolol, $CaCO_3$, $VitD_3$ | 14.74 | 12.8 | 19 | 15.9 | 75.9 | G5A1 | x |

**Calculated by the creatinine-based Chronic Kidney Disease in Children (CKiD) U25 Equation except for patients younger than 12 months, where eGFR is based on the Schwartz formula, and for patients older than 18 years, where eGFR is based on CKD-EPI 2021 guidelines; **CKD stage based on eGFR and albumin-to-creatinine (ACR) ratio as defined by the KDIGO 2024 Guidelines. Abbreviations: 3-MGA-uria: 3-methylgluthaconic aciduria; ACR: Albumin-to-creatinine ratio; AD: Autosomal Dominant; AR: Autosomal Recessive; ASD: Autism Spectrum Disorder; CAKUT: Congenital Anomalies of the Kidney and Urinary Tract; DM: Diabetes Mellitus; FS: Fanconi Syndrome; FTT: Failure to Thrive; GRACILE: Growth retardation, Renal (kidney) dysfunction, Aminoaciduria, Cholestasis, Iron overload, Lactate acidosis, Early death; GDD: Global Developmental Delay; in: intermittent; LS: Leigh Syndrome; MMC: Myelomeningocele; M: Male; NS: Nephrotic Syndrome; NSHL: Neurosensory Hearing Loss; OAs: organic acids chromatography; PKD: Polycystic Kidney Disease; Prot(u): proteinuria; SLSMD: single large-scale mtDNA deletions; UTI: Urinary Tract Infection; VUR: Vesicoureteral Reflux; X-L: X-linked; y: years; F: Female.*

## S4D. List of metabolites identified in the Metabolomics study

***Table S4. List of identified metabolites.*** *All metabolites listed were assigned with a confidence level of Putative identified metabolite (MSI Level 2) according to Metabolomics Standards Initiative - MSI), except those marked with #, tentative assignment. For each metabolite, the main metabolic pathway, HMDB and KEGG IDs, and spectral references are indicated. Peaks used for integration are underlined.*

| Compound Name | Main pathway | HMDB ID | KEGG ID | δ/ppm (multiplicity) |
|---|---|---|---|---|
| **Phenylalanine** | Aromatic AA metabolism | HMDB0000159 | C00079 | 3.19 (m); 4.00 (dd); 7.34 (m); 7.40 (m); 7.43 (m) |
| **Alanine** | Alanine metabolism | HMDB0000161 | C00041 | 1.48 (d); 3.78 (q) |
| **Dimethylamine (DMA)** | Amine metabolism | HMDB0000087 | C00543 | 2.71 (s) |
| **Acetate** | Beta-oxidation/ketone metabolism | HMDB0000042 | C00033 | 1.92 (s) |
| **Acetoacetate** | Beta-oxidation/ketone metabolism | HMDB0000060 | C00164 | 2.27 (s); 3.42 (s) |
| **Acetone** | Beta-oxidation/ketone metabolism | HMDB0001659 | C00207 | 2.23 (s) |
| **3-Methyl-2-oxovalerate (3M2OV)** | Branched-chain AA metabolism | HMDB0000491 | C00671 | 0.89 (d); 1.10 (d); 2.20 (m); 2.55 (m) |
| **Isoleucine** | Branched-chain AA metabolism | HMDB0000172 | C00407 | 0.94 (t); 1.02 (d); 1.28 (m); 1.46 (m); 1.99 (m); 3.67 (d) |
| **Leucine** | Branched-chain AA metabolism | HMDB0000687 | C00123 | 0.97 (t); 1.71 (m); 1.74 (m); 3.74 (t) |
| **Valine** | Branched-chain AA metabolism | HMDB0000883 | C00183 | 0.99 (d); 1.04 (d); 2.27 (m); 3.62 (d) |
| **α-Glucose** | Carbohydrate metabolism | HMDB0003345 | C00031 | 3.41 (m); 3.54 (dd); 3.72 (t); 3.77 (t); 3.83 (m); 5.23 (d) |
| **β-Glucose** | Carbohydrate metabolism | HMDB0000122 | C00221 | 3.24 (dd); 3.41 (m); 3.47 (m); 3.50 (t); 3.73 (dd); 4.65 (d) |
| **Glucose-1-phosphate**[#] | Carbohydrate metabolism | HMDB0001586 | C00103 | 3.37 (t); 3.46 (m); 3.48 (m); 3.70 (d); 3.89 (m); 5.51(dd) |
| ***myo*-inositol** | Carbohydrate metabolism | HMDB0000211 | C00137 | 3.26 (m); 3.52 (m); 3.61 (m); 3.64 (m) |
| **Creatine** | Creatine metabolism | HMDB0000064 | C00300 | 3.04 (s); 3.93 (s) |
| **Creatinine** | Creatine metabolism | HMDB0000562 | C00791 | 3.05 (s); 4.06 (s) |
| **Homovanillate (HVA)** | Dopamine metabolism | HMDB0000118 | C05582 | 3.43 (s); 6.76 (d); 6.94 (dd); 7.10 (d) |
| **Ethanol** | Ethanol metabolism | HMDB0000108 | C00469 | 1.17 (t); 3;65 (q) |
| **Glucuronate** | Glucoronate pathway | HMDB0000127 | C00191 | 3.30 (dd); 3.50 (m); 3.57 (dd); 4.09 (d); 4.65 (d); 5.27 (d) |
| **Glycine** | Glycine and serine metabolism | HMDB0000123 | C00037 | 3.57 (s) |
| ***cis*-Aconitate** | Glycolysis/Krebs cycle | HMDB0000072 | C00417 | 3.11 (d); 5.70 (s) |
| **Citrate** | Glycolysis/Krebs cycle | HMDB0000094 | C00158 | 2.52 (dd); 2.67 (dd) |
| **Fumarate** | Glycolysis/Krebs cycle | HMDB0000134 | C00122 | 6.52 (s) |
| **Malonate** | Glycolysis/Krebs cycle | HMDB0000691 | C00383 | 3.11 (s) |
| **Oxoglutarate** | Glycolysis/Krebs cycle | HMDB0000208 | C00026 | 2.45 (t); 3.00 (s) |
| **Pyruvate** | Glycolysis/Krebs cycle | HMDB0000243 | C00022 | 2.38 (s) |
| **Succinate** | Glycolysis/Krebs cycle | HMDB0000254 | C00042 | 2.41 (s) |
| **Glycolate** | Glyoxylate and dicarboxylate metabolism | HMDB0000115 | C03547 | 3.96 (s) |
| **5-Aminolevulinate (5-ALA)** | Heme biosynthesis | HMDB0001149 | C00430 | 2.42 (dt); 2.51 dt); 2.74 (t); 3.81 (s) |
| **1-Methylhistidine** | Histidine metabolism | HMDB0000001 | C01152 | 3.34 (s); 7.03 (s); 8.15 (s) |
| **Histidine** | Histidine metabolism | HMDB0000177 | C00135 | 3.15 (dd); 3.24 (dd); 3.98 (t); 7.10 (s); 8.08 (s) |
| **3-Hydroxybutyrate (3-HBA)** | Ketone metabolism | HMDB0000011 | C01089 | 1.23 (d); 2.24 (dd); 2.39 (dd); 4.15 (m) |

| | | | | |
|---|---|---|---|---|
| **3-Hydroxyisovalerate (3-HIVA)** | Leucine metabolism | HMDB0000754 | C20827 | 1.27 (s); 2.35 (s) |
| **2-Aminoadipate** | Lysine degradation | HMDB0302754 | C00956 | 1.63 (m); 1.81 (m); 2.07 (m); 2.23 (t); 2.33 (m); 3.86 (m) |
| **Lysine** | Lysine degradation | HMDB0000182 | C00047 | 1.47 (m); 1.75 (m); 3.03 (m) |
| **Trimethylamine *N*-oxide (TMAO)** | Methylamine metabolism | HMDB0000925 | C01104 | 3.27 (s) |
| **Betaine** | Methylation metabolism | HMDB0000043 | C00719 | 3.26 (s); 3.90 (s) |
| **Trigonelline** | Nicotinate metabolism | HMDB0000875 | C01004 | 4.43 (s); 7.11 (d); 8.07 (d); 8.85 (s); 9.13 (s) |
| **Formate** | One carbon pool by folate | HMDB0000142 | C00058 | 8.46 (s) |
| ***N*-Phenylacetylglycine (*N*- PhAcGly)** | Phenylalanine metabolism | HMDB0000821 | C05598 | 3.64 (s); 3.82 (s); 7.36 (m); 7.43 (m) |
| **Hypoxanthine** | Purine metabolism | HMDB0000157 | C00262 | 8.19 (s); 8.22 (s) |
| **Inosine** [#] | Purine metabolism | HMDB0000195 | C00294 | 3.86 (dd); 3.93 (dd); 4.34 dd);4.38 (td); 4.78 (dd); 6.09 (d); 8.23 (s); 8.34 (s) |
| **Lactate** | Pyruvate metabolism | HMDB0000190 | C00186 | 1.34 (d); 4.11 (q) |
| ***N*-Methylnicotinamide (MNA)** | Nicotinate and nicotinamide metabolism pathway | HMDB0003152 | NA | 4.48 (s); 8.18 (m); 8.90 (d); 8.97 (d); 9.29 (s) |
| **2-Hydroxyisobutyrate (2-HIBA)** | Short-chain fatty acid metabolism | HMDB0242161 | NA | 1.26 (s); 1.36 (s) |
| **Methionine** | Sulfur-containing AA metabolism | HMDB0000696 | C00073 | 2.10 (s); 2.65 (t); 3.88 (t) |
| **Taurine** | Sulfur-containing AA metabolism | HMDB0000251 | C00245 | 3.27 (t); 3.43 (t) |
| **Threonine** | Threonine metabolism | HMDB0000167 | C00188 | 1.32 (d); 4.22 (m) |
| **4-Hydroxyphenylacetate (4-HPA)** | Tyrosine / Phenylalanine metabolism | HMDB0060390 | C13636 | 3.54 (s), 6.87 (d), 7.17 (d) |
| **Tyrosine** | Tyrosine metabolism | HMDB0000158 | C00082 | 3.06 (dd); 3.19 (dd); 3.93 (dd); 6.90 (d); 7.20 (d) |
| **Tryptophan** | Tryptophan metabolism | HMDB0000929 | C00078 | 7.29 (t); 7.55 (d); 7.75 (d) |
| **3-Indoxylsulfate** [#] | Tryptophan metabolism | HMDB0000682 | NA | 7.21 (dd); 7.28 (dd); 7.36 (s); 7.51 (d); 7.70 (d) |
| **Urea** | Urea cycle | HMDB0000294 | C00086 | 5.83 (br, s) |
| **3-Hydroxyisobutyrate (3-HIBA)** | Valine metabolism | HMDB0000023 | C06001 | 1.11 (d); 2.65 (m); 3.52 (m); 3.68 (m) |
| **3-Aminoisobutyrate (BAIBA)** | Valine metabolism | HMDB0003911 | C05145 | 1.20 (d); 3.55 (m); 3.01 (dd); 3.10 (dd) |
| **Hippurate** | Xenobiotic metabolism | HMDB0000714 | C01586 | 3.96 (d); 7.54 (m); 7;63 (tt) 7.83 (dd); 8.52 (br) |
| **Propylene glycol** [#] | Xenobiotic metabolism/Possible contaminant | HMDB0001881 | C00583 | 1.14 (d); 3.43 (dd); 3.53 (dd); 3.86 m |
| **Tartrate** [#] | | HMDB0000956 | C00898 | 4.35 (s) |

*Abbreviations: AA: amino acid; HMDB: Human Metabolome Database; KEGG: Kyoto Encyclopedia of Genes and Genomes. Multiplicity: s: singlet; d: doublet; dd: doublet of doublets; ddt: doublet of doublets of triplets; t: triplet; q: quartet; m: multiplet; br: broad signal.*

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